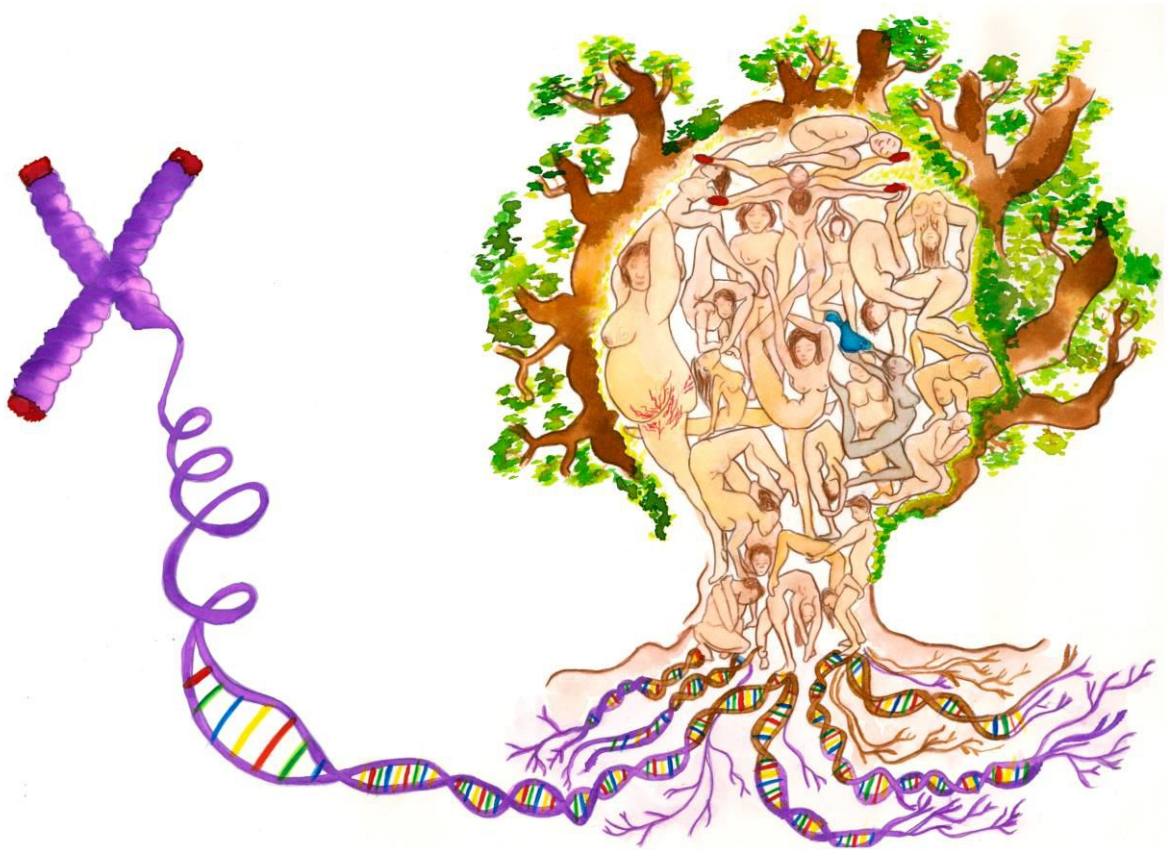
Universitat Autònoma de Barcelona

DOCTORAL THESIS

Investigation of the telomere maintenance system in Cushing's syndrome: A contribution to the phenomena of early ageing and specific morbidity



Anna Aulinas Masó

Thesis director: Prof. Susan Webb Youdale

Programa de Doctorat en Medicina Interna
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Summary

List of publications

This thesis is mainly based in the following two original articles:

Paper I:

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 Jul;171(1):21-9. doi: 10.1530/EJE-14-0098.

Paper II:

Aulinas A, Ramírez MJ, Barahona MJ, Valassi E, Resmini E, Mato E, Santos A, Crespo I, Bell O, Surrallés J, Webb SM. Dyslipidemia and chronic inflammation markers are correlated with telomere length shortening in Cushing's syndrome. PLoS ONE. 2015; 10(3): e0120185. doi:10.137/journal.pone.0120185.

According to the evaluation of Postgraduat subcomission, the following Reviews cannot be part of the main body of the thesis, since they are not original articles. However, the Reviews appear in Annex II, as they have been important to review the state of the art and the background of this thesis.

Review I:

Aulinas A, Santos A, Valassi E, Mato E, Crespo I, Resmini E, Roig O, Bell O, Webb SM. Telomeres, aging and Cushing's syndrome: are they related? Endocrinol Nutr. 2013 Jun-Jul;60(6):329-35.

Review II:

Aulinas A, Valassi E, Webb SM. Prognosis of patients treated for Cushing syndrome. Endocrinol Nutr. 2014 Jan;61(1):52-61

Review III:

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 Dec;79(6):751-9.

Abbreviations

ACEI	angiotensin-converting enzyme inhibitors
ACTH	adrenocorticotropin hormone
ANCOVA	Analysis of covariance
ATPIII	Adult Treatment Panel III
ATRX	ATP-dependent helicase
BMD	bone mineral density
BMI	body mass index
BP	base pairs
CD	Cushing's disease
CRH	corticotropin releasing hormone
CRP	C-reactive protein
CS	Cushing's syndrome
CTC1	conserved telomere maintenance complex component 1
CV	coefficient of variation
CVR	cardiovascular risk
DAXX	death associated protein 6
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GC	glucocorticoids
GH	Growth hormone
HDL	high-density lipoprotein
HPA	Hypothalamic-pituitary-adrenal
HRQoL	Helath-related quality of life
hTERT	telomerase reverse transcriptase
IFG-1	insulin-like growth factor type 1
IL-6	interleukin-6
IQR	interquartile range
KB	kilobase

LDL	low-density lipoprotein
LV	left ventricle
NCEP	National Cholesterol Educational Program
NS	non significant
OGTT	oral glucose tolerance test
PBMCs	peripheral blood mononuclear cells
RIA	radioimmunoassay
ROS	reactive oxygen species
SD	standard deviation
sTNF-R1	Plasma soluble TNFalfa-receptor 1
sTNF-R2	Plasma soluble TNFalfa-receptor 2
TC	total cholesterol
TERC	telomerase RNA component
TL	telomere length
TNFalfa	tumor necrosis factor-alpha
TRF	telomere restriction fragment
TSH	thyroid stimulating hormone
T2DM	type 2 diabetes mellitus

Introduction and background

Cushing's syndrome (definition, prevalence, etiology, clinical manifestations)

Cushing's syndrome (CS) is a chronic and systemic disease made up by the group of signs and symptoms due to excessive cortisol secretion. CS is a rare endocrine disease with an incidence ranging from 0.7 to 2.4 cases/million inhabitants per year (1), which would mean around 14 new cases of CS per year in Catalunya. Endogenous CS is more common in women than men.

This chronic exposure to endogenous hypercortisolism may be caused more frequently (70% of the cases) by a pituitary adrenocorticotropin (ACTH)-producing adenoma (causing Cushing's disease, CD). Adrenocortical tumors constitute around 20% of the cases (mostly benign adenomas, less frequently bilateral macronodular hyperplasia or an adrenal carcinoma) and more rarely CS can be due to an ectopic source of ACTH secretion or corticotropin releasing hormone (CRH) production (<10% of the cases) (2). Nevertheless, the most common cause of CS is the use of supraphysiological amounts of exogenous glucocorticoids (GC) for different inflammatory or autoimmune diseases (iatrogenic CS).

CS symptoms and signs are very prevalent in general population and not sufficiently specific; none of them is exclusive of hypercortisolism, making it difficult to diagnose CS. A few signs can more reliably help to distinguish CS from obesity or diabetes, due to protein wasting such as the presence of thin skin, easy bruising, proximal weakness, muscle atrophy, and decreased linear growth and obesity in children(1). When clinical presentation is florid, diagnosis is usually straightforward. However, mostly CS symptoms and signs are non-specific, so unfortunately CS is often diagnosed very late, months or years after the initiation of clinical manifestations(2). This remarkable delay in diagnosis, which implies excessive cortisol exposure for a long time, has implications in the persistence of morbidities and outcomes of CS in spite of being biochemically cured.

Chronic cortisol hypersecretion causes central adiposity, arterial hypertension, insulin resistance, dyslipidemia, impaired glucose tolerance or diabetes mellitus and also increased coagulability risk, manifestations which are part of the metabolic syndrome in patients

with CS(3,4). These abnormalities determine an increased cardiovascular risk (CVR) not only during the active phase of the disease but also long-term after biochemical remission. Some clinical features of CS are resistant to change after correction of cortisol excess.

Persistence of comorbidities after remission of CS (Review II, Annex 2)

Over the last decades, diagnosis and treatment of CS have made big progress, surgical procedures have improved and new medical treatments have become available. Despite these advances CS is a dreadful disease causing many co-morbidities and premature death if untreated. Additionally, in spite of biochemical "cure" of hypercortisolism and clinical improvement after effective treatment, many of these complications are only partially reversible and may not be associated with a full clinical recovery at short-, and long-term follow-up. The main comorbidities and clinical manifestations after remission of CS, are summarized in the following diagram (*Figure 1)* (3):

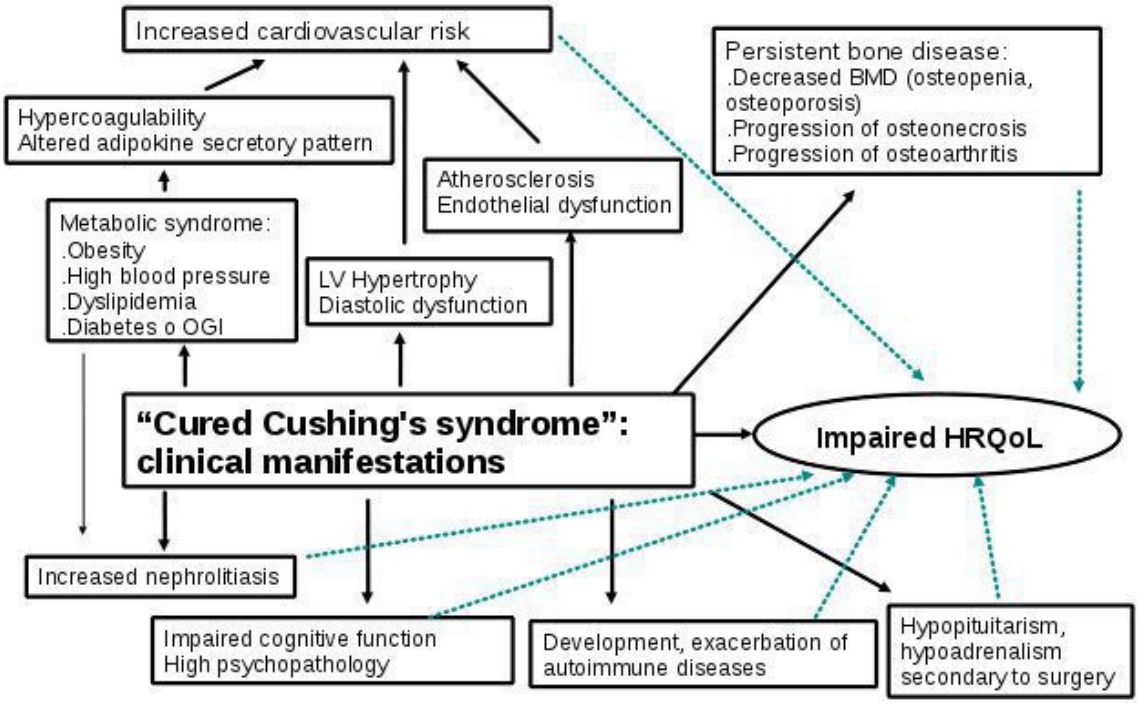


Figure 1: Main comorbidities and clinical manifestations after remission of CS.

*Abbreviations: BMD: bone mineral density, HRQoL: Health-related quality of life, LV: left ventricle, OGI: oral glucose intolerance.

Maintenance of visceral obesity and altered adipokine secretory patterns which perpetuate features of the metabolic syndrome (impaired glucose tolerance, dyslipidemia, atherosclerosis and hypercoagulability) contribute to the persistence of elevated CVR and deterioration of the cardiovascular system; in fact, cardiovascular disease is the most important cause of mortality in CS patients (5,6). CD patients present higher mortality rates than patients with nonfunctioning pituitary macroadenomas, suggesting that hypercortisolism and the length of exposure to hypercortisolism is associated with increased mortality (7). Persistence of increased central adiposity may link metabolic alterations and cardiovascular morbidity in CS patients, as adipose tissue secrete several adipokines which are involved in a wide variety of physiological or pathological processes (immunity, inflammation, endothelial damage, atherosclerosis, insulin signaling, bone remodeling...) (8,9). Elevated levels of pro-inflammatory molecules such as tumor necrosis factor-alpha (TNFalfa) and interleukin-6 (IL-6) and lower levels of adiponectin have been described in CS patients who had been surgically cured of hypercortisolism for an average of 11 years (10). This unbalanced concentrations of adipokines contribute to the persistent "low grade" inflammation state observed in CS patients.

Although significant decreases in blood pressure are observed in the postoperative period, there is still evidence of hypertension (around 20% of patients), associated with duration of hypertension during the active phase of hypercortisolism (11). An echocardiography study observed that hypertensive patients with CS presented more structural and functional cardiac abnormalities than normotensive patients and hypertensive controls (12).

Recovery of bone mineral density (BMD) seems to be only partial in "cured" CS patients (13). Previous exposure to high amounts of cortisol levels may be particularly detrimental in young patients who have not completed skeletal growth (14,15).

Moreover, previous exposure to chronic hypercortisolism may have irreversible effects on the structures of the central nervous system controlling cognitive function and mood (16,17). Hippocampus, amygdala, cerebral cortex are rich in GC receptors and they are particularly vulnerable to GC excess present in CS (18–20). A decrease in hippocampus and cerebellar cortex volum, as well as, an impairment of memory, visual and spatial information, reasoning, verbal learning, decision-making skills and language performance

have been described in “cured” CS (21–25). Increased prevalence of silent nephrolitiasis (around 30%) compared to general population have also been described in “cured” CS patients (26).

All these co-morbidities have a negative impact on quality of life and mortality. Worse health-related quality of life has been described in biochemically cured CS patients associated with physical and psychosocial impairments, especially in the presence of hypopituitarism, even long-term after control of hypercortisolism (27–29).

The exact mechanisms by which these abnormalities do not recover completely appear to be complex and are not currently well understood. Prognosis and comorbidities of patients treated for CS are described in more extensive detail in **Review II.**

Telomeres: physiology and biology

Telomeres (from the Greek *telos* [end] and *meros* [part]) are noncoding repetitive DNA sequences (from 4-15 Kb), composed of multiple repetitions of a guanine-rich sequence (TTAGGG), located at the end of linear chromosomes, and protecting them from erosion and end-to-end chromosome fusion (30). Their critical role in maintaining chromosomal stability was first described in the 1930s by McClintock (31) and Muller (32). In seventies the unusual nature of telomeres, with their simple repeated DNA sequences composing chromosome ends was discovered by Elisabeth Blackburn (33). Several years later, telomere-shortening was recognized as a mechanism that normally limits cells to a fixed number of divisions suggesting that this is reponsible for ageing at the cellular level and sets a limit on lifespans. The Nobel Prize in Physiology and Medicine in 2009 was awarded to Elizabeth Blackburn, Carol Greider and Jack Szostak for discovering the molecular structure of telomeres (enzyme telomerase complex), and how these protect chromosomes from degradation.

Telomeres are composed of thousands of tandem DNA repeat sequences: hexameric TTAGGG in the leading strand and CCCTAA in the lagging strand. It is now well

established that these sequences are covered by a protein complex called Shelterin, which stabilizes and protects them. Telomere structure are reflected in *Figure* 2:

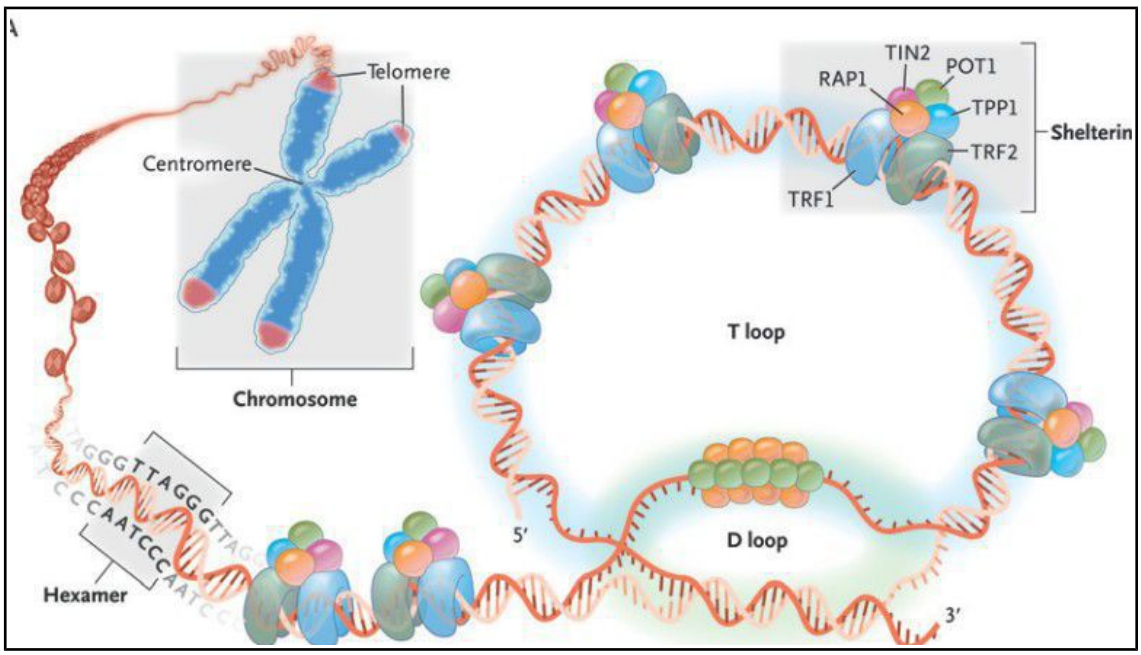


Figure 2: Telomere structure, located at the end of linear chromosomes, composed of hundreds of hexameric sequences TTAGGG. Protective proteins associated with telomere DNA are termed shelterin. Adapted from Calado et al. 2009(30)

Without telomeres, genetic material would be lost after every cell division; thus, when telomeres are critically short, cell division stops and senescence and apoptosis are induced (30). Their discoveries stimulated research in a new exciting field aiming to explore the role of telomeres in normal ageing, cancer and age-related diseases. The impact of their work from the early 1970s up to now is reflected by the increasing rate of publications in the field of telomeres over time. The evolution of indexed publications in Pubmed on "telomere" over the last decades (1980-2014) are reflected in the following graph (*Figure 3*):

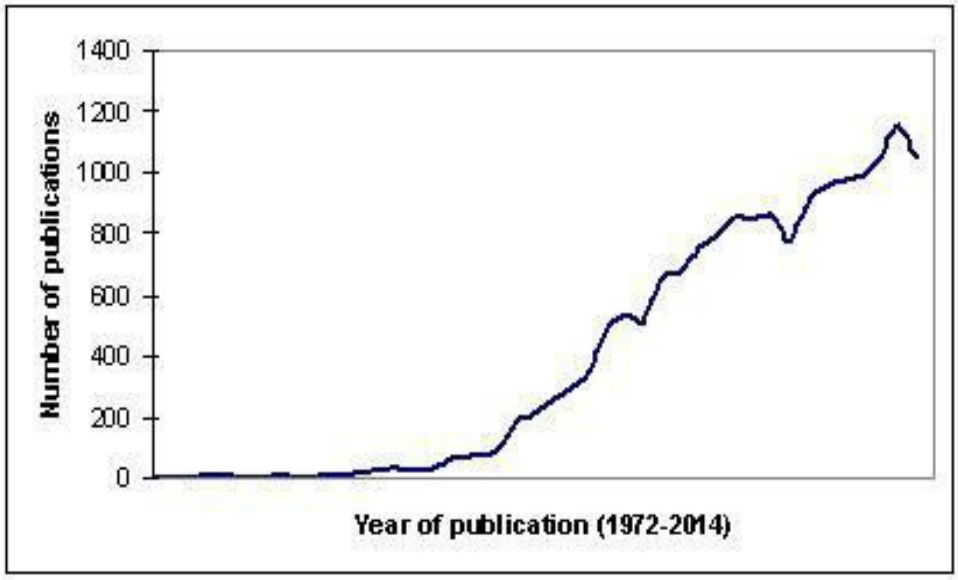


Figure 3: The evolution of indexed publications in Pubmed on "telomere" over the last decades.

To avoid telomere length (TL) attrition and to maintain TL, germ-line cells and a few somatic cells produce telomerase. Telomerase is a specific enzymatic complex involved in telomere repair and elongation. It catalyses telomeric DNA synthesis to reduce chromosomal end degradation after terminal DNA replication and thus maintains TL (34). Telomerase consists of several components (35) (*Figure 4*):

.Catalytic component with telomerase reverse transcriptase activity (hTERT)

.Telomerase RNA component (TERC) is used by hTERT as a template to synthesize telomere DNA

.Dyskerin complex stabilizes the whole telomerase machinery.

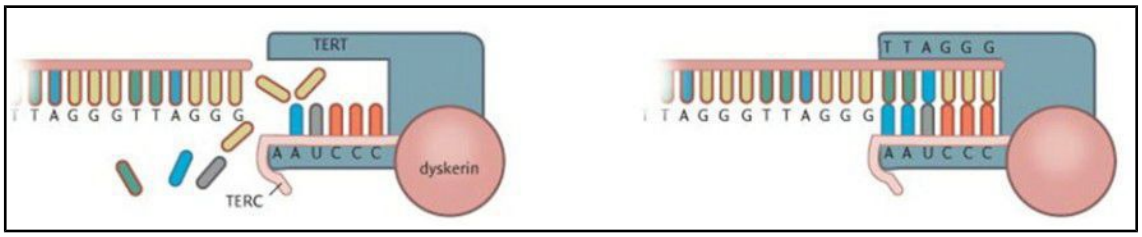


Figure 4: Schematic overview of telomerase complex, composed by two RNA complexes (TERC) and telomere reverse transcriptase (TERC) stabilized by dyskerin. Adaptated from Zhu et al.2011(36)

TL typically decreases with ageing, but the shortening rate is not uniform for all kind of tissues and cells; for example, brain cells and cardiomyocytes show little attrition (36–38). Undifferentiated stem cells have longer TL, while in more differentiated cells, TL are shorter. Even in “nondividing” cells, telomeres can be shortened by oxidative stress, which preferentially damages guanine-rich sequences as telomeres, to a greater extent than nontelomeric DNA (39,40).

Moreover, TL is considerably heterogeneous, even in the same cell and for individuals of similar age. Recent studies revealed that TL changes could be dependent on baseline TL (newborns). In early life, inheritance seems to be and important point, being one of the main determinants of TL. However, the inherited impact decreases with increasing age, due to the effect of environmental factors on TL (41,42). Genetic, epigenetic and environmental factors can regulate telomerase function. These include socio-economic status, lifestyle, autoimmunity, histone methylation and acetylation, stress level, drugs (angiotensine-converting enzyme inhibitors and resveratrol), personal habits (smoking, diet, physical exercise), growth factors and stress hormones (**cortisol**, catecholamines and sex hormones), which can influence and modulate telomerase dynamics and activity (43–51). Processes known to modulate telomerase dynamics and to affect TL either by shortening or lengthening are summarized in *Figure 5* (52).

Moreover, telomere biology can be involved in the pathophysiology of several clinical entities such as cancer (53–55), premalignant lesions (56), aplastic anaemia (57), fibrosis of the lungs and liver (58), dyskeratosis congenita (59), ageing (60) and as a risk factor for cardiovascular disease (poor lipid profile, high systolic blood pressure, fasting glucose, smoking, greater abdominal adiposity) (61–68).

Whether a common molecular mechanism is causally involved in the development of these human conditions requires further research with prospective, longitudinal and interventional studies. Nevertheless, measuring TL may contribute to the understanding of its clinical and biological significance, as it can be used as an indicator of chromosome stability, telomerase activity, proliferative capacity and cellular ageing.

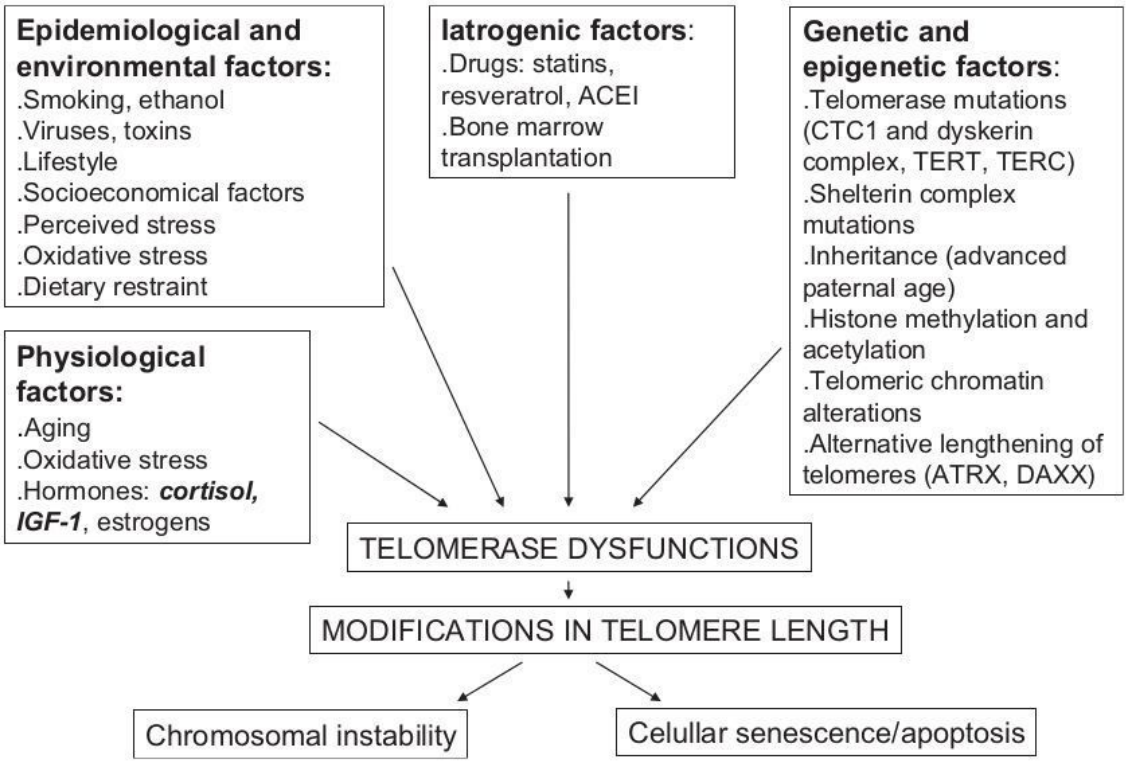


Figure 5: Processes known to modulate telomerase dynamics and to affect telomere length.

*Abbreviations: CTC1: conserved telomere maintenance complex component 1; IFG-1: insulin-like growth factor type 1; TERT: telomerase reverse transcriptase; TERC: telomerase RNA component; ATRX: ATP-dependent helicase; DAXX: death associated protein 6; ACEI: angiotensin-converting enzyme inhibitors

Hypothalamic-pituitary-adrenal (HPA) axis and the telomere system (Review I-III, Annex II)

Some endocrine diseases like adrenal and GH dysfunction are associated with ageing-like processes and increased cardiovascular risk, but the underlying mechanisms are complex and not always clear.

GCs cause different levels of oxidative stress in different tissues, with the brain being the most susceptible to damage (69). Chronic psychological stress is perceived by the cortex of the brain, inducing secretion of CRH, leading to increases in ACTH and cortisol levels (70). This dysregulation of HPA axis leads to cortisol-induced changes such as reduced availability of intracellular glucose energy store, neurotoxic effects (mainly in prefrontal

cortex and hippocampus), excitotoxicity, neuroinflammation and accelerated cell ageing via effects on the telomere/telomerase maintenance systems (71,72). Possible relationships and mechanisms which relate hypercortisolism and celular ageing are reflected in the following diagram (*Figure 6*) (73):

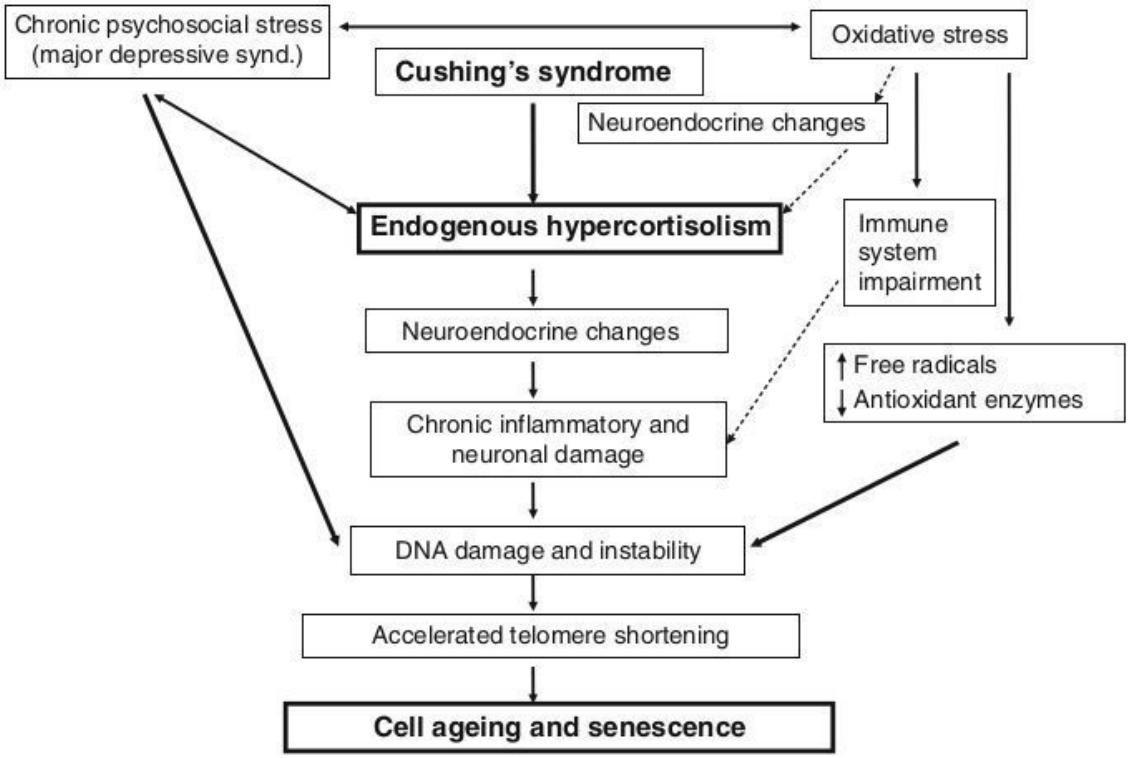


Figure 6: Possible relationships and mechanisms which relate hypercortisolism and celular ageing.

In fact, in patients with major depression, hippocampal atrophy is often reported, similarly to the lesions observed in patients with CS, suggesting a possible "pro-ageing" effect of GCs in certain cells of the body (18,22,72,74).

Given the role of telomeres in some of these mechanisms, we decided to review what evidence there was to associate the telomere system with endocrine dysfunctions of the HPA axis(52) (**Review III, Annex II)**

To our knowledge, telomere dysfunction has not been described in endogenous hypercortisolism, nor in the more common situation of exogenous hypercortisolism after

GC therapy. In contrast, it has been evaluated in different psychiatric diseases, like acute and chronic stress and post-traumatic stress disorder, where hypercortisolism is often present, representing another model of endogenous hypercortisolism (75,76). These situations are associated with poor health indexes, and TL has been found to be shorter than that in matched controls (42). In this line, accelerated telomere shortening has been related to higher levels of urinary catecholamines, and free urinary cortisol in situations with high perceived psychological stress (in sisters of patients with breast cancer, in acute mental stress) (50). Similarly, shorter buccal cell TL in children was observed in 6-year-old children exposed to laboratory stressors, with higher levels of salivary cortisol and higher autonomic reactivity (77).

Greater cortisol responses and dysregulated patterns of daily cortisol secretion were associated with shorter telomeres in peripheral blood mononuclear cells (PBMCs) in 14 postmenopausal women caring for a partner with dementia, compared with age- and body mass index (BMI)-matched noncaregivers (78). Consistent with these observations, significant reductions of telomerase activity in T lymphocytes after exposure to high hydrocortisone levels comparable with those that might be reached in vivo during stress, have been observed in in vitro studies (by as much as 50% 3 days later) (79). A rapid and dynamic loss of telomeric sequences in dexamethasone-treated mice thymocytes has also been observed(80). Recently, a longitudinal study evaluating the association between coexisting changes in cortisol and telomerase activity in PBMCs has been published. The authors examined whether participation in a mindfulness-based interventions and improvements in psychological distress, eating behavior and metabolic factors were associated with increases in telomerase activity in PBMCs. They observed that changes in chronic stress, anxiety, dietary restraint, cortisol and glucose 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 (81).

The research linking chronic stress, telomere system and HPA function is sometimes contradictory(82), and we should be careful in reaching conclusions about telomere dynamics due to the complexity of neuropsychiatric conditions (concurrent changes in stress hormones, neurotransmitters, autonomic activity, cytokines, inflammation, oxidative

factors...) which can influence final TL. Studies examining the relationship between TL and the HPA axis are summarized in Table 1 of **Review III** (52).

Diagnosis or type of stress	References	Total *n*	Findings
Perceived stress (Breast cancer sisters)	36	647	Accelerated telomere shortening with higher levels of urinary catecholamines and urinary free cortisol
Laboratory stressors	37	78	Shorter buccal cell TL in children with higher levels of salivary cortisol and higher autonomic reactivity
High caregiver stress (dementia caregiver)	38	14	Shorter telomeres in PBMCs were associated with greater cortisol responses and dysregulated patterns of daily cortisol secretion
	39	22	Telomerase activity increased during acute stress associated with greater salivary cortisol increases in response to stressor
Acute mental stress	48	62	Lower leucocyte telomerase activity was associated with exaggerated autonomic reactivity and with increased excretion of stress hormones (catecholamines and cortisol) in response to acute mental stress
Dietary restraint	40	56	Premature telomere shortening was observed in women with dietary restraints linked to greater perceived stress and elevated salivary and urinary cortisol
High hydrocortisone levels *in vitro*	45		50% reduction of telomerase activity of T lymphocytes was observed with exposure to high hydrocortisone levels *in vitro*
Embryonic exposure to corticosterone	47	60 (eggs)	Shorter telomeres were observed with exposure to exogenous corticosterone during embryonic development (domestic chickens)
Mindfulness-based intervention for stress eating	49	47	Changes in telomerase activity were negatively associated with changes in serum morning cortisol levels (after intervention)
Dexamethasone administration in mice thymocytes	49		Rapid and dynamic loss of telomeric sequences in dexamethasone-treated thymocytes
Major Depressive Disorder and hypocortisolism	52	91	Shorter TL was associated with depression and hypocortisolaemic state (low post-DST cortisol and high percentage of cortisol reduction after the DST)

Table 1: Studies examining the relationship between telomere length and the hypothalamic-pituitary-adrenal axis. Reference's numbers are those referred in bibliography of Review III (Annex II)(52)

*Abbreviations: DST: dexamethasone; PBMCs: peripheral blood mononuclear cells.

Cardiovascular risk, inflammation markers and telomere length

Premature cell senescence and oxidative stress are both cause and consequence of several CVR factors and their complications. In humans it is widely accepted that TL is affected by oxidative stress and might be considered a novel marker of cardiovascular risk and cardiovascular ageing (62,83,84) .

An association between TL shortening and age-related human disorders, like type 2 diabetes mellitus (T2DM), poor lipid profile, high blood pressure have been reported (65,67,68,85). Recently, in a longitudinal evaluation in a large group of patients, shorter baseline TL was significantly associated with unfavorable scores of most metabolic syndrome components at the 6-year follow-up (86), suggesting that cellular aging might

play a role in the onset of various ageing-related somatic diseases inducing metabolic alterations.

On the other hand, CS increases CVR factors, including impaired glucose tolerance, atherosclerosis, hypertension, dyslipidemia, hypercoagulability, obesity, increase visceral adiposity and insulin resistance (3,4). This increased visceral adiposity is associated with altered production of adipocytokines, which determines a "low grade" inflammatory state, promoting a cascade of metabolic aberrations leading to permanent cardiovascular risk (10). Low levels of adiponectin in CS, and increased release of pro-inflammatory adipocytokines and inflammatory markers, like soluble tumor necrosis factor-α receptors (sTNF-R1, sTNF-R2), interleukin-6 (IL6) and C-reactive protein (CRP) also confer an inflammatory state and increased morbidity and mortality observed in CS (8,10).

Increased inflammation biomarkers, such as C-reactive protein (CRP) and IL6, and oxidative stress in different study populations (boilermakers exposed to heat and fumes, adolescents, healthy older adults) have also been associated with short telomeres (87–89). In the same line, in a large population study evaluating dysregulated physiological stress systems and cellular ageing, the authors observed that a pro-inflammatory state (increased levels of CRP, IL6) together with hyperactivation of the HPA axis and a high heart rate were associated with shorter TL (90). Moreover, increased circulating inflammation markers and adipocytokines have been related to leukocyte turnover stimulation and increased reactive oxygen species (ROS), causing cell damage and telomere attrition (61). These data might indicate that dysregulation of physiological stress and inflammatory systems are intertwined with cellular ageing processes and may influence each other bidirectionally. In fact, oxidative stress, inflammation and increased cell turnover associated with CVR factors are major determinants of accelerated telomere shortening.

Although several studies have shown significant association between shorter TL and altered metabolic syndrome components or impaired adipocytokine profile, some studies did not confirm this. Most of the research has often been performed with small samples, limited age range, or some did not adjust for important confounding factors (lifestyle and clinical factors); therefore, it is largely unknown how the telomere system and its maintenance might influence on somatic disease processes.

Most studies on TL and inflammation markers have been performed in healthy subjects, T2DM, cardiovascular disease or psychiatric conditions. However, no study has reported data on TL in CS related to metabolic or inflammatory state.

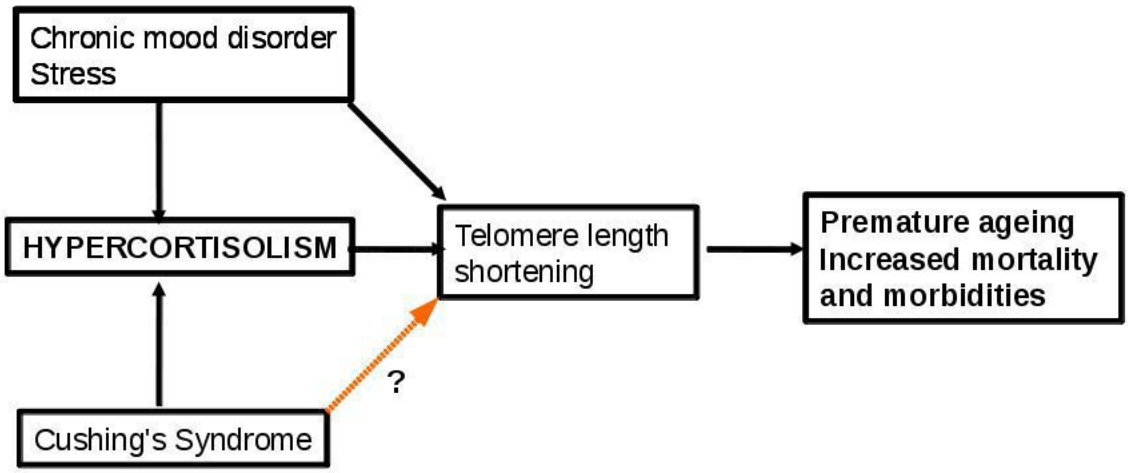
Hypothesis

Stimulation of the HPA axis occurs in different clinical situations. Hypercortisolism is often present in chronic psychosocial stress and psychiatric conditions which are related with impaired health status and increased cellular damge. This conditions have been associated to telomere system dysfunctions, inducing TL shortening. On the other hand, increased CVR and increased inflammatory state have also been associated with premature ageing and TL shortening. TL is considered to be an indicator of chromosome stability, proliferative capacity, and cellular ageing; therefore, measuring TL could contribute to the understanding of clinical and biological processes in patients with endogenous hypercortisolism.

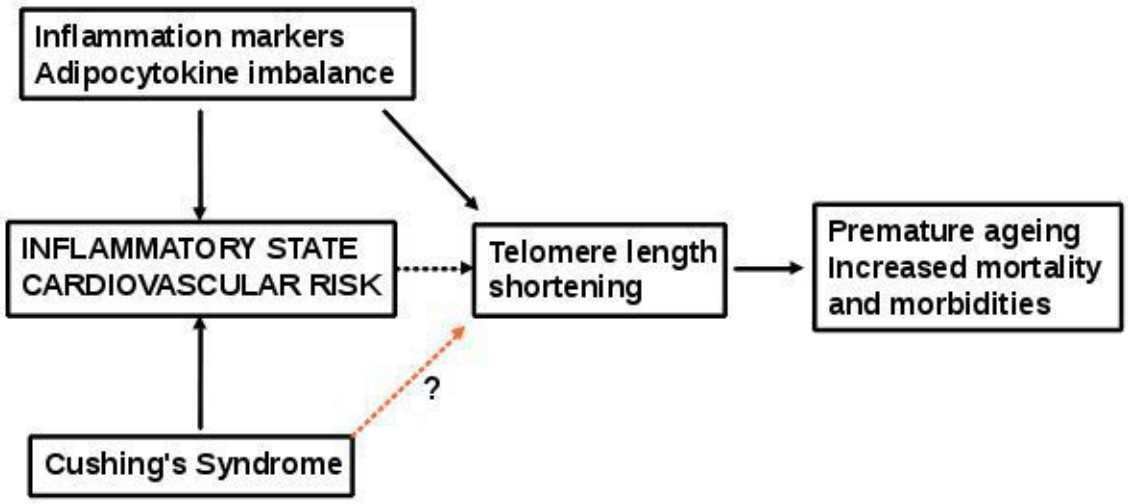
CS (a paradigm of chronic endogenous hypercortisolism) determines increased morbidity and features of premature ageing. Furthermore, an imbalance of adipocytokine production, with increased inflammation markers and increased CVR have been found in CS compared to matched controls, even after biochemical remission of hypercortisolism.

These evidences led us to hypothesize:

1. Telomere length 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.



2. Telomere length shortening might be involved in this “low grade” inflammatory state and higher prevalence of CVR factors observed in CS, even when hypercortisolism is biochemically cured. Therefore, it could be possible that the increased CVR and increased inflammatory state present in CS, mediate this increased morbidity through TL shortening.



In other words, it could be possible that hypercortisolemia contributes to premature ageing by inducing accelerated TL shortening, which in turn would determine the persistent morbidity and clinical consequences associated with CS, even years after being biochemically cured of hypercortisolism.

To the best of our knowledge, telomere dysfunction has not been evaluated in CS patients before.

Objectives

1. To investigate TL in patients diagnosed with CS, compared with sex-, age-, and smoking-matched healthy controls **(Paper I)**

2. To evaluate whether normalization of the HPA axis after treatment reverses possible abnormalities **(Paper I)**

3. To evaluate the relationship between TL and CVR factors in patients with CS **(Paper II)**.

4. To evaluate the relationship between TL and inflammation markers in patients with CS **(Paper II)**.

5. To investigate the major determinants of TL in patients with CS **(Papers I and II)**.

Material and methods

Subjects included:

Patients

Papers I and II: Seventy-seven patients with endogenous CS followed in our institution since 1982 were eligible. Patients with adrenal carcinoma were excluded. Fourteen were men (18%) and 63 women (82%). Mean age at the time of the study was 48.6±12.8 years. Fifty-nine patients were of pituitary origin (77%), 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%) had active disease at the time of the study and 56 (73%) were cured; median time of remission of hypercortisolism was 3.6 years (IQR 11.6). Eight active CS patients (38%) were treated with metyrapone, 6 (29%) with ketoconazole and 3 (14%) with both drugs. Median duration of hypercortisolism was 62 months (IQR 70.5). 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). Median period between onset of symptoms and biochemical diagnosis of CS was 24 months (IQR 37). Twenty-two patients (29%) had received pituitary radiotherapy and 71 (92%) had undergone surgery. Fifty-three % (n=41) were cured after initial treatment and had no recurrence and 19% (n=15) were cured after further therapies for recurrent disease. Fifteen cured patients (19%) 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 (12%) patients were GH-deficient (4 of which were replaced with recombinant human GH); 8 women (10%) were gonadotropin-deficient (all on estrogen/progesterone hormone replacement therapy), and 15 patients (19%) 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.

Paper I:

Longitudinal evaluation: 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 (27%) 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 (47%) were postmenopausal at remission but no gonadotropin-deficiency was observed (n=8).

Control subjects:

Papers I and II: 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 (91). Females usually present longer TL than males, since estrogens stimulate telomerase activity and protect DNA from ROS-induced damage (92). Cigarette smoke constituents increase cumulative and systemic oxidative stress and inflammation, which induce increased white blood cell turnover, resulting in accelerated TL shortening (93). Four controls (6%) were on antihypertensive therapy, another 4 (6%) were receiving statin treatment for dyslipidemia, and 3 (4%) were treated with calcium and vitamin-D.

2) Exclusion criteria

Adrenal carcinomas were excluded due to their worse prognosis.

Medical history and physical examination excluded any control subject who reported glucocorticoid exposure, severe and/or acute diseases and severe psychiatric alterations (however, anxiety and mild depression were not exclusion criteria).

All participants provided a blood sample for DNA extraction, fasting blood measurements and gave written informed consent to the study. The study was approved by the Comité Ético de Investigación Clínica of the Hospital de la Santa Creu i Sant Pau (Code: 10/031/1070).

Methods

Design:

Paper I: cross-sectional case-control study and a longitudinal evaluation.

Paper II: cross-sectional case-control study.

Clinical variables collected and definition (**Papers I and II**):

Anthropometry (weight, height, body mass index and waist/hip ratio) was measured in patients and controls. Obesity was defined as BMI ≥ 30 kg/m2. Increased abdominal circumference was defined as >102 centimeters (cm) in men and >88 cm in women. 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 (5.8 mmol/l), low-density lipoprotein (LDL) >130 mg/dl (3.4 mmol/l), triglyceride levels ≥150 mg/dl (1.7 mmol/l) or treatment with lipid-lowering medication. Diabetes mellitus was confirmed with fasting glucose levels >126 mg/dL (7 mmol/l) in two consecutive determinations or a 2-hour glucose after an oral glucose tolerance test (OGTT) >200 mg/dL (11.1 mmol/l). Adult patients were considered osteopenic when T score was <-1 and >-2.5 or osteoporotic when T score was <-2.5 SD. The presence of metabolic syndrome was defined by the criteria of the National Cholesterol Educational Program (NCEP) Adult Treatment Panel III (ATPIII) (94), as modified by the American Heart Association/National Heart, Lung and Blood Institute

(95). Alcohol consumption was divided into non/mild (intake <110 gr/week for women, <170 gr/week for men), or moderate/severe (intake >110 gr/week for women, >170 gr/week for men).

Genomic DNA extraction from total leukocytes(**Papers I and II)**

DNA extraction from leukocytes was carried out using an adapted *Proteinase K and Phenol protocol (96)*. 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 blood 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(**Papers I and II)**:

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 Hinfl 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 (97). The accuracy of Southern Blot technique is

up to ± 300 base pairs (bp) (34). 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 (*Figure 7*). The mean TL for Hela cells was 4113 bp with a standard deviation of ±210bp, which is in the range of the accuracy of Southern Blot technique.

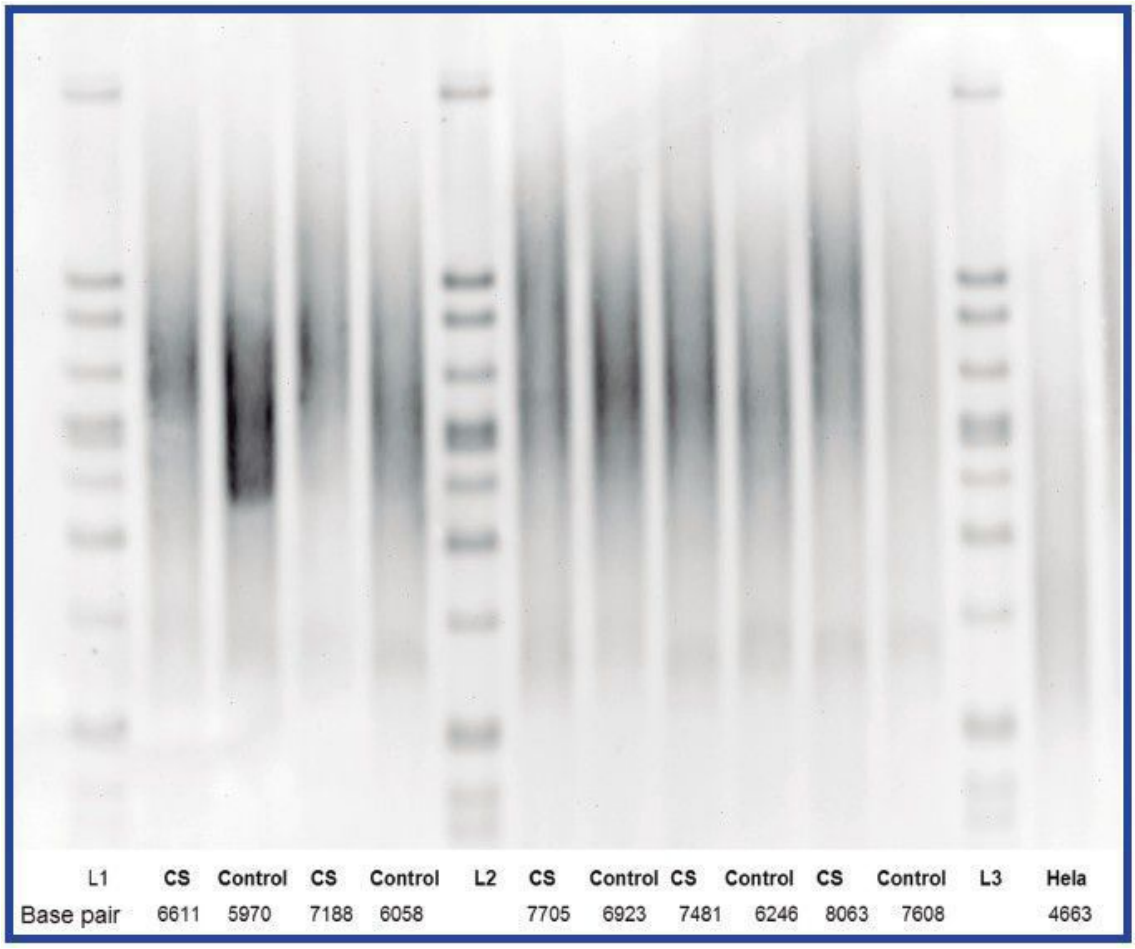


Figure 7: An example of Southern blot results of telomere length measurement of CS patients and their matched controls. *Abreviations: CS: Cushing's syndrome, L1,L2,L3: ladders

Paper II: Using the same films as in mean TRF analysis, the proportion of short telomeres (<5 kilobase (kb)) were calculated in each sample. Total chemiluminiscence intensity of each sample and that below molecular size marker 5 kb were measured. Background was fixed as the signal at the nadir of the low molecular weight region.

Biochemistry and hormone analyses

Papers I and II: Fasting samples for routine determinations by standard automated laboratory methods were obtained for glucose, TC, high-density lipoprotein (HDL) and LDL cholesterol and triglycerides. Blood counts were performed using automated cell counters. Twenty-four-hour urinary free cortisol was measured with a commercial radioimmunoassay (RIA) (Coat-A- Count Cortisol, Siemens) with prior extraction with an organic solvent; intra and interassay coefficients of variation (CV) were 5.1 and 6.4% respectively. Plasma ACTH was measured by chemiluminiscent immunometric assay (Immulite 2000®, Siemens Healthcare Diagnostics Products Ltd., Llanberis, UK; intra and interassay CV of 9.5 and 10%). Serum cortisol was measured by electro-chemiluminescent immunoassay (Modular Analytics E170 ®, Roche Diagnostics GmbH, Mannheim, Germany; intra and interassay CV of 1.7 and 2.8%).

Paper II: Adiponectin was determined by enzyme-linked immunosorbent assay (ELISA) (EZHADP-61K; PromoCell GmbH, Heidelberg, Germany; intra and interassay CV of 3.4 and 5.7%). Serum IL-6 was determined by high sensitivity ELISA (Bender MedSystems GmbH, Vienna, Austria; intra and interassay CV of 6.9% and 8%). Plasma soluble TNFalfa-receptor 1 (sTNF-R1) and sTNF-R2 were evaluated by solid phase Enzyme Amplified Sensitivity Immunoassays (Biosource Europe S.A., Fleunes, Belgium; intra and interassay CV <8%). Serum CRP was measured by immunoturbidimetric assay (Modular DPE®, Roche Diagnostics GmbH, Mannheim, Germany; intra and interassay CV of 2.76 and 4.61%).

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. Statistical significance was accepted at $p < 0.05$.

Paper I: 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.

Paper II: ANCOVA was performed to evaluate TL after adjustment for age (as covariate). Multiple linear regression analysis (stepwise) including variables correlated with TL in a univariate analysis and other clinically relevant as potential predictive factors for TL (dependent variables) was performed.

Results

1) Comparison between CS and matched controls (**Paper I**)

Main baseline characteristics of CS patients and controls are summarized in *Table 1* (*Annex 1*). As expected for the initial matching, no differences in age, gender and smoking habit were observed among CS and controls. CS patients had more hypertension, diabetes, dyslipidemia, osteoporosis and psychiatric history than their matched controls ($p<0.05$).

No differences were observed between males and females (7732±1242 vs. 7540±1361 base pairs (bp), respectively). TL did not differ between CS and controls (7667±1260 vs. 7483±1214, respectively). TL did not differ between active CS, cured CS (with or without secondary adrenal insufficiency) and their matched controls (*Figure 1, Annex 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, Annex 1*). 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, no differences in TL according to the use or not of steroidogenesis inhibitors were observed (metyrapone 8258±1178 vs ketoconazole 7896±1432, NS).

2) Longitudinal analysis in CS patients evaluated both during active disease and in remission (**Paper I**)

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, Annex 1*), but was minimal in 2 and of doubtful relevance, since it was around

the detection limit of the Southern blot technique, of 300 bp (around 4%) TL's variation in our population(38). 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, Annex 1*), in sharp contrast with TL shortening usually observed as age increases. No significant differences in the presence of hypertension, dyslipidemia, T2DM 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 ($R = 0.494$, $p=0.061$).

3) TL according to the presence of individual CVR factors in patients with CS (**Paper II**)

Clinical and biochemical characteristics of the subjects included in the study are shown in *Table 2 (Annex 1)*. TL declined with age as expected ($r= -0.400$, $p<0.001$). Mean TL was strongly correlated with the proportion of short telomeres (<5 kb) ($r= -0.917$, $p < 0.001$) (*Figure 4, Annex 1*). No differences in TL were observed related to disease activity nor was there any correlation between duration of hypercortisolism and TL ($r= -0.082$, NS).

Mean TL after adjustment for age depending on the presence or absence of CVRF were analyzed *(Figure 5, Annex 1)*. CS with dyslipidemia had shorter TL than those without (7328±1274 vs 7957±1137, p 0.024). Dyslipidemic CS also had a higher proportion of short telomeres (<5 kb) compared to non-dyslipidemic CS patients (31.7±2.2 vs 24.8±2.03 %, p 0.029). Patients with CS plus obesity or hypertension or metabolic syndrome showed shorter TL than those without, although these differences lost statistical significance after adjusting for age.

When cured and active CS were evaluated separately, those with dyslipidemia, independently of being active or in remission of hypercortisolism, presented shorter TL and a higher proportion of short telomeres than those without dyslipidemia *(Figure 6, Annex 1)*. When clinical characteristics (age, gender, smoking, hypertension, T2DM, activity of disease, cardiovascular disease, obesity, menopausal status) between

dyslipidemic and non-dyslipidemic CS patients were compared, to explain shorter TL in dyslipidemic CS patients, the former were older (dyslipidemic 53±11.7 years vs non dyslipidemic 45±12.7 years, $p < 0.05$) and more frequently obese (49% vs. 34% in non-dyslipidemic CS patients, $p < 0.05$); these differences persisted after adjustment for BMI and age (7313±1210 vs 7873±1182 bp, $p < 0.05$). We did not observe differences in TL in patients taking or not statin therapy. No differences in other CVR factors (hypertension, T2DM, metabolic syndrome, obesity, smoking) according to activity of the disease were observed.

Twenty-one CS presented both dyslipidemia and hypertension; after adjustment for age and BMI, TL was shorter compared to CS patients without dyslipidemia and/or hypertension (7132±1041 bp vs 7868±1191 bp, respectively, $p < 0.05$). Fifteen CS patients presented with three CRV factors (dyslipidemia, hypertension and obesity); TL was shorter compared to those without three concomitant CVR factors (6956±1280 vs 7860±1180, respectively, $p < 0.001$) *(Figure 7, Annex 1)*. TL did not differ related to disease activity. No differences in TL according to the presence or absence of T2DM, smoking habit and increased abdominal circumference were observed. No differences between TL according to the presence or absence of CVR factors (dyslipidemia, hypertension and metabolic syndrome) after adjustment for age and BMI were observed in the control group.

Correlations between TL and dyslipidemic-related parameters *(Table 3, Annex 1)* in 60 patients not treated with statins showed a negative correlation of total cholesterol and triglycerides with TL (r-0.279 and r–0.259, respectively, $p<0.05$). No correlations were found with HDL (r-0.236), or with LDL (r-0.05). In 17 dyslipidemic CS patients on statin therapy, no correlations were found with any lipid parameter.

4) Correlations between TL, adipocytokines and inflammation markers: (**Paper II**)

In 32 CS (25 cured, 7 with active disease), evaluation of adipocytokines and inflammation markers was possible (*Table 2, Annex 1*).

A negative correlation between CRP and TL was observed (r -0.412, $p = 0.019$) *(Figure 8, Annex 1).* Also, a negative correlation between IL6 and TL was found (r -0.441, $p = 0.016$). No other significant correlations were observed between other adipocytokines and TL

(adiponectin r 0.131, sTNF-R1 r -0.186 and TNF-R2 r -0.128, NS). The proportion of short telomeres also correlated positively with CRP (r 0.437, p 0.012) and IL6 (r 0.328, p 0.036), but not with adiponectin, sTNF-R1 or sTNF-R2.

5) Predictors of TL in CS patients (**Papers I and II**)

Paper I: In the multiple linear regression analysis performed to identify potential predictive factors of TL in the study population, 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 4, Annex 1*), 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).

Paper II: A multiple linear regression analysis to evaluate determinants of TL in CS included age, gender, T2DM, hypertension, dyslipidemia, smoking, obesity, duration of hypercortisolism and disease activity in the model, to find predictors of TL. Age (ß -32, t -3.01, p = 0.004) and dyslipidemia (ß -310, t -2.10, p = 0.030) were the only negative independent predictors of TL (R^2 0.21).

Discussion

Previous evidences have demonstrated that:

-CS determines increased morbidity and mortality, even after therapy compared to background population (summarized in Review I) and is associated with premature aging processes (summarized in Review II)

-Hyperstimulation of the HPA axis due to psychosocial stress has been related to telomere length shortening (explained in more detail in Review III)

-Telomere lenght shortening has also been associated with cardiovascular disease, CVR factors and chronic inflammation processes

These previous evidences took us to hypothesize that 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. Additionaly, telomere length shortening might be involved in the “low grade” inflammatory state and higher prevalence of CVR factors observed in CS, even when hypercortisolism is biochemically cured.

Therefore, we designed this research to answer our hypothesis, and results are reflected in Papers I and II. To the best of our knowledge, this is the first research to evaluate TL in this rare disease and with a relatively large series of CS patients, which could provide a unique opportunity to examine the effects of hypercortisolism on telomere maintenance.

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 (1). They were carefully matched for age, gender and smoking status with controls.

Comparison between CS and matched controls (Paper I)

CS determines increased morbidity and mortality, especially in the untreated state but also after therapy (3,4).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 (98). 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 (50,99) .

Against our initial hypothesis, 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. The relatively small group and subgroups of patients 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) (30). Additionally, TL may be affected by what is known as a "pseudolengthening" mechanism; specifically, TL of lymphocytes becomes increasingly shorter than those of granuylocytes over the years (100). And since a redistribution of leukocyte cell types is often seen in hypercortisolism (lymphopenia and neutrophilia) this may also affect the measured TL obtained from the total leukocyte count (101). 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 (101). However, after adjustment for total leukocyte count (as a covariate) no differences in TL between CS and their healthy controls were identified.

Longitudinal analysis in CS patients evaluated both during active disease and in remission (Paper I)

To further investigate TL system in endogenous hypercortisolism we evaluated whether normalization of the HPA axis after treatment could reverse possible abnormalities and we planned a longitudinal study. When investigated longitudinally, our preliminary data show

that patients with active CS have a shorter TL, which becomes longer after hypercortisolism disappeared with effective treatment. Fifteen patients were included in the longitudinal study, they were evaluated twice, during hypercortisolism and again after remission, adjusting for age (as a covariate). We could not include the remaining 6 active patients (21 active patients in the initial evaluations), because 4 of them still presented active disease and 2 patients were lost to follow up. Our initial hypothesis was confirmed, 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 requires caution before reaching 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 (50). Similarly, shorter buccal cell TL was observed in children exposed to laboratory stressors with higher levels of salivary cortisol and higher autonomic reactivity (77). 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 (78). 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 (79). 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 probable that when cortisol normalizes, a recovery of telomerase activity takes place, increasing TL or lowering attrition rates.

Contrary to these evidences and to our results, a recent publication showed telomere shortening associated with hypocortisolism 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 (102). 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 to that seen in a recent longitudinal intervention study with Mediterranean diet, where BMI was inversely correlated with changes in TL (103).

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)(100). 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.

Although the results of the longitudinal evaluation are the opposite to that expected by increasing age and therefore is an interesting result, this finding is certainly preliminary based on a small group of patients. A larger group of patients, as well as large groups of patients followed longitudinally would clearly strengthen the conclusion of our findings.

Relationship between TL and CVR factors in patients with CS (Paper II)

Our approach was to investigate the relationship between TL and classical cardiovascular risk factors, since our hypothesis was that TL shortening might be involved in the higher prevalence of CVR factors in CS, even when hypercortisolism is biochemically cured. The

main finding is the negative impact on TL maintenance of dyslipidemia, further worsened if hypertension and or obesity coexisted.

Dyslipidemia after adjusting for age and disease activity was the main factor negatively related to telomere lengthening in CS, even after controlling for other clinical and metabolic confounders. Other individual CVR factors (hypertension, smoking, T2DM, obesity) were not correlated with TL. CS patients with dyslipidemia had shorter TL in all stages (active, in remission or adrenal insufficient after surgery) compared to CS without dyslipidemia. Differences in TL between dyslipidemic and non-dyslipidemic patients persisted after adjustment for BMI (greater in dyslipidemic patients), as suggested by some authors (64,103). However, TL shortening was not associated with dyslipidemia in the control group, probably due to the low prevalence of dyslipidemia observed in controls (n=15), which reduced statistical power and prevented firm conclusions in this group of healthy controls. Additional TL shortening was found in patients with both dyslipidemia and hypertension. Not surprisingly TL was even shorter when these patients were also obese, since excessive adiposity results in a metabolic imbalance, with an increased inflammatory state and oxidative stress, phenomena associated to accelerated telomere shortening (61,64).

Available literature on the relation between TL, lipids and other CVR factors is often discordant. Our findings, namely a negative correlation of total cholesterol and triglycerides and TL are consistent with several but not all previous studies *(Table 5, Annex 1)*. Similar to our findings, in a healthy young population at low cardiovascular risk, an inverse correlation between triglycerides and TL was observed (104,105). In T2DM patients, an association between shorter TL and oxidative stress was reported (65), as well as an inverse correlation between TL and total cholesterol, LDL-cholesterol, BMI, triglycerides and CRP (61,85). However, other studies observed no relation between TL and CVR factors in a population without cardiovascular disease (105,106). Similar findings were reported in obese children (107), stable coronary artery disease (108) or myocardial infarction (109).

We were unable to demonstrate effects of CS activity (active or cured hypercortisolism), hypopituitarism or hydrocortisone replacement on TL. Since statin therapy prevents TL

erosion of endothelial progenitor cells in healthy subjects (109), cholesterol lowering medications may preserve or even elongate TL; since our study was not longitudinal, this preservation effect of statins on TL could not be evaluated.

Although the mechanisms involved are unclear, we propose the following hypothesis. Elevated cholesterol and triglycerides are atherogenic, determining repeated mechanical, hemodynamic, and/or immunological injury, increasing cell turnover and production of ROS in certain cells (as in subclinical chronic inflammation) (110). The link between cholesterol and TL may be through this increased cell damage and turnover, leading cells to their maximum replicative capacity and translating into shortened TL and cell ageing (61). Unfavourable lipid phenotypes would then determine increased oxidative stress, accelerated senescence and cell aging, which in turn could explain our finding in CS.

Most studies only report mean TL. Increasing evidence suggests that regardless of mean TL, the presence of a few critically short telomeres may cause a cell to enter senescence (111,112). Therefore, we measured the proportion of short telomeres, as it may provide additional information, since short telomeres may be crucial for cellular senescence. CS patients with dyslipidemia exhibited a higher proportion of short telomeres. We observed a strong negative correlation between mean TL and proportion of short telomeres; consequently, measurement of the proportion of short telomeres has not provided much extra information on that provided by TL alone. Nevertheless, it has been useful to confirm our results and to reinforce our conclusions and findings with mean TL measurement. Whether short TL imply a higher risk of dyslipidemia or if dyslipidemia hastens shortening of telomeres is currently unknown.

Relationship between TL and adipocytokines and inflammation markers in patients with CS (Paper II)

Our approach was to investigate the relationship between TL and inflammation markers in CS patients, since we hypothesized that TL shortening might be involved in the “low grade” inflammatory state observed in CS patients, even after remission of hypercortisolism. We observed a negative impact of inflammation markers (CRP and IL-6)

on TL maintenance. Elevations of CRP and IL6, after adjusting for age and disease activity, were the inflammation markers negatively related to telomere lengthening in CS.

Similar findings were reported in 2500 healthy Caucasians supporting that chronic systemic inflammation promotes both atherogenesis and telomere attrition (105). Also in 36 healthy women where optimism and pessimism were evaluated, a strong negative correlation between TL and IL6 was observed in the pessimist state (113). Another recent study showed that adipocytes under oxidative stress had shortened telomeres, increased mRNA protein expression of IL6 and sTNF, with decreased expression of adiponectin (114). Recently a large-scale study with almost 3000 adult subjects showed that increased inflammation markers (IL6, CRP and TNFalfa) together with higher awakening cortisol response were associated with shorter leukocyte TL, especially when these dysregulation stress systems were cumulative (90). Adiponectin has anti-atherogenic and anti-inflammatory properties, protective against metabolic phenomena known to accelerate ageing. Glucocorticoids inhibit adiponectin secretion (8); thus, as expected, lower adiponectin was observed in CS compared to matched controls (10). Interestingly, a correlation has been observed between telomere shortening and hypoadiponectinemia in obesity (115), and we also found a trend, which was not statistically significant, probably due to the limited sample size.

Predictors and major determinants of TL (Papers I and II)

In the multiple regression analysis including all the subjects of the study, leukocytes count together with age and the presence of dyslipidemia were predictive factors for TL, explained 23 percent of the TL present in our study population (**Paper I**). When we evaluate predictors of TL including classical CVR factors in CS population, again age and the presence of dyslipidemia were negative independent predictors of TL, explaining 21 percent of the TL present in our CS patients (**Paper II**).

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 populations (91,116). 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)(116).

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 (83,99), although not all results published in the literature are concordant (117). 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 cannot 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.

Study limitations

This research 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, individual variability of possible drug effects on telomere attrition, etc.
- 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. It would be interesting to evaluate TL in other tissues such as the pituitary or the adrenal, even in vascular cells or adipocytes in CS; however, this would be even more difficult than obtaining peripheral leukocytes for TL evaluation. We cannot ensure that our findings are reproducible in cells of the cardiovascular system, because glucocorticoids induce changes in the immune system. Nevertheless, although there is ongoing debate about the comparability of TL in different subtypes of leukocytes, mean leukocyte TL seems to correlate strongly with other body tissues according to recent literature (118).
- We could not measure telomerase activity, which probably could provide a more direct approach on both the telomere system and its dynamics.
- Due to its cross-sectional nature causality cannot be inferred, limiting conclusions on the potential relationship between TL and dyslipidemia or inflammatory markers. Even though most cross-sectional studies on telomere biology and ageing are much larger, large-scale, longitudinal, prospective and well-designed studies in general population are still lacking, so that the influence of different physiological states on TL still have to be elucidated.

Final conclusions

- Patients with Cushing's syndrome did not differ in TL when compared to healthy controls.

- In the cross-sectional study of CS and controls, no difference in TL was found; however, in the longitudinal evaluation, patients with active CS had shorter TL than those with biochemical control of hypercortisolim, despite being on average 3 years older.

- CS patients with dyslipidemia had shorter TL than both controls and CS patients without dyslipidemia

- In CS, TL is shortened in those with dyslipidemia, independently of disease activity; further shortening of TL was observed if obesity and/or hypertension coexisted.

- In CS, TL is negatively correlated with increased inflammation markers (CRP and IL6).

- Increased lipids and “low” grade of inflammation may contribute to TL shortening and consequently to premature ageing and increased morbidity in CS.

- These preliminary results suggest that hypercortisolism, increased lipids and “low” grade of inflammation might negatively impact telomere maintenance, contributing to TL shortening, a feature of premature ageing, leading to increased morbidity, circumstances which are often present in CS patients.

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Publications

Paper I:

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 Jul;171(1):21-9. doi: 10.1530/EJE-14-0098.

Paper II:

Aulinas A, Ramírez MJ, Barahona MJ, Valassi E, Resmini E, Mato E, Santos A, Crespo I, Bell O, Surrallés J, Webb SM. **Dyslipidemia and chronic inflammation markers are correlated with telomere length shortening in Cushing's syndrome**. PLoS ONE. 2015; 10(3): e0120185. doi:10.137/journal.pone.0120185.

Telomere length analysis in Cushing's syndrome

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Abstract

Introduction: Hypercortisolism in Cushing's syndrome (CS) is associated with increased morbidity and mortality. Hypercortisolism also occurs in chronic depressive disorders and stress, where telomere length (TL) is shorter than in controls. We hypothesized that shortening of telomere might occur in CS and contribute to premature aging and morbidity.
Aim: To investigate TL in CS patients compared with controls.
Methods: Seventy-seven CS patients (14 males, 59 pituitary, 17 adrenal, and one ectopic; 21 with active disease) were compared with 77 gender-, age-, and smoking-matched controls. Fifteen CS were evaluated longitudinally, during active disease and after remission of hypercortisolism. Leukocyte TL was measured by telomere restriction fragment–Southern technique. Clinical markers were included in a multiple linear regression analysis to investigate potential predictors of TL.
Results: Mean TL in CS patients and controls was similar (7667 vs 7483 bp, 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 was a positive predictor for TL ($P<0.05$). As expected, a negative correlation was found between TL and age (CS, $R=-0.400$ 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 hypercortisolism. These preliminary results suggest that hypercortisolism might negatively impact telomere maintenance. Larger studies are needed to confirm these findings.

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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), 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 (HPA) axis also resulting in hypercortisolism may also occur in psychiatric diseases such as acute and chronic stress and post-traumatic stress

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disorder (2, 3). These situations are associated with poor health indexes, and telomere length (TL) has been found to be shorter than that 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 in 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 to be 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 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 aging in patients with CS. Hypercortisolemia could contribute to premature aging 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. As TL is an indicator of chromosome stability, proliferative capacity, and cellular aging, 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 with 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, six (28.5%) with ketoconazole, and three (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 percent ($n=41$) 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 and range 10–20). Nine patients (11.7%) were GH deficient (four of which were replaced with recombinant human GH (rhGH)); eight women (10.4%) were gonadotropin deficient (all on estrogen/progesterone hormone replacement therapy); and 15 patients (19.4%) were hypothyroid, ten due to thyroid-stimulating hormone (TSH) deficiency and five due to primary

hypothyroidism (all on L-thyroxine (L-T_4) replacement). CS was considered in remission if either adrenal insufficiency was demonstrated (basal morning cortisol <100 nmol/l (<4 μg/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 carried out once they were in remission. In this longitudinal study, three 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±2.2 mg and range 10–20); four patients (26.6%) were hypothyroid, two due to TSH deficiency and two due to primary hypothyroidism (all on L-T_4 replacement). None of the cured patients were GH-deficient; seven 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, because estrogens stimulate telomerase activity and protect DNA from reactive oxygen species-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 four (5.7%) were receiving statin treatment for dyslipidemia, and three (4.3%) were treated with calcium and vitamin D.

Anthropometry (weight, height, BMI, 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, LDL >130 mg/dl, triglyceride levels ≥150 mg/dl, or treatment with lipid-lowering medication. Diabetes mellitus (DM) was confirmed with fasting glucose levels >126 mg/dl in two consecutive determinations or 2-h glucose after oral glucose tolerance test (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 S.D.

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 ▸ Genomic DNA extraction from total leukocytes was carried out 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 before processing. The white blood cell layers were harvested and digested with buffer containing 0.1 M $MgCl_2$, 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 passing through a blunt 20-gauge needle (0.9 mm diameter) at 4 °C 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 quantified by spectrophotometric analysis. The quality of genomic DNA was checked for high molecular weight by 1% agarose gel electrophoresis.

TL measurements ▸ TL measurements were carried out 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 RsaI and HinfI for 2 h at 37 °C. Samples were loaded on a 0.5% Seakem Gold Agarose gel and were run for 21 h at 35 V. The gels were treated with HCl, denaturalized and neutralized, and then transferred to a nylon membrane by capillarity for 12–18 h. After fixation with u.v., hybridization was carried out with a DIG-labeled telomeric probe (3 h at 42 °C). Finally, restriction washes, incubation with anti-DIG-AP antibody, and detection by chemiluminescence were carried out. Images were analysed with the program Quantity One. TRF mean was

calculated using the formula: TRF mean = ΣOD*i*/Σ(OD*i*/*Li*), where OD*i* is the chemiluminescent signal and *Li* is the length of the TRF fragment at position *i* (15). A control sample, 2 μg 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 4113 bp, with a S.D. of ±210 bp, which is in the acceptable range of accuracy of the Southern blot technique. The accuracy of southern blot technique is up to ±300 bp (16).

Biochemical, hormone, and bone analyses ▸ Routine serum determinations were carried out by standard automated laboratory methods: fasting glucose, TC, HDL and LDL cholesterol and triglyceride levels. Blood counts were made using automated cell counters. Twenty-four hours urinary free cortisol was measured with a commercial RIA with prior extraction with an organic solvent. Plasma ACTH, serum cortisol, and insulin-like growth factor 1 (IGF1) levels were measured using a commercial chemiluminescent immunometric assay. Lumbar spine and whole-body bone mineral density and bone mineral content were measured by DXA scanning (Delphi QDR 4500; Hologic); the mean precision error (coefficient of variation) was 1%.

Statistical analysis

Statistical analyses were carried out using the SPSS 19.0 Statistical Package for Windows (SPSS, Inc.). Initially a descriptive analysis of all variables was carried out in order to verify correct introduction of data in the database. Quantitative data are expressed as mean and S.D. (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 carried out where necessary to normalize the distribution of a particular measure. Comparison between two groups was made using Student's *t*-test (Gaussian distribution) or Mann–Whitney's *U* (non-Gaussian distribution)-test. A χ^2 test was performed for categorical variables. Fisher's exact test was performed when appropriate. Pearson's correlation coefficient was used to estimate linear association between two quantitative variables. Analysis of covariance was performed to evaluate TL after adjustment for age and for total leukocyte count (as covariates). Multiple linear regression analysis including age, gender, BMI, type 2 DM, dyslipidemia, hypertension, psychiatric history, duration of hypercortisolism, current hypercortisolism, total leukocytes, and 24-h 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

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 Fig. 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 (Fig. 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 ($R=-0.292$, $P<0.01$) (Fig. 2). A positive correlation was found between IGF1 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

Table 1 Baseline characteristics of patients with Cushing's syndrome (CS) and controls. Data are presented as percentage and mean±S.D.

	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
BMI (kg/m^2)	28±5.6	26.4±4.9	<0.05
Waist:hip ratio	0.92±0.07	0.85±0.07	<0.05
24-h Urinary free cortisol (nmol/24 h)	266±180	132±59	<0.001
Morning serum cortisol (nmol/l)	450±259	375±120	<0.05
Leukocytes (×10^9/l)	7.3±2.3	5.8±1.7	<0.05
Neutrophils (×10^9/l)	4.4±2.0	3.5±1.2	<0.05
Lymphocytes (×10^9/l)	2.1±0.8	1.9±0.4	NS

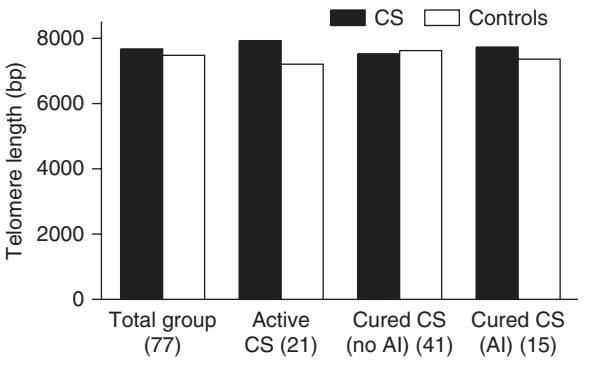

Figure 1

TL in the whole group of 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. CS, Cushing's syndrome; AI, adrenal insufficiency; TL, telomere length.

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-h 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 observe differences in TL depending on the use of steroidogenesis inhibiting drugs (treated with 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 five (33%) patients, TL decreased after remission (Fig. 3), but was minimal in two and of doubtful relevance, because it was around the detection limit of 300 bp (around 4%) TL's variation in our population (17). 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 (Fig. 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 have increased TL during remission and those who did not have increased TL. Patients who incremented TL also decreased their BMI more after remission than those who did not increase TL (−2.3 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

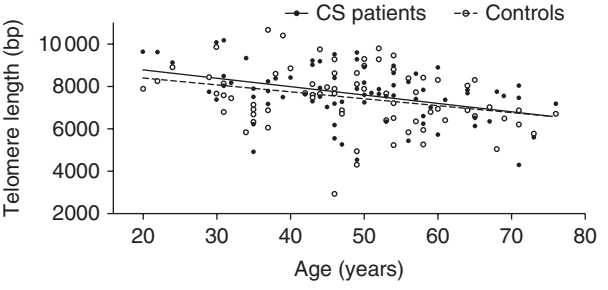

Figure 2

Telomere length in relation to age in patients with Cushing's syndrome (closed circle) and controls (open circle). Telomere length is shortened with advancing age in both CS ($R=-0.400$, $P<0.001$) and controls ($R=-0.292$, $P<0.01$).

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

	CS	Controls	*P*
Leukocytes in active disease ($\times 10^9$/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 ($\times 10^9$/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 ($\times 10^9$/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

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 with background population (1, 18). Severe morbidities are also increased even in the 3 years before diagnosis when compared with the normal population, and are not completely reversible after endocrine cure (18). 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 that 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 aging 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/million inhabitants per year (19). 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, socioeconomic status, lifestyle, growth factors, etc.) (5). In addition, TL may be affected by what is known as a 'pseudolengthening' mechanism (20); specifically, TL of lymphocytes becomes increasingly shorter than those of granulocytes over the years (17). And as 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 were lower than that observed 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 with 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 the predictive factors for TL, explaining 23% 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, in which 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-h urinary free cortisol, certain cardiovascular risk

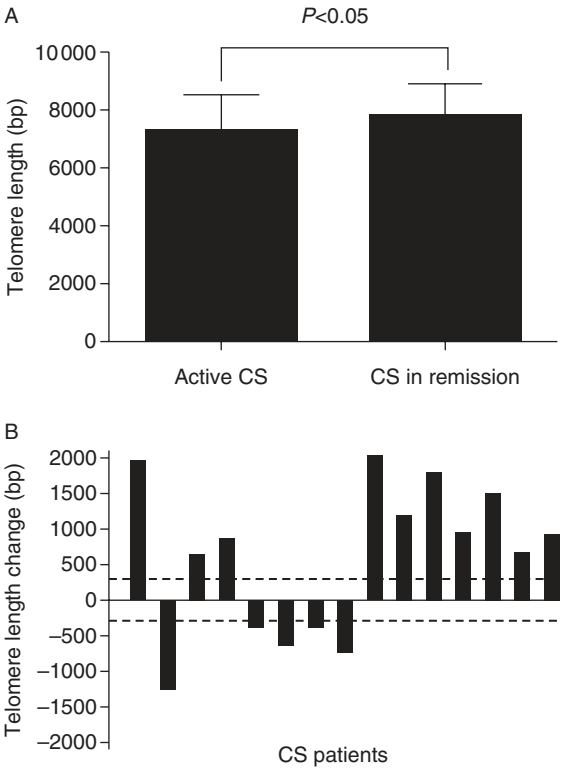

Figure 3

(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). (B) TL increased in 10/15 patients, with increasing age. The dotted line shows the detection limit of the Southern blot technique. CS, Cushing's syndrome.

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 they did not seem to have impact on TL in the case–control regression analysis, with the exception of dyslipidemia which negatively affected TL, we cannot 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, because 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 preliminary based on a small number of patients, and should be confirmed in the future in larger studies, 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 with matched noncaregiver controls (27). Consistent with this and with our longitudinal results, one *in vitro* study observed how exposure to high hydrocortisone levels, comparable with 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 that 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 hypercortisolemic 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 hypocortisolemic patients compared with cured patients without secondary adrenal insufficiency; an explanation could be that all adrenal-insufficient patients were correctly replaced with hydrocortisone.

Lifestyle modifications such as 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 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, similar to that seen in a recent longitudinal intervention study with Mediterranean diet, where BMI was inversely correlated with changes in 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 (20).

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 opposite to what is expected by increasing age, and is an interesting result, it is certainly preliminary based on a small group of patients. We could not include the remaining six active patients, because four of them still had active disease and two were lost to follow-up. A larger group of patients, as well as a longer longitudinal follow-up, would clearly strengthen the conclusion of these 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 aging 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, because glucocorticoids induce changes in the immune system; however, this would be even more difficult than obtaining peripheral leukocytes for TL evaluation. In addition, we could not measure telomerase activity (which required fresh processing), which would provide a more direct approach to both the 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 aging improve when disease is controlled. However, in the entire group of CS patients, no difference in TL was observed when compared with healthy controls, pointing to the fact that many other factors determine TL apart from age, including dyslipidemia, healthier lifestyles or differences in leukocyte subsets cell counts. Larger prospective studies are required to confirm these changes in TL in CS patients and to investigate the implications of these abnormalities further.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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RESEARCH ARTICLE

Dyslipidemia and Chronic Inflammation Markers Are Correlated with Telomere Length Shortening in Cushing's Syndrome

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Abstract

Introduction

Cushing's syndrome (CS) increases cardiovascular risk (CVR) and adipocytokine imbalance, associated with an increased inflammatory state. Telomere length (TL) shortening is a novel CVR marker, associated with inflammation biomarkers. We hypothesized that inflammatory state and higher CVR in CS might be related to TL shortening, as observed in premature aging.

Aim

To evaluate relationships between TL, CVR and inflammation markers in CS.

Methods

In a cross-sectional study, 77 patients with CS (14 males, 59 pituitary-, 17 adrenal- and 1 ectopic-origin; 21 active disease) and 77 age-, gender-, smoking-matched controls were included. Total white blood cell TL was measured by TRF-Southern technique. Clinical data and blood samples were collected (lipids, adrenal function, glucose). Adiponectin, interleukin-6 (IL6) and C-reactive protein (CRP) were available in a subgroup of patients (n=32). Correlations between TL and clinical features were examined and multiple linear regression analysis was performed to investigate potential predictors of TL.

Results

Dyslipidemic CS had shorter TL than non-dyslipidemic subjects (7328±1274 vs 7957±1137 bp, $p<0.05$). After adjustment for age and body mass index, cured and active CS dyslipidemic patients had shorter TL than non-dyslipidemic CS (cured: 7187±1309 vs 7868±1104;

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active: 7203±1262 vs 8615±1056, respectively, $p<0.05$). Total cholesterol and triglycerides negatively correlated with TL (r-0.279 and -0.259, respectively, $p<0.05$), as well as CRP and IL6 (r-0.412 and -0.441, respectively, $p<0.05$). No difference in TL according the presence of other individual CVR factors (hypertension, diabetes mellitus, obesity) were observed in CS or in the control group. Additional TL shortening was observed in dyslipidemic obese patients who were also hypertensive, compared to those with two or less CVR factors (6956±1280 vs 7860±1180, respectively, $p<0.001$). Age and dyslipidemia were independent negative predictors of TL.

Conclusion

TL is shortened in dyslipidemic CS patients, further worse if hypertension and/or obesity coexist and is negatively correlated with increased inflammation markers. Increased lipids and a "low" grade inflammation may contribute to TL shortening and consequently to premature ageing and increased morbidity in CS.

Introduction

Cushing's syndrome (CS) due to chronic exposure to endogenous hypercortisolism may be caused by a pituitary adenoma, an adrenocortical tumor or ectopic adrenocorticotropic hormone (ACTH) or corticotropin-releasing hormone (CRH) production [1]. Nevertheless, the most common cause of CS is the use of exogenous glucocorticoids. CS increases cardiovascular risk factors (CVRF), including impaired glucose tolerance, atherosclerosis, hypertension, dyslipidemia, hypercoagulability, obesity, increased visceral adiposity and insulin resistance [2]. This increased visceral adiposity is associated with altered production of adipocytokines, which determines a "low grade" inflammatory state, promoting a cascade of metabolic aberrations leading to permanent cardiovascular risk [3]. Low levels of adiponectin in CS, and increased release of pro-inflammatory adipocytokines and inflammatory markers, like soluble tumor necrosis factor-α receptors (sTNF-R1, sTNF-R2), interleukin-6 (IL6) and C-reactive protein (CRP) [3, 4] also confer an inflammatory state and increased morbidity and mortality observed in CS.

Telomeres are nucleoprotein structures at the end of eukaryotic chromosomes, made up of several thousand repetitive DNA sequences (TTAGGG) coated by capping proteins. They protect the genome from damage providing chromosome stability. Telomeres shorten with repeated cell division, and cells enter senescence followed by apoptosis when a critically short telomere length (TL) is reached [5]. As telomere shortening is approximately the same in different tissues, circulating leukocytes from blood cells are used as easily accessible surrogate tissue for TL assessment when analysing systemic effects of chronic diseases, like cardiovascular disease [6,7]. Even in "nondividing" cells, telomeres are shortened by oxidative stress, which preferentially damages guanine-rich sequences to a greater extent (as found in telomeres) than nontelomeric DNA. Increasing evidence suggests that one critically short telomere may cause a cell to enter senescence regardless of mean TL [8]. This supports that measurement of the proportion of short telomeres in an individual may provide additional information, since short telomeres may be crucial for cellular senescence.

Premature cell senescence and oxidative stress are both cause and consequence of several CVRF and their complications. In humans it is widely accepted that TL is affected by oxidative stress and considered a novel marker of cardiovascular risk [9,10]. An association between TL

shortening and age-related human disorders, like type 2 diabetes mellitus (T2DM), poor lipid profile and high blood pressure have been reported [11,12,13]. Also, short telomeres are associated with increased oxidative stress and inflammation biomarkers, such as CRP and IL6 [14]. Increased circulating inflammation markers and adipocytokines are related to leukocyte turnover stimulation and increased reactive oxygen species (ROS), causing cell damage and telomere attrition [15]. In fact, oxidative stress, inflammation and increased cell turnover associated with CRVF are major determinants of accelerated telomere shortening.

Thus, a major issue in telomere research is to understand what factors, in addition to age, influence TL, with its clinical and therapeutic implications. An imbalance of adipocytokine production and higher prevalence of CVRF have been reported in CS compared to controls [3,4]. TL shortening is also observed in inflammatory states and in cardiovascular disease. Based on these previous evidences which relate premature aging with TL shortening on the one hand, and increased cardiovascular risk and inflammatory state with TL shortening on the other hand, we devised our hypothesis. Since CS is also characterized by increased cardiovascular risk (hypertension, dyslipidemia, central obesity, diabetes. . .), and increased inflammatory state, we hypothesized that TL shortening may in part be behind and contribute to the increased morbidity and features of premature ageing observed in patients with CS. Therefore, we speculated that TL shortening might be involved in this "low grade" inflammatory state and higher prevalence of CVRF in CS, even when hypercortisolism is biochemically cured.

Most studies on TL have been performed in healthy subjects, T2DM, cardiovascular disease or psychiatric conditions. We recently reported no differences in TL in a cross-sectional comparison of CS and controls, but when patients with active CS were evaluated longitudinally after biochemical control, telomere lengthening was observed despite being on average 3 years older [16]. However, no study has reported data on TL in CS related to metabolic or inflammatory state. Thus, our aim was to evaluate the relationship between TL, CVRF and inflammation markers in patients with CS and investigate major determinants of TL.

Materials and Methods

Subjects

In this cross-sectional study, patients with CS followed since 1982 were eligible. Adrenal carcinomas were excluded. Seventy-seven CS patients and 77 controls, matched for gender, age and smoking participated. Fourteen were men (18%) and 63 women (82%). Mean age was 48.6 ±12.8 years. Fifty-nine patients were of pituitary origin (77%), 17 of adrenal origin (adenoma or bilateral macronodular hyperplasia) and in one the origin was unknown (ectopic ACTH secretion of unknown source). Twenty-one (27%) had active disease and 56 (73%) were cured (median time of remission was 3.6 years (IQR 11.6)). Eight with active CS (38%) were treated metyrapone, 6 (29%) with ketoconazole and 3 (14%) with both drugs. Median duration of hypercortisolism was 62 months (IQR 70.5). Since mortality and morbidity risk is increased in CS, even before diagnosis and treatment [17], duration of hypercortisolism was considered as the period between onset of symptoms (as referred by patients) and remission of hypercortisolism (in patients in remission) or the time of current analysis (in active patients). Median period between symptoms onset and biochemical diagnosis of CS was 24 months (IQR 37). Twenty-two patients (29%) had undergone pituitary radiotherapy and 71 (92%) surgery. Fifty-three % (n = 41) were cured after initial treatment without recurrence and 20% (n = 15) were cured after further therapies for recurrent disease. Fifteen cured patients (20%) were adrenal insufficient on substitution with hydrocortisone (mean dose 17.6±3.7 mg, range 10–20). Nine (12%) were GH-deficient (4 replaced with recombinant human GH); 8 women (10%) were gonadotropin-deficient (all on estrogen/progesterone hormone replacement), and 15 (19%) 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 (32%) were on antihypertensive medication, 17 (22%) on statin treatment for dyslipidemia, 12 (16%) were treated with calcium and vitamin-D and 7 (9%) for T2DM.

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, features known to affect TL. Glucocorticoid exposure, severe and/or acute diseases and severe psychiatric alterations were excluded (however, anxiety and mild depression were not exclusion criteria). Four (6%) were on antihypertensive therapy, 4 (6%) were receiving statin treatment, 3 (4%) were treated with calcium and vitamin-D and 1 (1%) with metformin.

Anthropometry (weight, height, body mass index and waist/hip ratio) was measured in all subjects. Obesity was defined as BMI $\geq$ 30 kg/m^2. Increased abdominal circumference was defined as >102 centimeters (cm) in men and >88 cm in women. 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) >5.8 mmol/l, low-density lipoprotein (LDL) >3.4 mmol/l, triglycerides $\geq$1.7 mmol/l or treatment with lipid-lowering medication. T2DM was confirmed by fasting glucose >126 mg/dL in two consecutive determinations or glucose 2-hour after an oral tolerance test >200 mg/dL. The presence of metabolic syndrome was defined by the criteria of the National Cholesterol Educational Program (NCEP) Adult Treatment Panel III (ATPIII) [18], as modified by the American Heart Association/National Heart, Lung and Blood Institute [19]. Alcohol consumption was divided into non/mild (intake <110 gr/week for women, <170 gr/week for men), or moderate/severe (intake >110 gr/week for women, >170 gr/week for men).

All participants gave written informed consent to the study, approved by the Comité Ético de Investigación Clínica of the Hospital de la Santa Creu i Sant Pau (Code: 10/031/1070), and provided a blood sample for DNA extraction and fasting blood measurements.

Methods

Genomic DNA extraction from total leukocytes was performed using an adapted Proteinase K and Phenol protocol [20]. Blood samples 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 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. Lysates were homogenized by passes through a blunt 20-gauge needle (0.9 mm diameter) at 4°C temperature and DNA was purified by phenol:chloroform:isoamilic alcohol (25:24:1) extraction, and ethanol precipitation. Genomic DNA was dissolved in Tris-EDTA buffer and 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 telomere restriction fragment assay (TRF) using the Telo TAGGG Telomere Length Assay Kit (Roche 12209136001); 1 μg DNA was digested with 20 units of Rsal and Hinfl for 2 h at 37°C. Samples were loaded on a 0.5% Seakem Gold Agarose gel and run for 21 h at 35 V. Gels were treated with HCl, denaturalized and neutralized, and transferred to a nylon membrane by capillarity for 12–18 h. After fixation with UV, hybridization was carried out with a DIG-labeled telomeric probe (3 h at 42°C). Restriction washes,

incubation with anti-DIG-AP antibody and detection by chemiluminiscence was carried out. Images were analyzed with the Quantity One program. TRF mean was calculated using the formula: TRF mean = $\Sigma OD_i/\Sigma(OD_i/L_i)$, where OD_i is the chemiluminiscent signal and L_i is the length of the TRF fragment at position i [21]. 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 4114 bp with a standard deviation of ±210 base pairs (bp), in the acceptable range of accuracy of the Southern Blot Technique (around 300 bp) [22]. Using the same films as in mean TRF analysis, the proportion of short telomeres (< 5 kb) were calculated in each sample. Total chemiluminiscence intensity of each sample and that below molecular size marker 5 kb were measured. Background was fixed as the signal at the nadir of the low molecular weight region.

Biochemistry and hormone analyses. Fasting samples for routine determinations by standard automated laboratory methods were obtained for glucose, total cholesterol, high (HDL) and LDL cholesterol and triglycerides. Blood counts were performed using automated cell counters. Twenty-four-hour urinary free cortisol was measured with a commercial RIA (Coat-A- Count Cortisol, Siemens) with prior extraction with an organic solvent; intra and interassay coefficients of variation (CV) were 5.1 and 6.4% respectively. Plasma ACTH was measured by chemiluminiscent immunometric assay (Immulite 2000, Siemens Healthcare Diagnostics Products Ltd., Llanberis, UK; intra and interassay CV of 9.5 and 10%). Serum cortisol was measured by electro-chemiluminescent immunoassay (Modular Analytics E170, Roche Diagnostics GmbH, Mannheim, Germany; intra and interassay CV of 1.7 and 2.8%). Adiponectin was determined by ELISA (EZHADP-61K; PromoCell GmbH, Heidelberg, Germany; intra and interassay CV of 3.4 and 5.7%). Serum IL-6 was determined by high sensitivity ELISA (Bender MedSystems GmbH, Vienna, Austria; intra and interassay CV of 6.9% and 8%). Plasma sTNF-R1 and sTNF-R2 were evaluated by solid phase Enzyme Amplified Sensitivity Immunoassays (Biosource Europe S.A., Fleunes, Belgium; intra and interassay CV <8%). Serum CRP was measured by immunoturbidimetric assay (Modular DPE, Roche Diagnostics GmbH, Mannheim, Germany; intra and interassay CV of 2.76 and 4.61%).

Statistical analysis

A descriptive analysis was performed to verify correct introduction of data in the database. Quantitative data are expressed as mean and SD (Gaussian distribution) or as median (p50) and interquartile range (IQR) (non-Gaussian distribution), and categorical data as percentages. Data distribution was analyzed by the Kolmogorov-Smirnov test. TL variable was normally distributed. Logarithmic transformations were performed where necessary to normalize distribution. 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 (as covariate).

Multivariate linear regression analysis (stepwise) including variables correlated with TL in a univariate analysis and others clinically relevant as potential predictive factors for TL (dependent variable) was performed.

Statistical analyses were performed using the SPSS 21.0 statistical package for Windows (SPSS Inc, Chicago Illinois). Statistical significance was accepted at $p<0.05$.

Results

Clinical and biochemical characteristics of the subjects included in the study are shown in Table 1. TL declined with age as expected (r = -0.400, p<0.001). Mean TL was strongly correlated with the proportion of short telomeres (<5 kb) (r = -0.917, p < 0.001) (Fig. 1). No differences in TL were observed related to disease activity nor was there any correlation between duration of hypercortisolism and TL (r = -0.082, NS).

Mean TL after adjustment for age depending on the presence or absence of CVRF were analyzed (Fig. 2). CS with dyslipidemia had shorter TL than those without (7328±1274 vs 7957±1137, p 0.024). Dyslipidemic CS also had a higher proportion of short telomeres (<5kb) compared to non-dyslipidemic CS patients (31.7±2.2 vs 24.8±2.03%, p 0.029). Patients with CS plus obesity or hypertension or metabolic syndrome showed shorter TL than those without, although these differences lost statistical significance after adjusting for age.

When cured and active CS were evaluated separately, those with dyslipidemia, independently of being active or in remission of hypercortisolism, presented shorter TL and a higher proportion of short telomeres than those without dyslipidemia (Fig. 3A-B). When clinical

Table 1. Clinical and biochemical characteristics of patients with Cushing's syndrome (CS) and controls.

	CS (n = 77)	Controls (n = 77)	p
Clinical characteristics			
Age (years)	48.6± 12.8	48.4± 12.6	NS
Smokers (%)	25%	19%	NS
Moderate alcohol consumption (%)	26%	27%	NS
Diabetes mellitus (%)	14%	1%	<0.05
Hypertension (%)	57%	13%	<0.001
Dyslipidemia (%)	46%	20%	<0.05
Osteoporosis (%)	30%	3%	<0.001
Psychiatric history (%)	38%	11%	<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
Metabolic syndrome n (%)*	40%	15%	<0.001
Lipid and metabolic profile**			
Triglycerides (mmol/liter)	1.2±0.6	1.09±0.7	0.089
Total cholesterol (mmol/liter)	5.4 ± 1.05	5.3±1.1	NS
HDL cholesterol (mmol/liter)	1.5±0.4	1.5±0.3	NS
LDL cholesterol (mmol/liter)	3.5±0.8	3.4±1.1	NS
Lpa (mg/liter)	410.7±451.1	264±310.8	0.06
Adipocytokines and inflammatory markers			
	CS (n = 32)	Controls (n = 32)	
Adiponectin (ng/ml)	14.6 ± 6.8	18.6 ± 10	0.053
IL6 (pg/ml)	1.18±2.1	0.37±0.33	<0.001
sTNF-R1 (ng/ml)	1.87±0.69	1.31±0.32	<0.001
sTNF-R2 (ng/ml)	3.71±2.08	3.09±0.91	NS
C-reactive protein (mcg/ml)	0.37±0.26	0.36±0.38	NS

Abbreviations: Lpa: lipoprotein a; sTNF-R1, sTNF-R2: soluble tumor necrosis factor-α receptors; IL6: interleukin-6.

*As described in references 16 and 17.

**49% of dyslipidemic CS patients and 26% of dyslipidemic controls were on lipid lowering medications.

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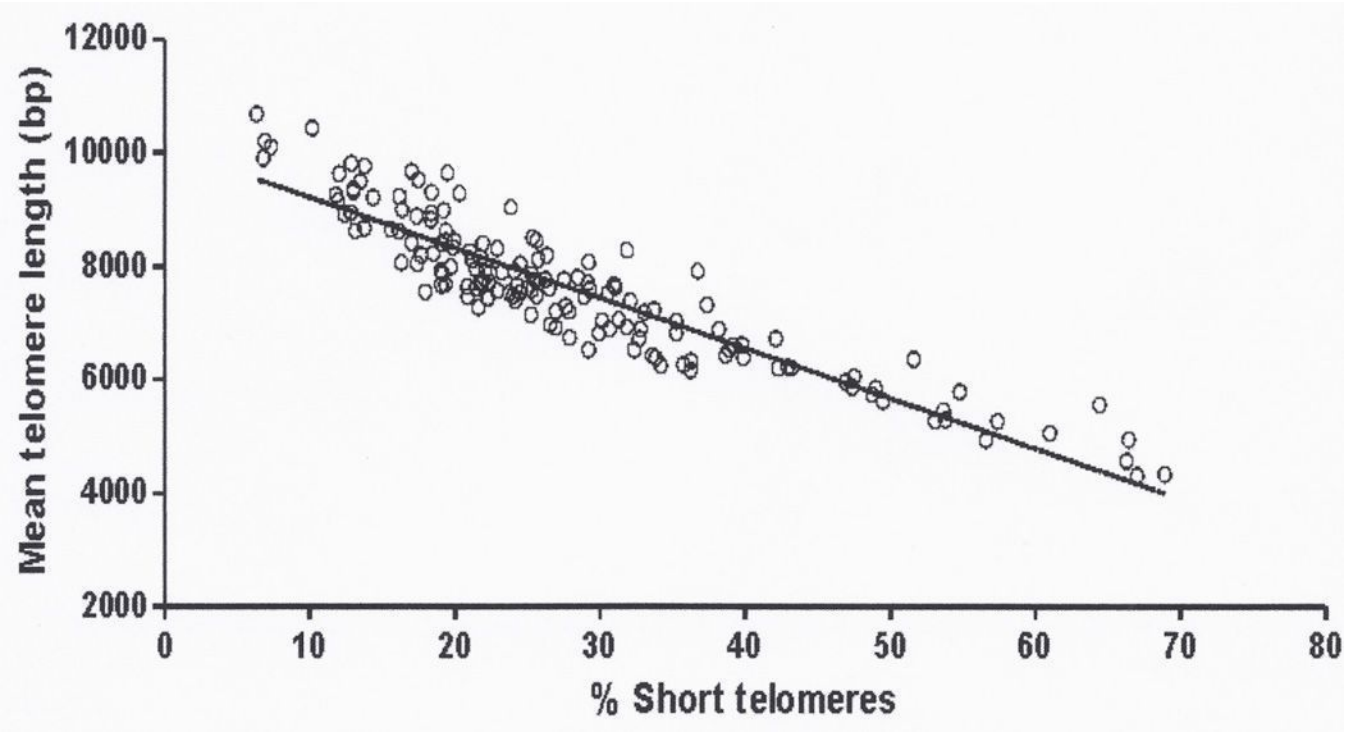

Fig 1. Correlation between mean telomere length and proportion of short telomeres (< 5kb) in the study population (r-0.917, p < 0.001).

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characteristics (age, gender, smoking, hypertension, diabetes, activity of disease, cardiovascular disease, obesity, menopausal status) between dyslipidemic and non-dyslipidemic CS patients were compared, to explain shorter TL in dyslipidemic CS patients, the former were older (dyslipidemic 53±11.7 years vs non dyslipidemic 45±12.7 years, $p < 0.05$) and more frequently obese (49% vs. 34% in non-dyslipidemic CS patients, $p < 0.05$); these differences persisted after adjustment for BMI and age (7313±1210 vs 7873±1182 bp, $p < 0.05$). We did not observe differences in TL in patients taking or not statin therapy. No differences in other CVRF according to activity of the disease were observed.

Twenty-one CS presented both dyslipidemia and hypertension; after adjustment for age and BMI TL was shorter compared to CS patients without dyslipidemia and/or hypertension (7132 ±1041 bp vs 7868±1191 bp, respectively, $p< 0.05$). Fifteen CS patients presented with three CRVF (dyslipidemia, hypertension and obesity); TL was shorter compared to those without three concomitant CVRF (6956±1280 vs 7860±1180, respectively, $p < 0.001$) (Fig. 4). TL did not differ related to disease activity. No differences in TL according to the presence or absence of T2DM, smoking habit and increased abdominal circumference were observed. No differences between TL according to the presence or absence of CVRF (dyslipidemia, hypertension and metabolic syndrome) after adjustment for age and BMI were observed in the control group.

Correlations between TL and dyslipidemic-related parameters (Table 2) in 60 patients not treated with statins showed a negative correlation of total cholesterol and triglycerides with TL (r-0.279 and r–0.259, respectively, $p<0.05$). No correlations were found with HDL (r-0.236), or with LDL (r-0.05). In 17 dyslipidemic CS patients on statin therapy, no correlations were found with any lipid parameter.

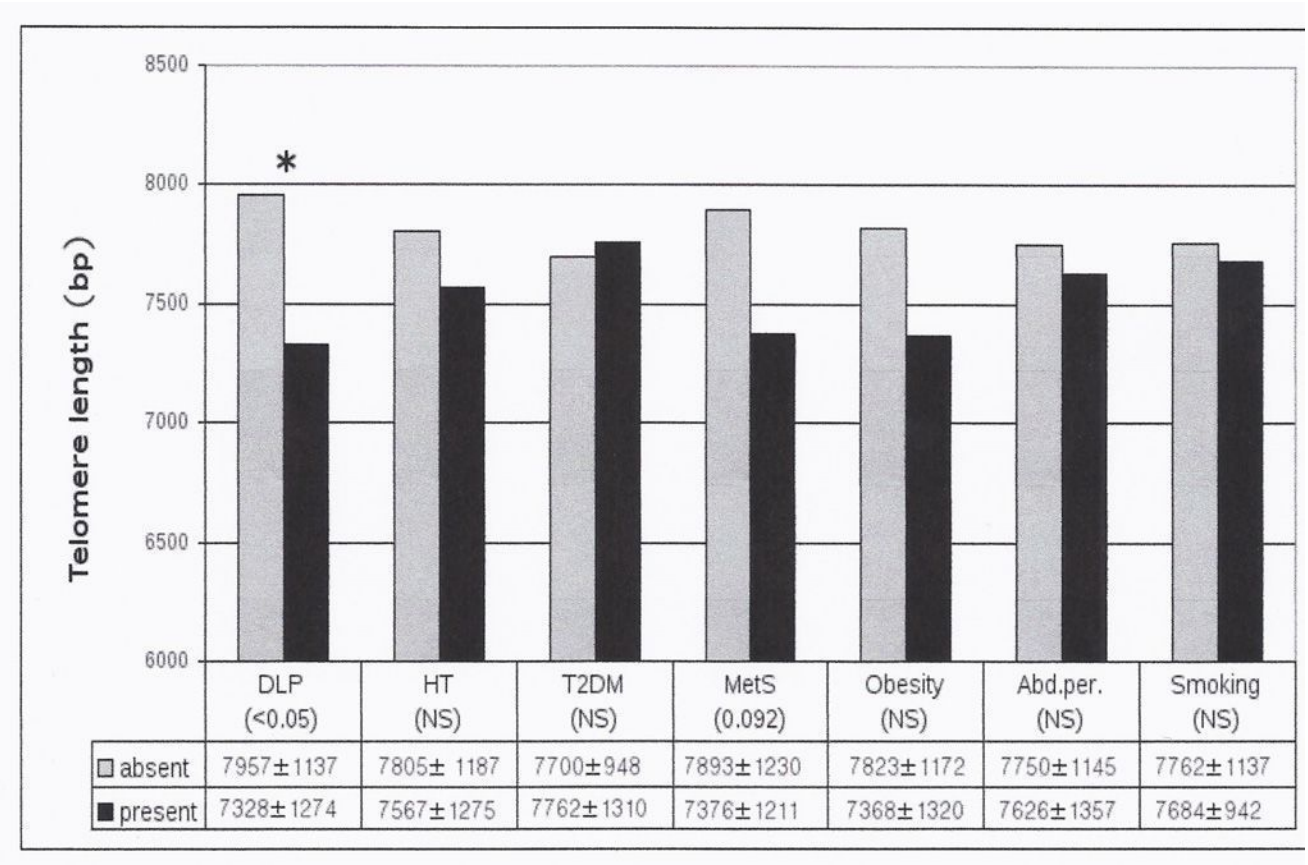

Fig 2. Mean telomere length according to different cardiovascular risk factors after adjustment for age in Cushing's syndrome patients. Abbreviations: bp, base pairs; DLP dyslipidemia, HT hypertension, T2DM Type 2 diabetes mellitus; MetS, metabolic syndrome; abd.per., increased abdominal perimeter.* $p<0.05$

doi:10.1371/journal.pone.0120185.g002

Correlations of TL, adipocytokines and inflammation markers

In 32 CS (25 cured, 7 with active disease), evaluation of adipocytokines and inflammation markers was possible (Table 1).

A negative correlation between CRP and TL was observed (r-0.412, p = 0.019) (Fig. 5). Also, a negative correlation between IL6 and TL was found (r-0.441, p = 0.016). No other significant correlations were observed between other adipocytokines and TL (adiponectin r 0.131, sTNF-R1 r-0.186 and TNF-R2 r-0.128, NS). The proportion of short telomeres also correlated positively with CRP (r 0.437, p 0.012) and IL6 (r 0.328, p 0.036), but not with adiponectin, sTNF-R1 or sTNF-R2.

TL determinants

A multiple linear regression analysis to evaluate determinants of TL in CS included age, gender, T2DM, hypertension, dyslipidemia, smoking, obesity, duration of hypercortisolism and disease activity in the model, to find predictors of TL. Age (ß-32, t-3.01, p = 0.004) and dyslipidemia (ß-310, t-2.10, p = 0.030) were the only negative independent predictors of TL (R^2 0.21).

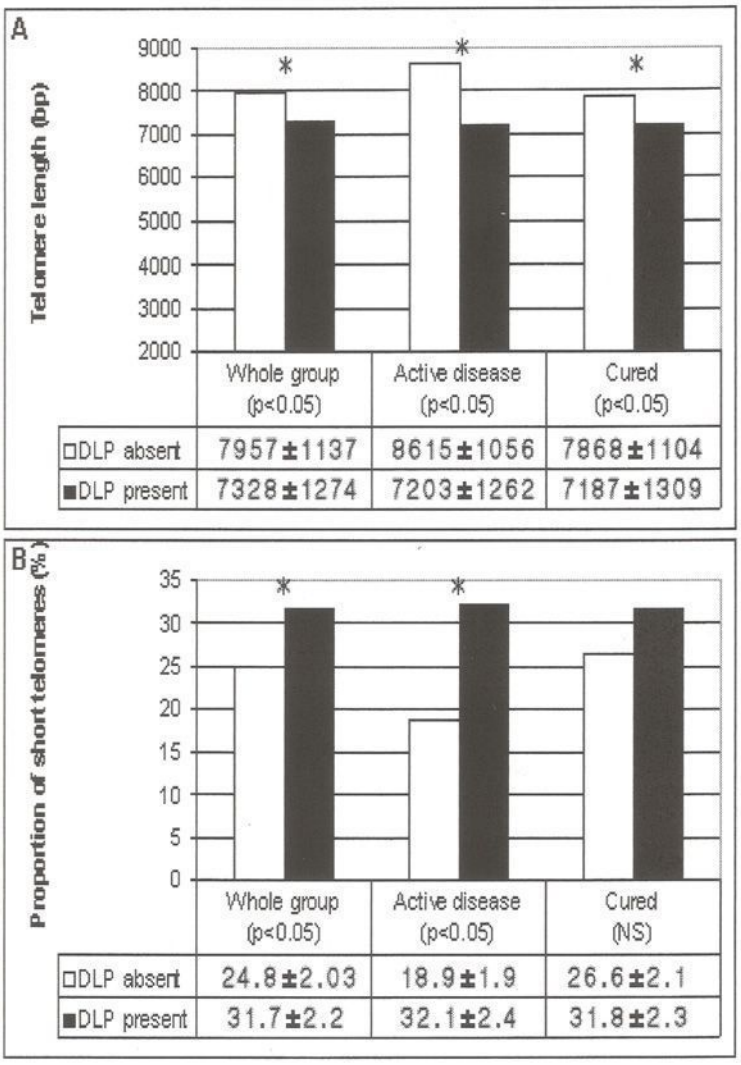

Fig 3. Mean telomere length (A) and proportion of short telomeres (<5kb) (B) in patients with Cushing's syndrome according to the presence or absence of dyslipidemia (*p < 0.05). Abbreviations: bp base pairs; DLP dyslipidemia.

doi:10.1371/journal.pone.0120185.g003

Discussion

Our initial hypothesis was that TL shortening might be involved in the "low grade" inflammatory state and higher prevalence of CVRF in CS, even when hypercortisolism is biochemically cured. For this reason, our approach was to investigate the relationship between TL, classical cardiovascular risk factors and inflammation markers in CS patients. The two main findings are the negative impact of dyslipidemia, further worsened if hypertension and or obesity coexist, and inflammation markers (CRP and IL-6) on TL maintenance. To the best of our knowledge this is the first study to evaluate the relation between individual CVRF and TL in CS.

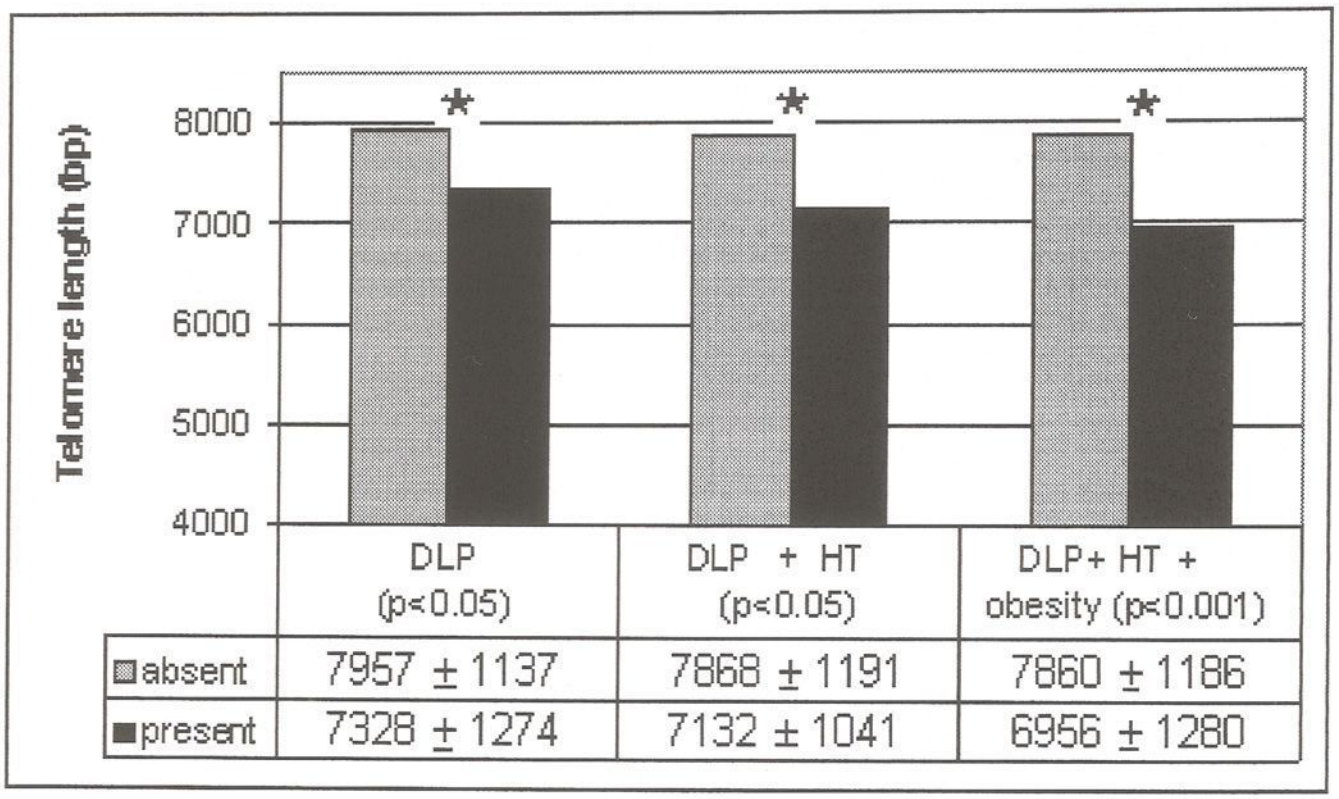

Fig 4. Mean Telomere length in patients with CS with several CVR factors. Dyslipidemic patients (n = 35) compared to those with normal lipids (n = 42); dyslipidemic and hypertensive patients (n = 21) compared to those who did not have both CVR factors (n = 56); patients with dyslipidemia, hypertension and obesity (n = 15) compared to those who did not have three CVR factors (n = 62). Abbreviations: DLP: dyslipidemia; HT: hypertension; bp: base pairs

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As expected, TL was inversely correlated with age, as described in much larger populations, supporting the reliability and validity of our results and the methodology used [23]. However, many factors affect TL, both individual and environmental (genetic, epigenetic, socio-economic status, lifestyle, growth factors, etc.), and should be taken into account when interpreting the results.

We found that dyslipidemia after adjusting for age and disease activity and elevations of CRP and IL6 were the main factors negatively related to telomere lengthening in CS, even after controlling for other clinical and metabolic confounders. Other individual CVRF

Table 2. Correlations of telomere length with lipid profile in patients with Cushing's syndrome without statin treatment (n = 60).

Parameter	r coefficient	p
Triglycerides	-0.259	< 0.05
Total cholesterol	-0.279	< 0.05
LDL cholesterol	-0.05	NS
HDL cholesterol	-0.236	NS

Abbreviations: LDL low density lipoprotein cholesterol; HDL high density lipoprotein cholesterol.

doi:10.1371/journal.pone.0120185.t002

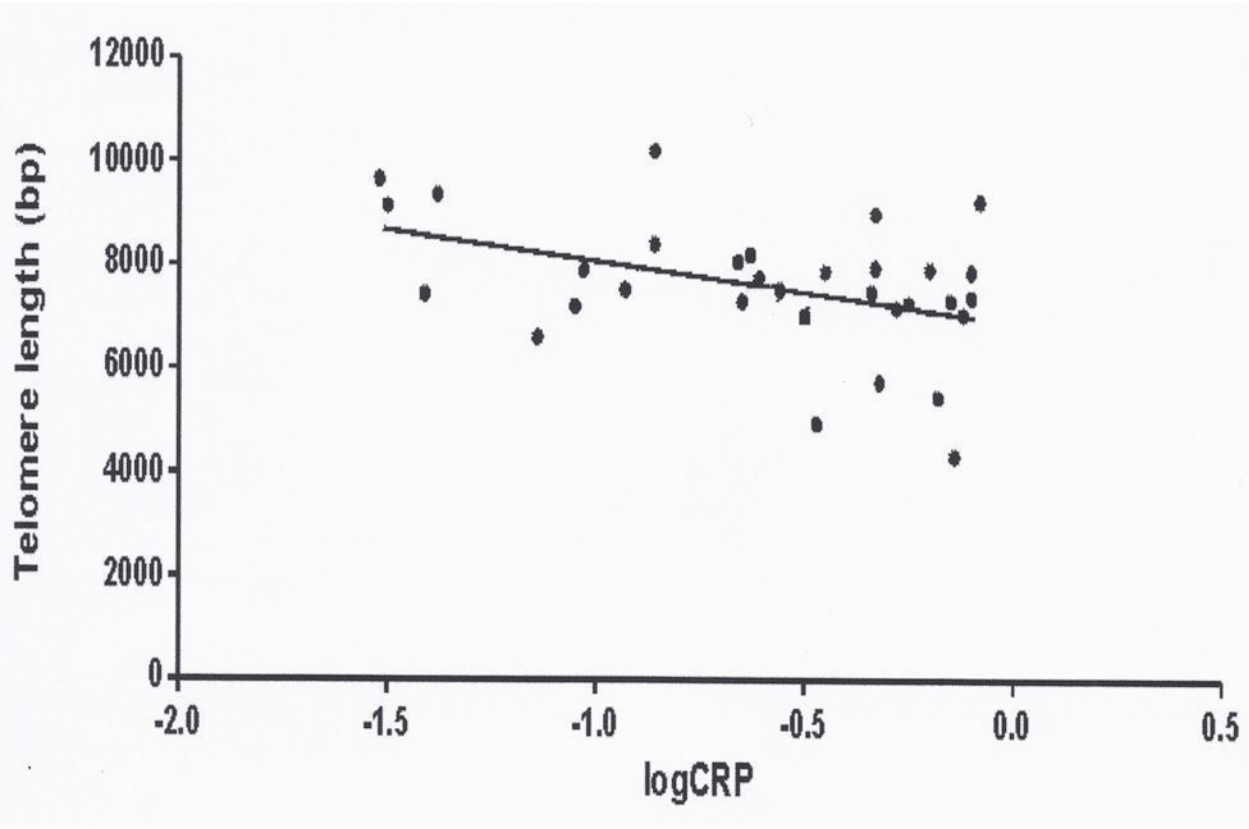

Fig 5. Correlations between C-reactive protein (expressed as logarithm) and telomere length (in base pairs = bp) in patients with Cushing's syndrome (r-0.412, p 0.019).

doi:10.1371/journal.pone.0120185.g005

(hypertension, smoking, T2DM, obesity) were not correlated with TL. CS patients with dyslipidemia had shorter TL in all stages (active, in remission or adrenal insufficient after surgery) compared to CS without dyslipidemia. Differences in TL between dyslipidemic and non-dyslipidemic patients persisted after adjustment for BMI (greater in dyslipidemic patients), as suggested by some authors [13, 24, 25]. However, TL shortening was not associated with dyslipidemia in the control group, probably due to the low prevalence of dyslipidemia observed in controls (n = 15), which reduced statistical power and prevented firm conclusions in this group of healthy controls. Additional TL shortening was found in patients with both dyslipidemia and hypertension. Not surprisingly TL was even shorter when these patients were also obese, since excessive adiposity results in a metabolic imbalance, with an increased inflammatory state and oxidative stress, phenomena associated to accelerated telomere shortening [13,14].

Available literature on the relation between TL, lipids and other CVRF is often discordant. Our findings, namely a negative correlation of total cholesterol and triglycerides and TL are consistent with several but not all previous studies (Table 3). Similar to our findings, in a healthy young population at low cardiovascular risk, an inverse correlation between triglycerides and TL was observed [26, 27]. In T2DM patients, an association between shorter TL and oxidative stress was reported [10], as well as an inverse correlation between TL and total cholesterol, LDL-cholesterol, BMI, triglycerides and CRP [12, 14]. However, other studies observed no relation between TL and CVRF in a population without cardiovascular disease [27, 28]. Similar findings were reported in obese children [29], stable coronary artery disease [30] or myocardial infarction [31].

Table 3. Studies examining relationships between the telomere system and lipid related parameters.

Study population	Reference	Number of subjects n	Main Findings
Studies reporting shorter TL with poor lipid profile			
South Asian T2DM (aged 45 to 60 years)	[12]	142	TL inversely correlated with triglycerides and total cholesterol
T2DM without complications	[14]	97M/96F	TL inversely correlated with BMI, LDL, total cholesterol, HOMA-IR, CRP levels.
Healthy adults	[26]	49M/33F	TL inversely correlated with waist circumference, triglycerides and directly correlated with HDL-cholesterol levels
Healthy adult people	[39]	1917	Higher LDL-cholesterol and CRP levels were observed in the shortest tertile group of TL
Studies not reporting relations between lipid profile and TL			
Caucasian T2DM	[10]	569	No correlations were found between total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and TL.
Subjects free of overt CVD	[28]	1218M/ 1291F	No correlations were found between total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and TL.
Patients from Helsinki Businessmen Study	[27]	436 M	No correlations between total cholesterol levels and TL were found in older ages.
T1DM patients	[36]	132	No correlations were found between BMI, LDL-cholesterol, CRP, duration of diabetes and TL
Patients with stable coronary artery disease	[30]	780	No differences in LDL-cholesterol, HDL-cholesterol were observed according to different quartiles of TL.
French obese and non-obese children	[29]	471/322	No correlations were found between total cholesterol, HDL-cholesterol and TL

Abbreviations: TL, telomere length; T2DM, type 2 diabetes mellitus; M, male; F, female; T1DM, type 1 diabetes mellitus; BMI, body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; CRP, C-reactive protein; LDL, low density lipoprotein; HDL, high density lipoprotein; CVD, cardiovascular disease.

doi:10.1371/journal.pone.0120185.t003

We were unable to demonstrate effects of CS activity (active or cured hypercortisolism), hypopituitarism or hydrocortisone replacement on TL. Since statin therapy prevents TL erosion of endothelial progenitor cells in healthy subjects [31], cholesterol lowering medications may preserve or even elongate TL; since our study was not longitudinal, this preservation effect of statins on TL could not be evaluated.

Another interesting finding is the negative correlation between inflammation markers (CRP and IL6) and TL. Similar findings were reported in 2500 healthy Caucasians supporting that chronic systemic inflammation promotes both atherogenesis and telomere attrition [27]. Also in 36 healthy women where optimism and pessimism were evaluated, a strong negative correlation between TL and IL6 was observed in the pessimist state [32]. Another recent study showed that adipocytes under oxidative stress had shortened telomeres, increased mRNA protein expression of IL6 and sTNF, with decreased expression of adiponectin [33]. Adiponectin has anti-atherogenic and anti-inflammatory properties, protective against metabolic phenomena known to accelerate aging. Glucocorticoids inhibit adiponectin secretion [4]; thus, as expected, lower adiponectin was observed in CS compared to matched controls [3]. Interestingly, a correlation has been observed between telomere shortening and hypoadiponectinemia in obesity [34], and we also found a trend, which was not statistically significant, probably due to the limited sample size.

Although the mechanisms involved are unclear, we propose the following hypothesis. Elevated cholesterol and triglycerides are atherogenic, determining repeated mechanical, hemodynamic, and/or immunological injury, increasing cell turnover and production of ROS in certain cells (as in subclinical chronic inflammation) [35]. The link between cholesterol and TL

may be through this increased cell damage and turnover, leading cells to their maximum replicative capacity and translating into shortened TL and cell ageing [14]. Unfavourable lipid phenotypes would then determine increased oxidative stress, accelerated senescence and cell aging, which in turn could explain our finding in CS.

Most studies only report mean TL. Increasing evidence suggests that regardless of mean TL, the presence of a few critically short telomeres may cause a cell to enter senescence [36, 37]. Therefore, we measured the proportion of short telomeres. CS patients with dyslipidemia exhibited a higher proportion of short telomeres. Whether short TL imply a higher risk of dyslipidemia or if dyslipidemia hastens shortening of telomeres is currently unknown.

The study has several limitations. Due to its cross-sectional nature causality cannot be inferred, limiting conclusions on the potential relationship between TL and dyslipidemia or inflammatory markers. The sample size, although respectable considering that CS is a rare disease, precludes analysis of different etiological subgroups of CS; neither does it allow controlling for all potential confounders, especially medical treatment during active disease, physical activity, individual variability of possible drug effects on telomere attrition, etc. Additionally, even in individuals of similar age, TL may show inter-individual variability [38]. It would be interesting to evaluate TL in other tissue samples (vascular cells, adipocytes) as we can not ensure that our findings are reproducible in cells of the cardiovascular system, because glucocorticoids induce changes in the immune system. However, this would be even more difficult than obtaining peripheral leukocytes for TL evaluation. Finally, even though most cross-sectional studies on telomere biology and ageing are much larger, large-scale, longitudinal, prospective and well-designed studies in general population are still lacking, so that the influence of different physiological states on TL still have to be elucidated.

In summary, in CS patients TL is shortened in those with dyslipidemia; if obesity and/or hypertension were also present, TL was even shorter than if dyslipidemia was present alone. Furthermore, reduced TL is negatively correlated with increased inflammation markers, suggesting that dyslipidemia and "low" grade of inflammation directly contribute to TL shortening, premature ageing and increased morbidities in CS. Larger prospective series and molecular and cellular functional studies are necessary to confirm these findings and to gain more insight on the pathogenesis of TL shortening in CS.

Supporting Information

S1 Dataset.
(XLS)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: AA MJR ER JS SMW. Performed the experiments: AA MJR MJB EM OB JS. Analyzed the data: AA MJR MJB EV ER IC AS JS SMW. Contributed reagents/materials/analysis tools: AA MJR EM OB JS SMW. Wrote the paper: AA MJR MJB EV ER EM AS IC JS SMW.

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Annex I

Tables and figures

Tables

Table 1. Baseline characteristics of patients with Cushing's syndrome (CS) and controls. Data are presented as % and mean ± SD.**(Paper I)**

	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

Table 2: Clinical and biochemical characteristics of patients with Cushing's syndrome (CS) and controls. Abbreviations: Lpa: lipoprotein a; sTNF-R1, sTNF-R2: soluble tumor necrosis factor-α receptors; IL6: interleukin-6. **(Paper II)**

	CS (n=77)	Controls (n=77)	p
Clinical characteristics			
Age (years)	48.6± 12.8	48.4± 12.6	NS
Smokers (%)	25%	19%	NS
Moderate alcohol consumption (%)	26%	27%	NS
Diabetes mellitus (%)	14%	1%	<0.05
Hypertension (%)	57%	13%	<0.001
Dyslipidemia (%)	46%	20%	<0.05
Osteoporosis (%)	30%	3%	<0.001
Psychiatric history (%)	38%	11%	<0.001
Body mass index (kg/m^2)	28 ± 5.6	26.4 ± 4.9	<0.05
Waist to hip ratio	0.92±0.07	0.85±0.07	<0.05
Metabolic syndrome n (%)*	40%	15%	<0.001
Lipid and metabolic profile**			
Triglycerides (mmol/liter)	1.2±0.6	1.09±0.7	0.089
Total cholesterol (mmol/liter)	5.4 ± 1.05	5.3±1.1	NS
HDL cholesterol (mmol/liter)	1.5±0.4	1.5±0.3	NS
LDL cholesterol (mmol/liter)	3.5±0.8	3.4±1.1	NS
Lpa (mg/liter)	410.7±451.1	264±310.8	0.06
Adipocytokines and inflammatory markers			
	CS (n=32)	Controls (n=32)	
Adiponectin (ng/ml)	14.6 ± 6.8	18.6 ± 10	0.053
IL6 (pg/ml)	1.18±2.1	0.37±0.33	<0.001
sTNF-R1 (ng/ml)	1.87±0.69	1.31±0.32	<0.001
sTNF-R2 (ng/ml)	3.71±2.08	3.09±0.91	NS
C-reactive protein (mcg/ml)	0.37±0.26	0.36±0.38	NS

*As described in references 16 and 17. **49% of dyslipidemic CS patients and 26% of dyslipidemic controls were on lipid lowering medications.

Table 3. Correlations of telomere length with lipid profile in patients with Cushing's syndrome without statin treatment (n=60). Abbreviations: LDL low density lipoprotein cholesterol; HDL high density lipoprotein cholesterol. **(Paper II)**

Parameter	r coefficient	p
Triglycerides	-0.259	< 0.05
Total cholesterol	-0.279	< 0.05
LDL cholesterol	-0.05	NS
HDL cholesterol	-0.236	NS

Table 4. Total leukocyte counts and leukocyte main subsets distribution (neutrophils and lymphocytes) of Cushing's syndrome (CS) patients during active disease and remission and their matched controls. Data are expressed as mean±SD. bp. base pairs **(Paper I)**

	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

Table 5. Studies examining relationships between the telomere system and lipid related parameters. Abbreviations: TL, telomere length; T2DM, type 2 diabetes mellitus; M, male; F, female; T1DM, type 1 diabetes mellitus; BMI, body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; CRP, C-reactive protein; LDL, low density lipoprotein; HDL, high density lipoprotein; CVD, cardiovascular disease. **(Paper II)**

Study population	Reference	Number of subjects n	Main Findings
Studies reporting shorter TL with poor lipid profile			
South Asian T2DM (aged 45 to 60 years)	[12]	142	TL inversely correlated with triglycerides and total cholesterol
T2DM without complications	[14]	97M/96F	TL inversely correlated with BMI, LDL, total cholesterol, HOMA-IR, CRP levels.
Healthy adults	[26]	49M/33F	TL inversely correlated with waist circumference, triglycerides and directly correlated with HDL-cholesterol levels
Healthy adult people	[39]	1917	Higher LDL-cholesterol and CRP levels were observed in the shortest tertile group of TL
Studies not reporting relations between lipid profile and TL			
Caucasian T2DM	[10]	569	No correlations were found between total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and TL.
Subjects free of overt CVD	[28]	1218M/ 1291F	No correlations were found between total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and TL.
Patients from Helsinki Businessmen Study	[27]	436 M	No correlations between total cholesterol levels and TL were found in older ages.
T1DM patients	[36]	132	No correlations were found between BMI, LDL-cholesterol, CRP, duration of diabetes and TL
Patients with stable coronary artery disease	[30]	780	No differences in LDL-cholesterol, HDL-cholesterol were observed according to different quartiles of TL.
French obese and non-obese children	[29]	471/322	No correlations were found between total cholesterol, HDL-cholesterol and TL

Figures

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. CS, Cushing's syndrome; AI, adrenal insufficiency; TL, telomere length **(Paper I)**

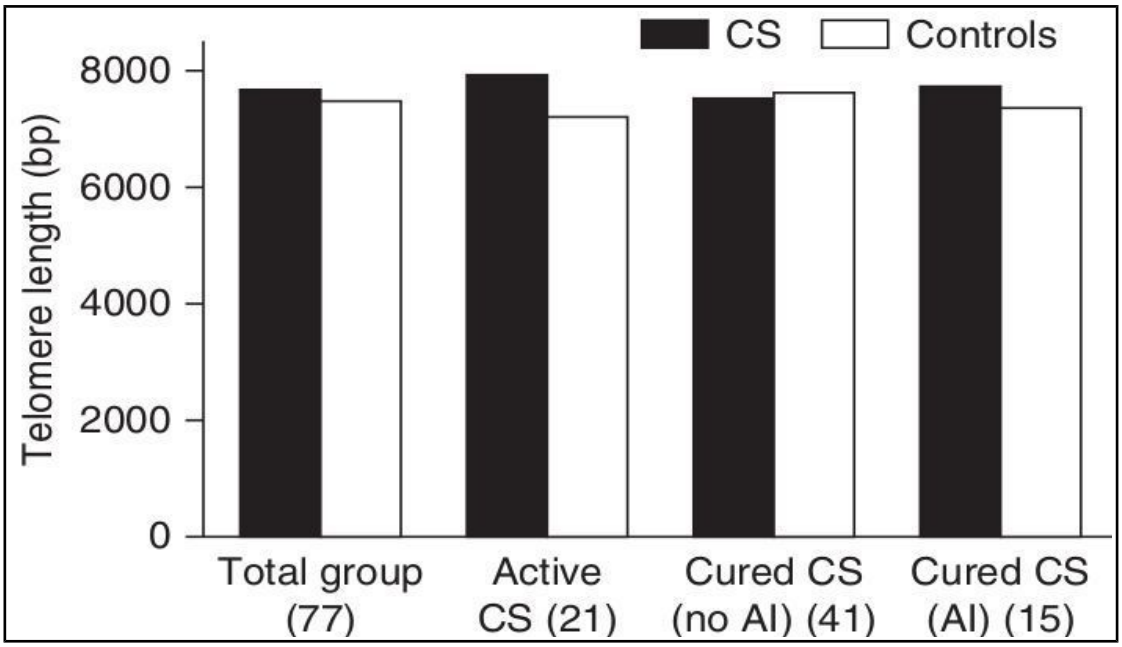


Figure 2. Telomere length in relation to age in patients with Cushing's syndrome (•) and controls (◦). Telomere length is shortened with advancing age in both CS (R = -0.400, p <0.001) and controls (R = -0.292, p <0.01). bp. base pairs.**(Paper I)**

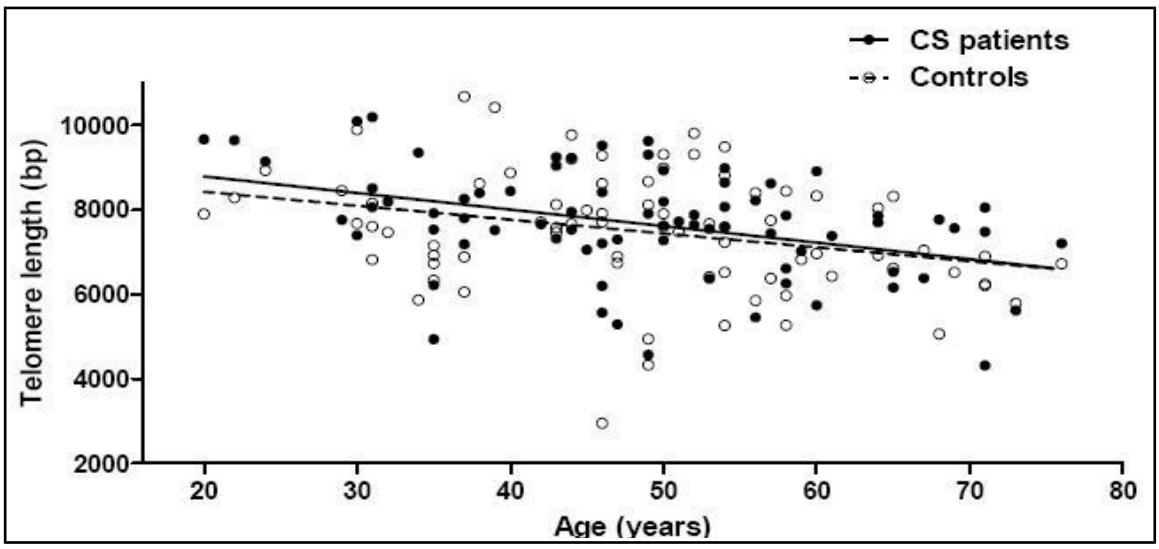


Figure 3. A: Changes in telomere length (TL) in 15 patients in whom samples were obtained both during active hypercortisolism (7273±1263 bp.) and after remission (7870±1039 bp.). **3B**: TL increased in 10/15 patients, increasing age. The dotted line shows the detection limit of the Southern Blot technique.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

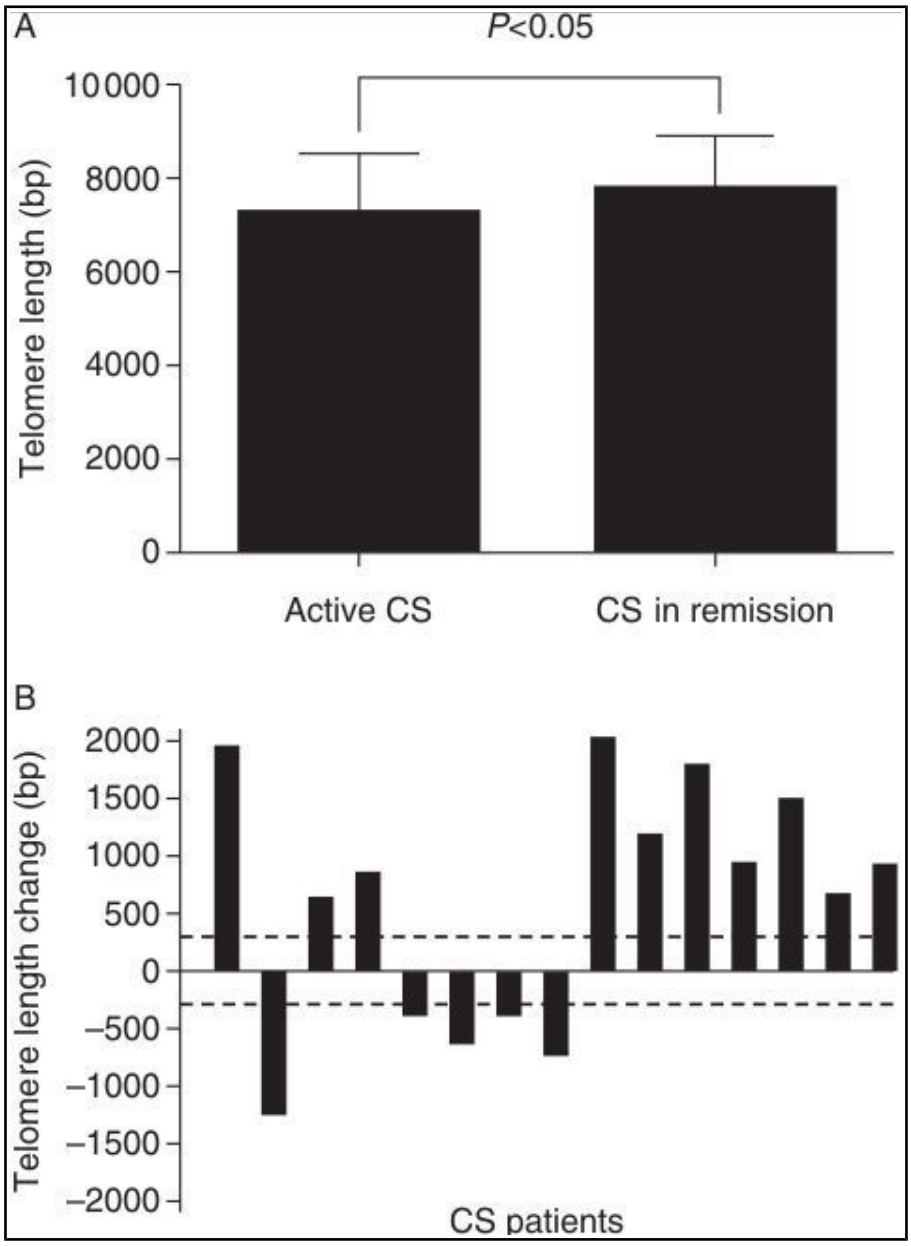


Figure 4: Correlation between mean telomere length and proportion of short telomeres (< 5kb) in the study population (r -0.917, p < 0.001). bp. base pairs **(Paper II)**

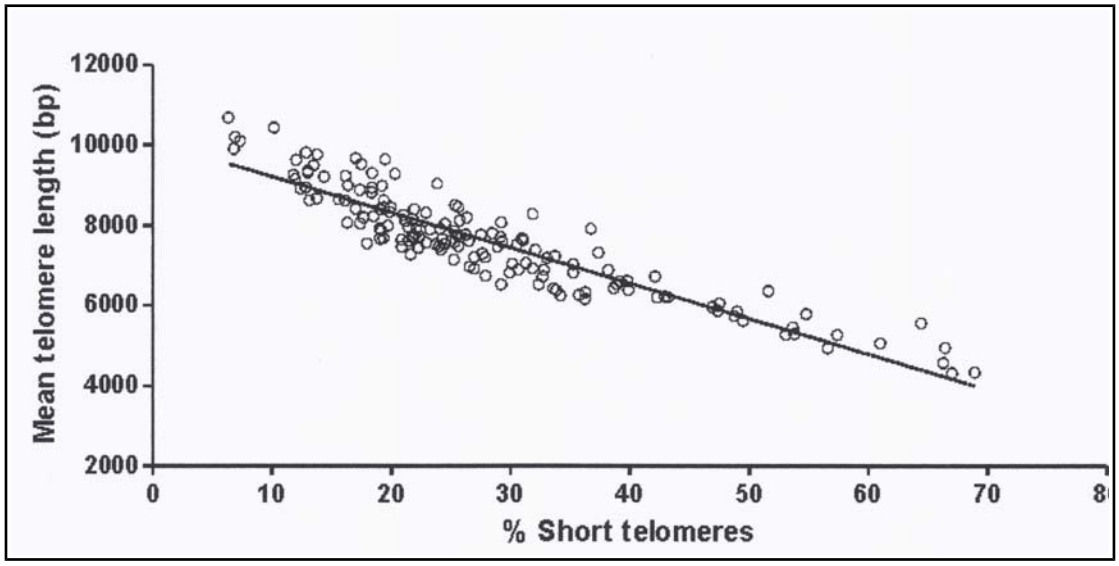
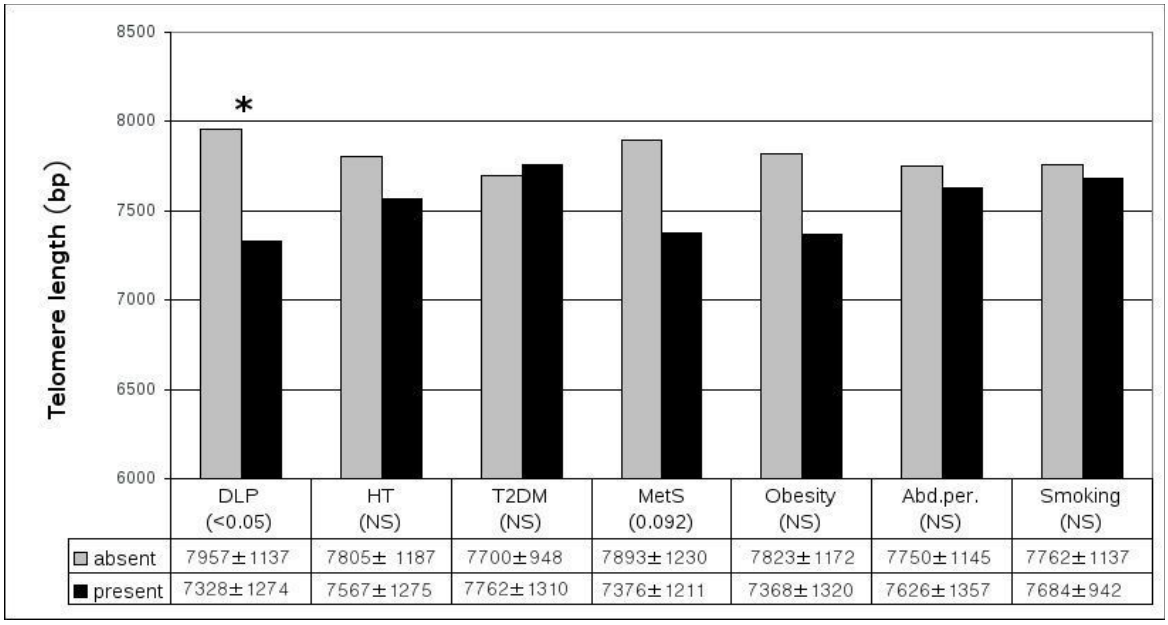


Figure 5: Mean telomere length according to different cardiovascular risk factors after adjustment for age in Cushing's syndrome patients. Abbreviations: bp, base pairs; DLP dyslipidemia, HT hypertension, T2DM Type 2 diabetes mellitus; MetS, metabolic syndrome; abd.per., increased abdominal perimeter.* p<0.05 **(Paper II)**



	DLP (<0.05)	HT (NS)	T2DM (NS)	MetS (0.092)	Obesity (NS)	Abd.per. (NS)	Smoking (NS)
absent	7957±1137	7805± 1187	7700±948	7893±1230	7823±1172	7750±1145	7762±1137
present	7328±1274	7567±1275	7762±1310	7376±1211	7368±1320	7626±1357	7684±942

Figure 6: Mean telomere length (A) and proportion of short telomeres (<5kb) (B) in patients with Cushing's syndrome according to the presence or absence of dyslipidemia (*p < 0.05). Abbreviations: bp base pairs; DLP dyslipidemia. **(Paper II)**

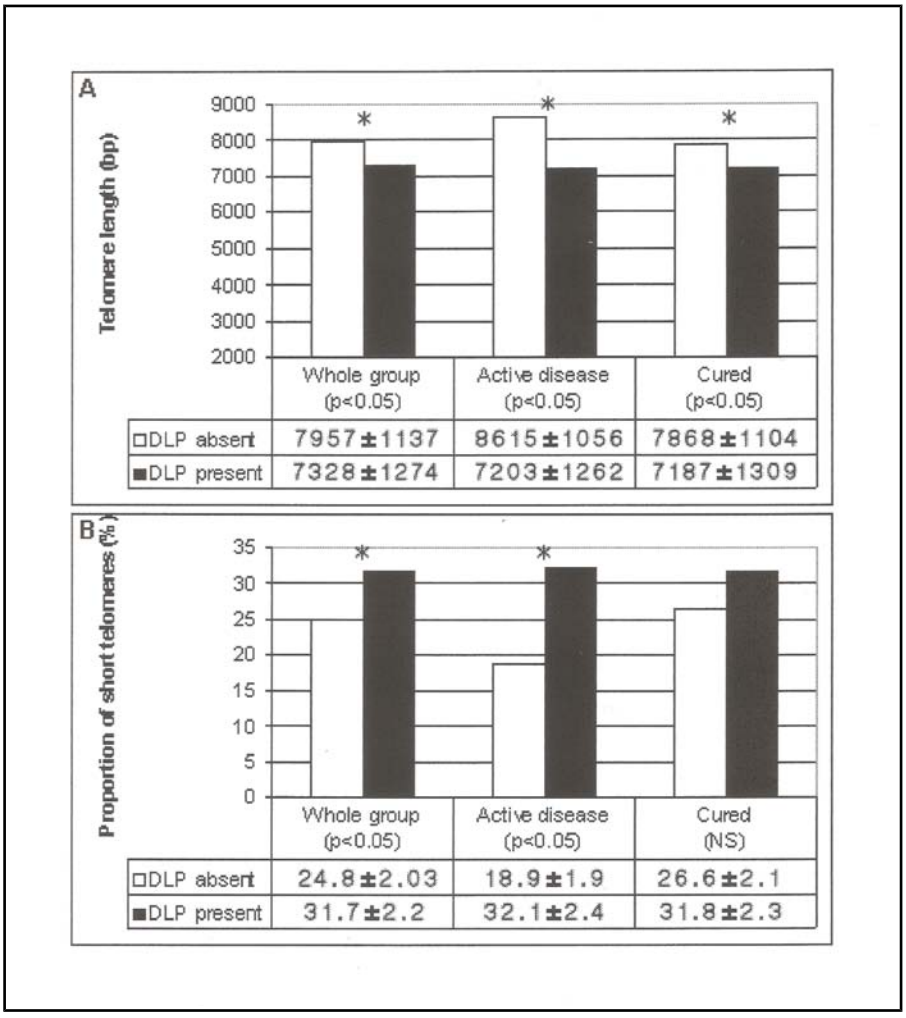


Figure 7. Mean Telomere length in patients with CS with several CVR factors. Dyslipidemic patients (n=35) compared to those with normal lipids (n=42); dyslipidemic and hypertensive patients (n=21) compared to those who did not have both CVR factors (n=56); patients with dyslipidemia, hypertension and obesity (n=15) compared to those who did not have three CVR factors (n=62). Abbreviations: DLP: dyslipidemia; HT: hypertension; bp: base pairs **(Paper II)**

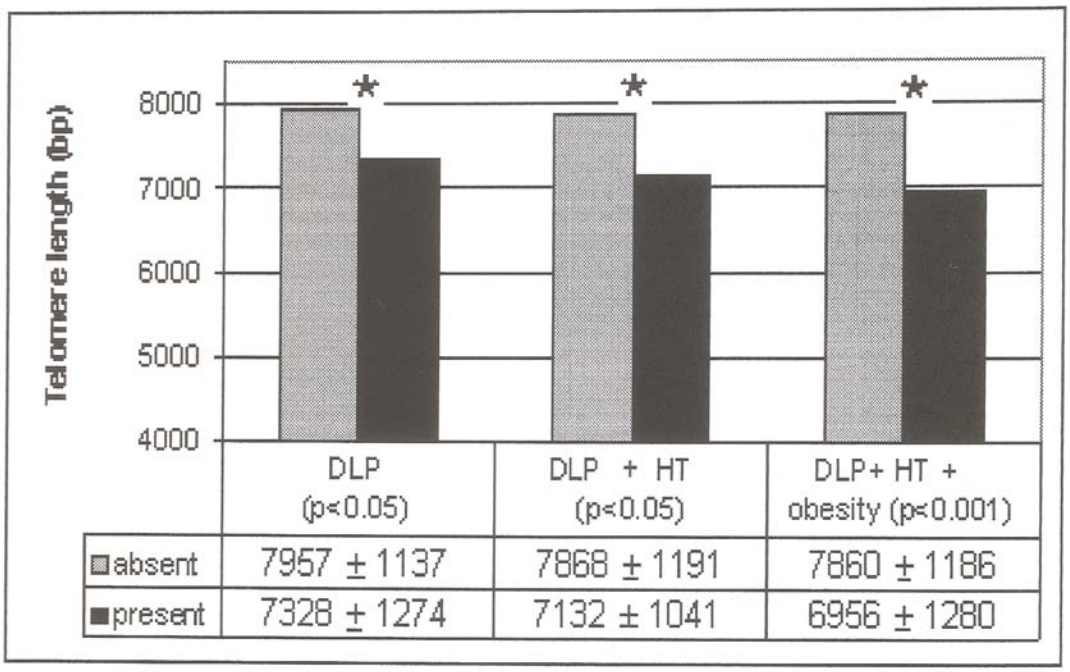
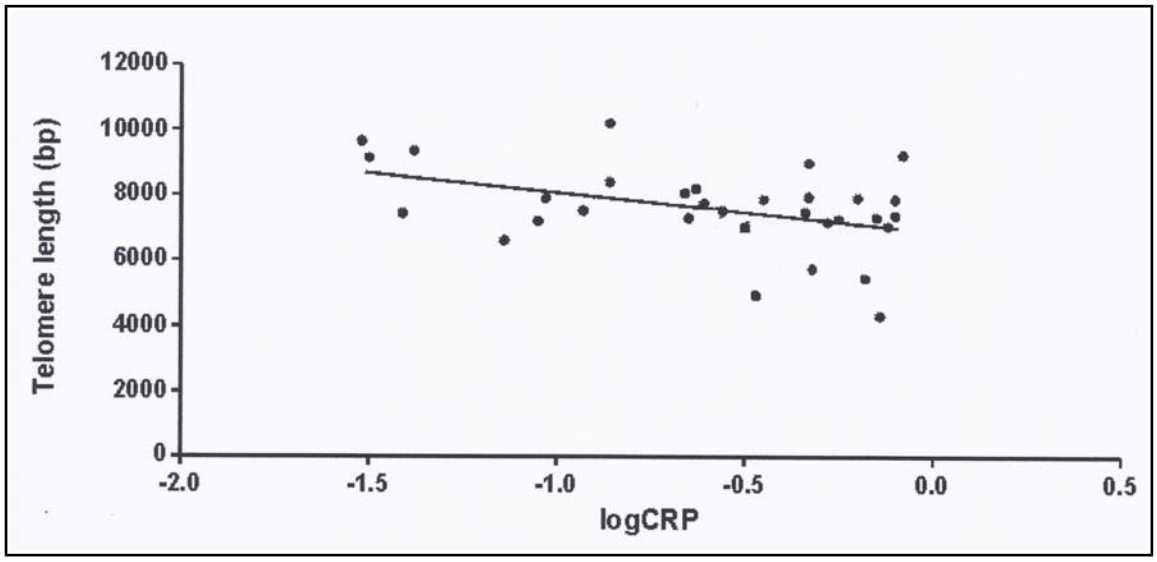


Figure 8: Correlations between C-reactive protein (expressed as logarithm) and telomere length (in base pairs = bp) in patients with Cushing's syndrome (r -0.412, p 0.019). **(Paper II)**



Annex II

Review I:

Aulinas A, Santos A, Valassi E, Mato E, Crespo I, Resmini E, Roig O, Bell O, Webb SM. **Telomeres, aging and Cushing's syndrome: are they related?** Endocrinol Nutr. 2013 Jun-Jul;60(6):329-35.

Review II:

Aulinas A, Valassi E, Webb SM. **Prognosis of patients treated for Cushing syndrome**. Endocrinol Nutr. 2014 Jan;61(1):52-61

Review III:

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 Dec;79(6):751-9.

Endocrinol Nutr. 2013;60(6):329–335

ENDOCRINOLOGÍA Y NUTRICIÓN

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REVISIÓN

Telómeros, envejecimiento y síndrome de Cushing: ¿están relacionados?

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PALABRAS CLAVE
Síndrome de Cushing;
Envejecimiento celular;
Sistema telomérico;
Estrés oxidativo

Resumen El síndrome de Cushing se debe a una hipersecreción de cortisol, asociado a una mayor mortalidad y una elevada morbilidad, que no es totalmente reversible a pesar del control bioquímico, presentando un conjunto de manifestaciones sistémicas similares a las que aparecen en el envejecimiento. El estrés crónico, que también conlleva una hiperestimulación del eje adrenal, se ha relacionado con el acortamiento telomérico acelerado, el daño oxidativo y el envejecimiento celular. A pesar de que el envejecimiento prematuro de los pacientes con síndrome de Cushing podría relacionarse con factores ambientales, no puede descartarse que la exposición crónica al hipercortisolismo determine un acortamiento telomérico y, por lo tanto, envejecimiento. En esta revisión se repasan las evidencias existentes que podrían relacionar el síndrome de Cushing y el envejecimiento celular prematuro.

KEYWORDS
Cushing's syndrome;
Cell aging;
Telomere system;
Oxidative stress

Telomeres, aging and Cushing's syndrome: Are they related?

Abstract Cushing's syndrome is due to excess cortisol secretion and is associated to increased mortality and severe morbidity that are not fully reversible despite biochemical control. The syndrome consists of a set of systemic manifestations similar to those found in aging. Chronic stress, which also causes hyperstimulation of the hypothalamic-pituitary-adrenal axis, has been related to accelerated telomere shortening, oxidative damage, and cell aging. Although premature aging in patients with Cushing's syndrome could be related to environmental factors,

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the possibility that chronic exposure to hypercortisolism causes telomere shortening, and thus premature aging, cannot be ruled out. This review discusses the available evidence supporting a link between Cushing's syndrome and cell aging.

Introducción

El síndrome de Cushing es una enfermedad endocrina rara causada por una hipersecreción de cortisol, ya sea de origen exógeno (iatrogénico) o endógeno, debido a un tumor hipofisario productor de ACTH (enfermedad de Cushing) y, menos frecuentemente, a un tumor adrenal productor de cortisol o a un tumor que determina secreción ectópica de ACTH. La incidencia anual del síndrome de Cushing endógeno se estima que es alrededor de $2/10^6$ habitantes. Se asocia a una importante morbilidad y al aumento de la mortalidad.

Las manifestaciones sistémicas que afectan mayormente a los pacientes con síndrome de Cushing son aquellas relacionadas con enfermedades cardiovasculares o bien con otros problemas asociados al propio envejecimiento[1]. Así, fenotípicamente, estos pacientes suelen presentar obesidad central (cara de luna llena y atrofia de extremidades), aumento de la masa grasa y reducción de la masa ósea y magra, debilidad muscular, aumento de la susceptibilidad a las infecciones, fragilidad capilar, hematomas, típicas estrías rojovinosas, hirsutismo, hipogonadismo, disminución de la líbido y características del síndrome metabólico (hipertensión, diabetes mellitus e insulinoresistencia, dislipidemia, enfermedad vascular, aterosclerosis, etc.) con aumento del riesgo cardiovascular, así como depresión, atrofia cerebral y alteración de la calidad de vida relacionada con la salud[1,2].

A pesar de que el hipercortisolismo se puede curar después de la cirugía (se consiguen controlar los niveles de cortisol hasta en el 90% de los sujetos operados) y con medicación, evidencias recientes muestran que estos pacientes permanecen con un elevado riesgo de padecer complicaciones cardiovasculares, osteopenia y alteraciones funcionales y estructurales cerebrales acompañadas de una baja calidad de vida relacionada con la salud[3].

Por otro lado, el enjevecimiento biológico se caracteriza por una pérdida progresiva y predecible de las funciones tisulares y celulares coordinadas, conduciendo a un riesgo creciente de vulnerabilidad y enfermedad. Inicialmente este deterioro es objetivado por la pérdida de la capacidad y la habilidad para mantener la homeostasis en situaciones de estrés y, en fases más avanzadas, incluso en situaciones de reposo[4]. Este proceso es específico de cada especie y los cambios producidos se manifiestan en múltiples órganos y sistemas, aunque los mecanismos que contribuyen al envejecimiento no son del todo conocidos.

Aunque es probable que el exceso de hipercortisolismo esté relacionado con estos procesos, los mecanismos exactos por los cuales esto pasa no están claros. En esta revisión se intentarán exponer las hipótesis y los mecanismos propuestos hasta la actualidad que relacionan el hipercortisolismo y el envejecimiento.

¿Reversibilidad de la morbilidad específica tras la resolución del hipercortisolismo?

Inicialmente se pensaba que la resolución del hipercortisolismo iba seguida de una normalización de la morbilidad causada por el mismo, pero en la última década se ha visto que esta normalización de la morbilidad no ocurre a pesar de resolverse el hipercortisolismo. El riesgo cardiovascular persiste elevado en el síndrome de Cushing bioquímicamente curado incluso 5 años después de la curación[5]. La recuperación completa de la densidad mineral ósea (DMO) es más controvertida. Algunos estudios muestran mejoría, incluso normalización[6,7], pero otros no[8,9]. Se ha observado que tanto la duración del hipercortisolismo endógeno como la duración del tratamiento sustitutivo con glucocorticoides exógenos después de un tratamiento quirúrgico eficaz del síndrome de Cushing se correlacionan negativamente con la DMO en mujeres 11 años después de la remisión del Cushing cuando se comparan con controles[10]. Se ha demostrado una persistente acumulación de grasa central en pacientes curados con un perfil desfavorable de adipoquinas (disminución de los niveles de adiponectina, elevación de los niveles plasmáticos de sTNF-R1 e interleuquina 6), dando lugar a un estado de inflamación de bajo grado[11]. Este «estado inflamatorio» puede determinar daño vascular, contribuyendo a la aterosclerosis y a la enfermedad cardiovascular en estos pacientes años después de la remisión. En el síndrome de Cushing se ha observado un incremento del estrés oxidativo y de la disfunción endotelial[12]. Se han demostrando niveles altos de aniones superóxido, de ciclooxigenasa-1 (COX-1) y de la sintetasa del óxido nítrico endotelial en los microvasos causantes de la disfunción endotelial y del aumento de la resistencia vascular periférica[13]. Asimismo, recientemente se ha demostrado que los glucocorticoides pueden activar de forma directa la vía de señalización de los receptores de los mineralocorticoides, independientemente de los niveles circulantes de aldosterona[14], sugiriendo que pueden contribuir a este daño vascular y endotelial objetivado en estos pacientes a través de las propiedades profibróticas de la aldosterona[15].

Además, tal y como se ha mencionado, el hipercortisolismo afecta al comportamiento, al estado de ánimo, a la actividad neuronal, a la memoria y a otros procesos del sistema nervioso central[1,2]. En estudios con RM de 3 Teslas se ha objetivado una mayor atrofia cerebral en comparación con los controles normales de la misma edad que no parece totalmente reversible a pesar de la normalización del cortisol. La psicopatología (sobretodo depresión atípica), muy prevalente en la enfermedad activa, mejora el primer año después del tratamiento pero a menudo persisten síntomas residuales[16]. Los niveles altos de cortisol se han asociado a alteraciones en las funciones de algunos neurotransmisores como una disminución de la síntesis cerebral de serotonina,

un aumento de la actividad noradrenérgica y niveles bajos de ácido 5-hidroxiindolacético en el líquido cefaloraquídeo, todos ellos relacionados con la patogenia de la depresión[17]. La cognición[18] y la calidad de vida relacionada con la salud tampoco parecen normalizarse después de la curación del hipercortisolismo, sugiriendo que todos estos cambios no son completamente reversibles[19,20]. Varios estudios reflejan que la hipersecreción de cortisol tiene un efecto envejecedor en el comportamiento cognitivo[21,22] pero los mecanismos por los cuales los glucocortidoides afectan al sistema cognitivo no están claros. Se formulan varias hipótesis entre las que cabe citar las alteraciones hipocampales objetivadas en ratas y la localización anatómica de gran cantidad de receptores de glucocorticoides en el cerebro, sobretodo en el hipocampo, la amígdala y los lóbulos prefrontales, que favorecerían cambios morfológicos en estas zonas como ocurre en el envejecimiento[23], o una reducción importante del metabolismo de la glucosa cerebral (visible por atrofia y muerte neuronal propias del envejecimiento). Estas alteraciones de la función cognitiva son evidentes incluso después de eliminar el efecto causado por la depresión, muy común en el hipercortisolismo y que puede interferir de forma clara en la función cognitiva. El motivo por el cual el hipercortisolismo crónico produce estas consecuencias a largo plazo a pesar de su normalización bioquímica no está claro pero sugiere un efecto neurotóxico poco reversible.

Estrés crónico e hipercortisolismo

El estrés crónico determina una hiperestimulación del eje hipotalámico-hipofisario-adrenal e hipercortisolismo endógeno. Además, se sabe que más de la mitad de los pacientes con depresión crónica tienen hiperactividad del eje adrenal, y representan otro modelo de hipercortisolismo endógeno[24].

El estrés crónico inferido por el córtex cerebral determina asimismo, a nivel hipotalámico, la secreción de corticorelina (CRH) con la consecuente activación del eje hipotalámico-hipofisario adrenal y, por lo tanto, la liberación de cortisol en la sangre. Se han objetivado niveles altos de CRH en el líquido cefaloraquídeo y de su RNAm en las regiones límbicas cerebrales de pacientes con depresión mayor crónica[25,26]. Asimismo, se ha observado que a menudo la remisión clínica del trastorno depresivo mayor va acompañada de una reversibilidad en las anormalidades del cortisol. En cambio, la sintomatología depresiva puede persistir a pesar de la resolución del síndrome de Cushing[27]. De forma contraria, también se ha sugerido que las elevaciones crónicas de cortisol, especialmente nocturnas (típicas del hipercortisolismo endógeno), podrían tener un papel importante en la patogenia de la depresión y en el estrés crónico[28]. Por otro lado, el papel de la 11-beta-hidroxiesteroide deshidrogenasa (11b-HSD), enzima reguladora del metabolismo de los glucocorticoides a nivel intracelular, ha sido sugerido como uno de los posibles mecanismos por los cuales el hipercortisolismo podría producir efectos deletéreos en el sistema nervioso y en el comportamiento. Existen 2 isoformas distintas de 11b-HSD: la tipo 1, que aumentaría los niveles intracelulares de cortisol (en el hígado, el tejido graso y el cerebro), y la tipo 2, que inactivaría los glucocorticoides (de cortisol a una molécula inactiva de cortisona)[29]. En las células del hipocampo únicamente se expresan la 11b-HSD tipo 1, aumentando por tanto los niveles de cortisol. Debido a que la 11-HDS tipo 2 no se expresa en el hipocampo, el cortisol se une a los receptores de mineralcorticoides (situados en el hipocampo y en las regiones límbicas) con una elevada afinidad (10 veces superior a la de los receptores de glucocorticoides), pudiendo tener un efecto importante en la patogenia de la depresión, en el estrés crónico[30] y también contribuir en la patogenesis del síndrome metabólico (por el aumento de la acción de la isoforma tipo 1 observado en el hígado y el tejido graso)[31].

Asimismo, el estrés crónico se relaciona con un bajo índice de salud, incluyendo un aumento de los factores de riesgo cardiovascular y una alteración de la función inmunitaria, similar a lo que ocurre en los pacientes con síndrome de Cushing; sin embargo los mecanismos exactos por los cuales esto pasa están aún por descubrir.

Parece ser que el estrés crónico podría afectar a la salud y al envejecimiento celular por distintos mecanismos no excluyentes entre sí y aún no bien definidos. Este produciría una alteración en la función inmunológica a través del estrés oxidativo (respuesta neuroendocrina con un hipercortisolismo secundario al estrés que aumentaría el daño neuronal y disminuiría las enzimas antioxidantes) y, finalmente, provocaría una alteración en la actividad de la telomerasa (disminución de su función) (fig. 1)[32].

Actividad telomérica y envejecimiento celular

El daño oxidativo celular y el severo estrés psicosocial se han relacionado con el acortamiento telomérico. Por lo tanto, el estrés crónico asociado a las alteraciones del estado de ánimo podría contribuir al exceso de vulnerabilidad a enfermedades propias del envejecimiento, tales como las enfermedades cardiovasculares (a través de un envejecimiento acelerado del organismo). Actualmente, una forma ampliamente aceptada de evaluar el envejecimiento celular es a través de la medición de la longitud telomérica[33]. Los telómeros son secuencias de DNA no codificante que en el caso de los humanos y en otros vertebrados están formados por repeticiones en tándem de una secuencia rica en guaninas TTAGGG (5'→3'). Están situados en los extremos de los cromosomas formando una estructura que protege a los mismos de la erosión que tiene lugar de forma natural durante la replicación de las moléculas lineales de DNA en cada división de la célula eucariota (fig. 2)[34]. Sin telómeros el material genético podría perderse cada vez que la célula se dividiese[35]. Cuando llegan a una longitud críticamente corta se paran las divisiones celulares, las células entran en un proceso de senectud y finalmente mueren. Para evitar este desgaste telomérico las células germinales, las células hematopoyéticas y especialmente las células madre adultas producen telomerasa (niveles elevados en el embrión que van disminuyendo a partir del nacimiento), una enzima que cataliza la síntesis de DNA para mantener la longitud telomérica y evitar así la senescencia y la apoptosis celular[35,36]. En cambio, la actividad de la telomerasa es detectada a niveles muy bajos y no uniforme en las células somáticas y en el resto de los tejidos.

Los telómeros pueden acortarse con la exposición acumulada al estrés oxidativo, a los estresantes genotóxicos, a los estímulos nocivos y tras el estrés psicosocial crónico,

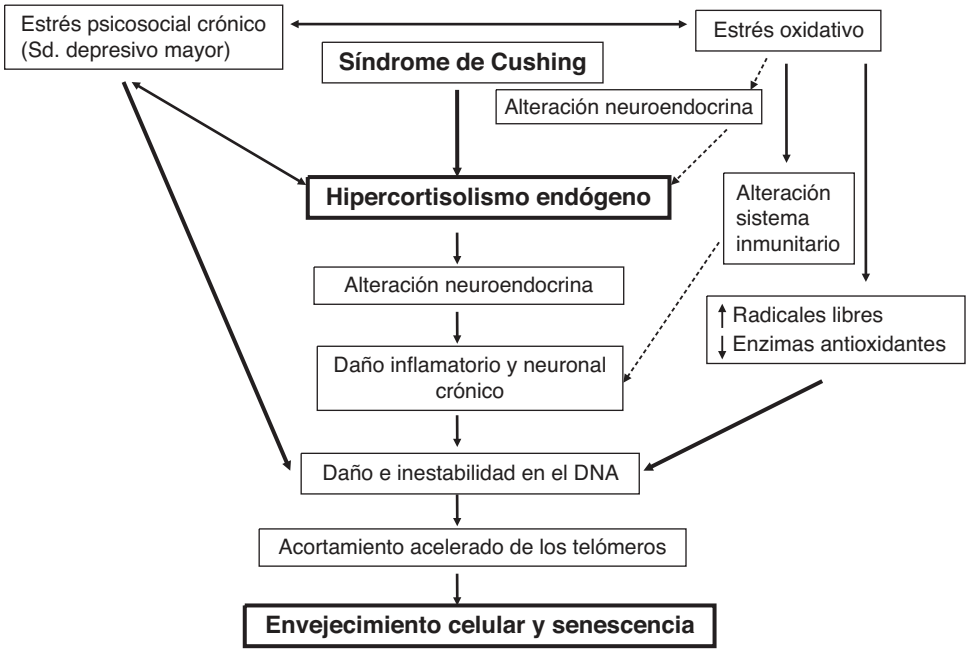

Figura 1 Relaciones entre el síndrome de Cushing y el envejecimiento celular.

aunque este acortamiento telomérico se ha visto que no es uniforme para todos los tipos celulares y tejidos[37].

La disfunción del sistema telomérico conlleva importantes consecuencias clínicas. Las enfermedades causadas por la alteración y las anormalidades del sistema telomérico representan un amplio espectro de manifestaciones clínicas: anemia aplástica adquirida (mutaciones en el complejo de la telomerasa), disqueratosis congénita (mutación en la disquerina, una proteína esencial para el funcionamiento telomérico), fibrosis pulmonar idiopática (el 15% tienen mutaciones en el complejo de la telomerasa), etc[35].

Asimismo, el acortamiento telomérico se ha relacionado con procesos de envejecimiento prematuro (p. ej.: aterosclerosis, enfermedad de Alzheimer, osteopenia o diabetes mellitus tipo 2), aumento del riesgo de enfermedad cardiovascular, de inflamación y de transformación maligna (p. ej.: cáncer colorectal, enfermedad inflamatoria intestinal, esófago de Barret, etc.), mayor mortalidad en pacientes con enfermedad coronaria, infarto de miocardio prematuro e incluso se ha relacionado con el engrosamiento de la íntima carotídea y la mortalidad prematura[38,39].

Es probable que el papel de las alteraciones de la biología telomérica en la enfermedad humana esté

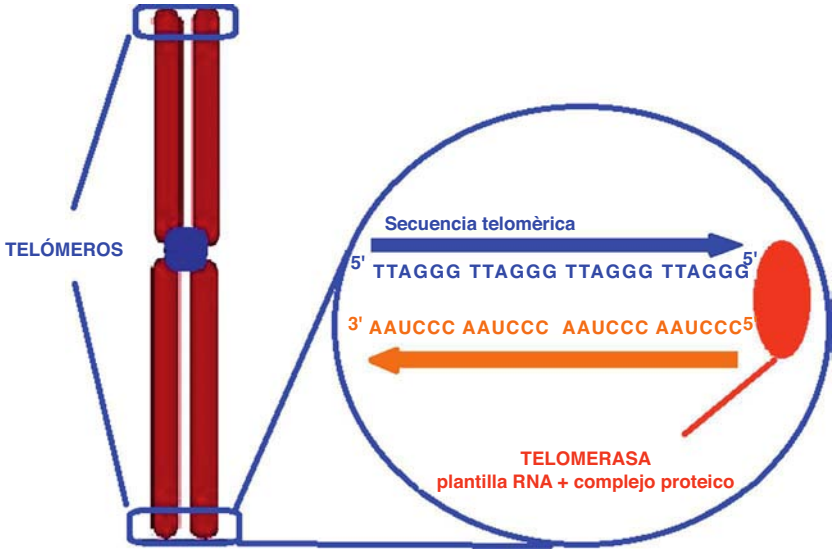

Figura 2 Representación esquemática de un cromosoma con la estructura telomérica en su extremo formado por múltiples repeticiones de secuencias hexaméricas de DNA (TTAGGG).

actualmente infraestimado, quedando aún mucho camino por investigar.

Estrés y envejecimiento: ¿se relacionan con los telómeros?

El trastorno depresivo mayor se asocia a una elevada morbimortalidad con un aumento de enfermedades relacionadas con el envejecimiento (cardiovasculares, cáncer, etc.). El estrés causado por estas enfermedades se considera una amenaza para la homeostasis corporal, generando una respuesta crónica al estrés (para recuperar el equilibrio) que puede inducir adaptaciones y cambios que determinen daño biológico a largo plazo.

La hipótesis de que el estrés tiene impacto en la salud modulando la tasa de envejecimiento celular también está respaldada por la evidencia que asocia el estrés psicológico al elevado estrés oxidativo (activación autonómica y respuesta neuroendocrina al estrés), la disminución de la actividad de la telomerasa y el acortamiento de la longitud telomérica, todos ellos conocidos como determinantes de la senescencia y la longevidad celular[40]. Estos efectos son evidentes a mayor duración y exposición al estrés. Se ha confirmado que el estrés psicosocial crónico afecta a la longitud telomérica; se analizaron pacientes con severo estrés crónico (madres cuidadoras de hijos crónicamente enfermos) y se observó que una mayor duración del estrés, así como una mayor percepción de los niveles de estrés se correlacionaban de forma significativa con un acortamiento de la longitud telomérica y con una menor actividad de la telomerasa en comparación con los controles apareados por edad y sexo, representando aproximadamente el equivalente a unos 10 años de envejecimiento biológico acelerado[41]. En pacientes con depresión mayor crónica (que frecuentemente presentan hipercortisolismo) se han encontrado resultados similares. En otro estudio de 647 mujeres con hermanas afectas de cáncer de mama se evaluó la relación entre la longitud telomérica (marcador de envejecimiento celular), el estrés percibido por estas mujeres y el nivel de hormonas de estrés (cortisol y metanefrinas) en la orina. Se objetivó una tendencia a un mayor acortamiento de los telómeros en las mujeres con un mayor índice de estrés percibido, aunque estas diferencias únicamente fueron significativas en mujeres mayores de 55 años, mujeres con un importante estrés psicosocial reciente y con mayores niveles de catecolaminas en la orina. Se encontró una tendencia a un mayor acortamiento telomérico con mayores niveles de cortisoluria sin alcanzar la significación estadística[42]. El impacto biológico de estos resultados en cuanto a la longitud telomérica no está claro dado el amplio rango de variación en la longitud telomérica de la población general, lo que dificulta su interpretación. Todavía quedan muchas incógnitas por aclarar en la relación entre la longitud telomérica, el envejecimiento y el riesgo de enfermedad.

Se ha visto que hay personas que tienen una resistencia fisiológica al estrés oxidativo, muy probablemente debido a variantes genéticas aún no bien conocidas, lo que aumentaría su esperanza de vida. Por otro lado, también se postula que esta resistencia fisiológica al estrés oxidativo (que cursaría con menor hipercortisolismo, menor alteración del sistema telomérico e inmunológico, etc.) esté relacionada con la resistencia psicológica al estrés, en cuyo caso no sería tan evidente que el acortamiento telomérico pudiera ser una secuela de una exposición prolongada a un estrés psicológico[40].

A pesar de la cantidad de trabajos que relacionan el estrés oxidativo, el estrés psicosocial crónico y el hipercortisolismo con la longitud mediana de los telómeros, recientemente algunos autores consideran que quizás el porcentaje de telómeros cortos podría ser un marcador más sensible de envejecimiento celular que la longitud media telomérica en situaciones de estrés agudo[41]. Se basan en el hecho de que el acortamiento de la longitud telomérica no es uniforme para todas las células y tejidos e incluso para los cromosomas de una misma célula. Mediante una técnica recientemente introducida (Universal STELA: Single Telomere Length Assay) se ha observado en cultivos celulares in vitro que la medición del porcentaje de telómeros cortos se correlaciona de forma positiva con marcadores bien conocidos de senescencia celular como la betagalactosidasa pero no siendo así con la longitud media de los telómeros[42]. Por lo tanto, parece que este nuevo método aporta más información, ayudando a conocer mejor la biología telomérica y su papel en el envejecimiento y el cáncer.

Hipercortisolismo y acortamiento de telómeros

Algunos estudios demuestran que la exposición a niveles altos de cortisol se asocia a una reducción significativa de la actividad de la telomerasa de los linfocitos T[36], pudiendo ser uno de los mecanismos por los cuales los pacientes con hiperestimulación del eje adrenal por estrés crónico, o bien por síndrome de Cushing, tienen alterada la inmunidad celular y, por tanto, mayor susceptibilidad a padecer infecciones.

Poco se sabe sobre los mecanismos biológicos del exceso de morbimortalidad asociados a las alteraciones del estado de ánimo, pero en la depresión crónica mayor existen evidencias que asocian las alteraciones de los sistemas biológicos relacionados con el estrés (sistema neuroendocrino, sistema telomérico, etc.) como uno de los mecanismos que contribuyen a su exceso de morbimortalidad.

Como ocurre en el síndrome de Cushing endógeno, el estrés crónico también determina una hiperestimulación del eje hipotalámico-hipofisario-adrenal e hipercortisolismo; más de la mitad de los pacientes crónicamente deprimidos con estrés crónico importante se sabe que tienen hiperactividad del eje adrenal y representan un modelo de hipercortisolismo endógeno[35,43,44].

Por lo tanto, teniendo en cuenta que las alteraciones del estado de ánimo (trastornos depresivos) se asocian a una elevada morbilidad, una mayor mortalidad y un envejecimiento celular, se podría hipotetizar que el acortamiento telomérico podría estar detrás tanto de la morbilidad aumentada como del envejecimiento prematuro, paradigmas del hipercortisolismo endógeno[45]. Es interesante el hecho de que se haya asociado el acortamiento de la longitud telomérica leucocitaria con una elevada excreción urinaria nocturna de cortisol[46]. En la misma línea, y más recientemente, un estudio in vitro en el que se aplicó cortisol a linfocitos T se objetivó una reducción significativa de la actividad de la telomerasa en forma de dosis dependiente[36].

Pero hasta el momento, según nuestros conocimientos, no se ha evaluado el sistema telomérico en la situación más típica de hipercortisolismo endógeno como es el síndrome de Cushing. Únicamente ha sido evaluado en situaciones de seudocushing (depresión mayor crónica y estrés psicosocial crónico).

Papel del acortamiento de telómeros en las complicaciones del síndrome de Cushing

El «link» biológico entre el síndrome de Cushing (caracterizado por el clúster de complicaciones sistémicas mencionadas anteriormente), el aumento del riesgo cardiovascular y el distrés neuropsicológico es la hipersecreción de cortisol, capaz de inducir diferentes mecanismos patogénicos, dando lugar al daño cardiovascular, a un envejecimiento prematuro y a un aumento de la mortalidad. Por lo tanto, podría ser que los niveles elevados de glucocorticoides contribuyeran a un envejecimiento prematuro. Tampoco se puede descartar que los factores ambientales determinen un envejecimiento prematuro.

¿Sería posible que la exposición previa crónica al hipercortisolismo determinara en estos pacientes un envejecimiento prematuro a través del acortamiento de los telómeros? ¿Podría el mecanismo de desgaste telomérico observado en el estrés crónico o en las alteraciones severas del estado de ánimo acontecer también en pacientes con síndrome de Cushing y contribuir a su envejecimiento prematuro, a su aumentada morbilidad residual y mortalidad? Que sepamos, hasta ahora no se ha evaluado el funcionamiento del sistema telomérico en el paradigma más típico de hipercortisolismo endógeno como es el síndrome de Cushing.

En resumen, el síndrome de Cushing es una enfermedad rara que, a pesar de que bioquímicamente se controle el hipercortisolismo mediante tratamiento, se asocia a un envejecimiento prematuro con mayor morbimortalidad. Los mecanismos exactos por los cuales esto pasa no están claros. Si bien los factores ambientales pueden tener un papel en estas consecuencias desfavorables, no se puede descartar que la exposición crónica al hipercortisolismo determine un acortamiento telomérico acelerado y, por tanto, un envejecimiento prematuro. Aclarar estos mecanismos podría ser el primer paso para la prevención y la mejoría de las consecuencias clínicas de estos pacientes.

Conflicto de intereses

Los autores declaran no tener ningún conflicto de intereses.

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REVISIÓN

Pronóstico del paciente tratado de síndrome de Cushing

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PALABRAS CLAVE
Síndrome de Cushing;
Enfermedad de Cushing;
Remisión;
Calidad de vida relacionada con la salud;
Riesgo cardiovascular;
Osteoporosis;
Mortalidad

Resumen El síndrome de Cushing (SC), debido a un adenoma hipofisario productor de ACTH, a tumores suprarrenales o a una secreción ectópica de ACTH, determina hipercortisolismo. Se asocia a mayor morbilidad, especialmente complicaciones metabólicas, cardiovasculares, osteoporosis, alteraciones psiquiátricas y deterioro cognitivo. A pesar de la «curación» bioquímica del hipercortisolismo y la mejora clínica tras tratamiento eficaz, estas complicaciones solo son parcialmente reversibles, observándose también una exacerbación de enfermedades autoinmunitarias. Todo ello conlleva un deterioro de la calidad de vida y un aumento de la mortalidad. En esta revisión se repasan las principales comorbilidades y consecuencias del SC a largo plazo, a pesar de su curación clínica y bioquímica.

KEYWORDS
Cushing syndrome;
Cushing disease;
Remission;
Health-related quality of life;
Cardiovascular risk;
Osteoporosis;
Mortality

Prognosis of patients treated for Cushing syndrome

Abstract Cushing syndrome (CS), due to an ACTH-secreting pituitary adenoma, adrenal tumors, or ectopic ACTH secretion, causes hypercortisolism. CS is associated with major morbidity, especially metabolic and cardiovascular complications, osteoporosis, psychiatric changes, and cognitive impairment. Despite biochemical ''cure'' of hypercortisolism and clinical improvement after effective treatment, these complications are only partially reversible. Exacerbation of prior autoimmune diseases is also seen. All of these lead to quality of life impairment and increased mortality. This review addresses the main comorbidities and long-term consequences of CS despite clinical and biochemical ''cure''.

Introducción

El síndrome de Cushing (SC) endógeno es consecuencia de la exposición crónica a concentraciones elevadas de

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glucocorticoides. El cortisol es el producto final de la estimulación del eje hipotalámico-hipófiso-adrenal (HHA), producido por la corteza adrenal en respuesta a la acción de la hormona adrenocorticotropa (ACTH).

La causa más frecuente de SC es una excesiva liberación de ACTH por un adenoma hipofisario (enfermedad de Cushing [EC]), aunque también puede existir una secreción ectópica de ACTH por una neoplasia. Por otro lado, la propia glándula suprarrenal puede secretar más cortisol por uno o varios adenomas, por hiperplasia adrenal bilateral, por un carcinoma, y más raramente por una displasia micronodular suprarrenal (en el síndrome de Carney) o por la presencia de receptores anómalos que determinan una hiperplasia macronodular bilateral independiente de la ACTH[1]. Característicamente en el SC desaparece el ritmo circadiano normal de la secreción de cortisol, con concentraciones máximas por la mañana y mínimas (prácticamente indetectables) a medianoche, así como el *feedback* fisiológico del eje HHA entre cortisol, ACTH y el péptido hipotalámico CRH.

En las últimas décadas se acumulan evidencias de que a pesar de corregir el hipercortisolismo tras tratamiento eficaz del SC, no se produce una remisión completa de las alteraciones clínicas asociadas[2-4]. Independientemente de la causa, la hipersecreción crónica de cortisol produce obesidad central, atrofia muscular y fatigabilidad, osteopenia, hipertensión arterial, intolerancia a la glucosa, hiperlipidemia, hipercoagulabilidad, altibajos emocionales y depresión, entre otros problemas. Esto conlleva un aumento del riesgo cardiovascular durante la fase activa de la enfermedad y muy probablemente también a largo plazo. Esto se ha ido evidenciando cada vez más en los últimos años, de manera que las comorbilidades y las complicaciones de la fase activa del SC, aunque mejoran claramente tras cirugía o tratamiento médico, persisten en parte tras el tratamiento, con consecuencias negativas sobre el sistema cardiovascular, el hueso, el cerebro, la calidad de vida relacionada con la salud (CVRS) e incluso mayor mortalidad[2-4]. El hecho de que se demore el diagnóstico correcto frecuentemente entre 2 y 5 años hace que el hipercortisolismo ejerza su efecto deletéreo durante mucho tiempo, antes de ser diagnosticado y tratado[5]. Por lo tanto, el seguimiento a largo plazo de estos pacientes es obligado para controlar las complicaciones debidas a la exposición crónica previa a concentraciones elevadas de cortisol.

Esta revisión se centra en el pronóstico y las consecuencias clínicas que presentan pacientes diagnosticados de SC y que han sido «curados» tras tratamiento adecuado, y el impacto que estas comorbilidades tienen en la CVRS y la mortalidad (fig. 1).

Sistema cardiovascular

El hipercortisolismo determina aumento de factores de riesgo cardiovascular como obesidad central, intolerancia a la glucosa, hipertensión, dislipidemia e hipercoagulabilidad (asociadas al síndrome metabólico), y mayor incidencia de aterosclerosis. Esto impacta en su morbimortalidad y determina que la enfermedad cardíaca sea la principal causa de muerte en los pacientes con SC.

Este riesgo cardiovascular persiste elevado incluso 5 años después de estar bioquímicamente curado el hipercortisolismo[6]. Se observó mayor índice de masa corporal (IMC), índice cintura/cadera, hipertensión arterial, concentración de glucemia e insulinemia en ayunas, fibrinógeno y un perfil lipídico desfavorable en comparación con el grupo control apareados por edad y sexo. Esto se acompañaba de incremento del grosor de la íntima media y menor coeficiente de distensibilidad, en comparación con el grupo control, incluso cuando se apareaban por IMC, confirmando una elevada prevalencia de aterosclerosis y factores de riesgo cardiovascular, similar a lo observado en la enfermedad activa, asociado a obesidad abdominal residual y/o a resistencia a la insulina. Un estudio de este mismo año observa que a pesar de la remisión del hipercortisolismo desde hace un promedio de 11 años, existe mayor patología cardiovascular especialmente en mujeres (42% SC vs 18% controles; $p < 0,05$) y pacientes menores de 45 años. Incluso después de excluir pacientes con hipopituitarismo o dislipidemia, los pacientes menores de 45 años presentan mayor prevalencia de calcificaciones coronarias y/o placas no calcificadas ateromatosas en comparación con controles sanos apareados (30% SC vs 0% controles; $p < 0,01$)[7].

En la misma línea, otro trabajo objetiva persistencia del aumento del perímetro abdominal en pacientes con SC (independientemente de la causa) tras un año de remisión hormonal[8]. Recientemente se ha comparado la composición corporal con resonancia magnética de cuerpo entero antes de la intervención y en remisión, 6 meses después de no requerir ya tratamiento con glucocorticoides para tratar la inhibición del eje HHA. Aunque se produjo una reducción en gran parte de los depósitos grasos, en la mayoría de los pacientes persistía sobrepeso u obesidad, a pesar de la remisión de la EC. Asimismo, se redujo la insulinemia y la leptinemia, sin mejora de la adiponectina ni de los parámetros lipídicos (cHDL, cLDL, triglicéridos)[9].

En un estudio caso-control también observamos que tras un promedio de 11 años de remisión, pacientes con SC endógeno seguían presentando mayor masa grasa total y obesidad central que los controles ajustados por edad y sexo[10].

El aumento de la obesidad central y de la grasa visceral es una característica fenotípica del SC y determina una producción alterada de adipocinas. Estas adipocinas pueden contribuir a la patogénesis de complicaciones vasculares, metabólicas e inflamatorias tales como daño endotelial, hipertensión arterial, alteración del remodelado óseo, aterosclerosis e inflamación de bajo grado[11]. El aumento de leptina, resistina y citocinas proinflamatorias, como el factor de necrosis tumoral alfa (TNF-α) y la interleucina-6 (IL-6), se asocian a mayor riesgo cardiovascular. En estudios in vitro se ha visto que la leptina aumenta la actividad del enzima 11 β-hidroxiesteroide deshidrogenada tipo 1 (11β-HSD1), que convierte la cortisona inactiva en cortisol[12]. Por tanto, esta y otras adipocinas y factores humorales podrían estimular más los niveles circulantes de cortisol, contribuyendo a las características típicas del síndrome metabólico, obesidad visceral e hiperleptinemia[13]. Sin embargo, el hecho de que 10 días después de la cirugía de la EC se haya encontrado elevación de leptina a pesar de una caída del cortisol sugiere que otros factores, aparte del cortisol, juegan un papel en la hipersecreción de leptina, como por ejemplo la persistencia de una distribución anómala de la grasa[14]. A largo plazo, con la remisión del hipercortisolismo, la leptina

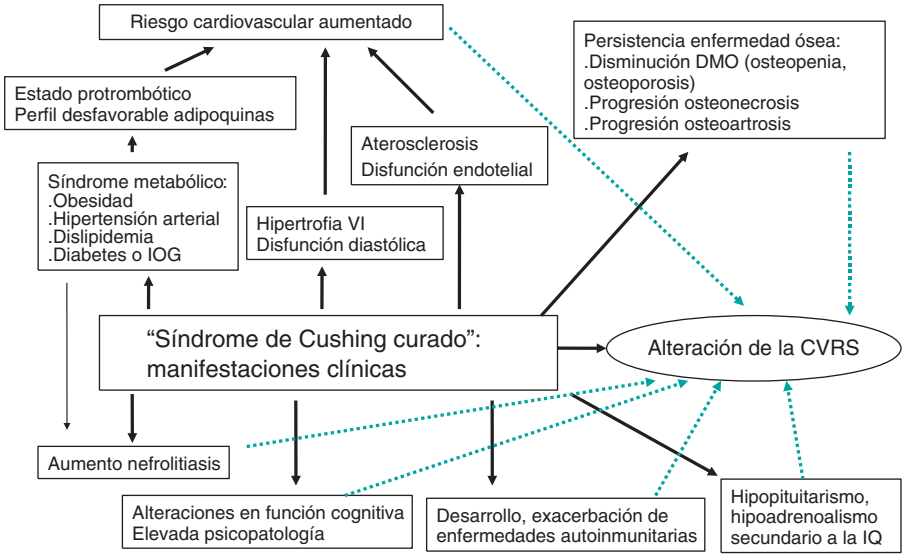

Figura 1 Representación esquemática de las posibles manifestaciones y consecuencias clínicas a pesar de la «curación» del hipercortisolismo.CVRS: calidad de vida relacionada con la salud; IG: intolerancia oral a la glucosa; IQ: intervención quirúrgica; VI: ventrículo izquierdo.

disminuiría de forma progresiva, en paralelo a la disminución del IMC, la masa grasa y la insulina.

La adiponectina tiene actividad antiaterogénica y antiinflamatoria, y está disminuida en la obesidad y en situaciones de insulinorresistencia. Se ha observado que la adiponectina es baja en pacientes con SC activo y en pacientes curados desde hace una media de 11 años, comparados con los controles[10]. El TNF-α es una citocina proinflamatoria con efectos reguladores sobre el metabolismo lipídico, la función de los adipocitos y la señalización insulínica. Su elevación se ha asociado a mayor riesgo de isquemia aguda coronaria recurrente. Se ha observado aumento del TNF-α y de la IL-6 en pacientes con SC activo pero también tras años de curación[10]. Este perfil desfavorable de adipocinas podría contribuir a este estado de inflamación de «bajo grado» presente en pacientes curados de su SC, con el consiguiente incremento persistente del riesgo cardiovascular[10,15]. Si coexiste déficit concomitante de hormona de crecimiento (GH) tras cirugía hipofisaria, empeora todavía más el riesgo cardiovascular y las alteraciones metabólicas[10]; estos parámetros metabólicos, las concentraciones de adipocinas y la composición corporal anómala pueden mejorar tras tratamiento sustitutivo con GH[16,17].

Las *alteraciones en el metabolismo de la glucosa* (resistencia a la insulina, intolerancia a la glucosa y diabetes mellitus) son otros factores de riesgo cardiovascular importantes, y los glucocorticoides favorecen su desarrollo. La prevalencia de estas alteraciones es variable según las series, oscilando entre el 20 y el 47% los pacientes que tienen diabetes mellitus, y entre el 21 y el 64% los pacientes que presentan intolerancia a la glucosa[6,18]. Aunque su prevalencia se reduce con la curación bioquímica, a menudo persisten, observándose concentraciones más elevadas de insulina que en la población control[10].

La *hipertensión arterial* moderada persistente, a pesar de un tratamiento eficaz del SC, parece estar asociada a mayor duración de la hipertensión en la fase activa del hipercortisolismo. Su patogenia parece ser multifactorial: inhibición del sistema vasodilatador, activación del sistema renina-angiotensina-aldosterona e inhibición del catabolismo periférico de las catecolaminas[19]. Además, el aumento de las concentraciones de cortisol puede exceder la capacidad de la enzima 11β-HSD2 (que inactiva el cortisol), facilitando la unión del cortisol a los receptores de mineralocorticoides, lo que conlleva un aumento del efecto de la aldosterona y de la fibrosis del miocardio. En los pacientes en remisión del hipercortisolismo en los que persiste la hipertensión arterial se han observado más alteraciones estructurales y funcionales cardíacas que en sujetos hipertensos controles, lo que sugiere que una exposición previa a hipercortisolismo empeora los efectos negativos de la hipertensión arterial[19].

En esta misma línea, varios grupos han descrito lesiones cardíacas funcionales y estructurales, como hipertrofia ventricular izquierda, disfunción diastólica y disminución del rendimiento sistólico en pacientes con SC activo. Recientemente se ha observado aumento significativo de la fibrosis miocárdica en pacientes con SC activo comparado con controles sanos y con hipertensión arterial, sugiriendo por tanto que el hipercortisolismo podría tener un efecto directo sobre la fibrosis miocárdica independiente de la hipertrofia del ventrículo izquierdo (VI) y de la hipertensión arterial. Parece ser que esta fibrosis es uno de los factores más importantes para el desarrollo de disfunción cardíaca, y también uno de los factores que más condicionará el grado de regresión de la cardiomiopatía observada en el SC[20]. Se objetivó que a los 18 meses del tratamiento exitoso del SC se producía una mejoría de la función sistólica y diastólica del VI en paralelo con una reducción de fibrosis miocárdica.

En un estudio en el que se compararon 15 pacientes con SC y disfunción del VI subclínica con 30 controles apareados por edad, sexo, fracción de eyección e hipertensión, objetivaron que estas anomalías en la estructura y en la función del VI fueron reversibles a los 18 meses después

de la normalización del hipercortisolismo[21]. Un trabajo más reciente observó que los parámetros anormales de la masa ventricular izquierda observados en el 70% de los pacientes con SC activo mejoraron considerablemente durante el seguimiento promedio de 4 años tras remisión del hipercortisolismo, aunque seguían siendo mayores que en los controles[22]. En 25 pacientes con EC se objetivó persistencia del síndrome metabólico, más daño vascular y presencia de mayor número de placas ateroscleróticas en la arteria carótida común comparado con los controles (31,2% vs 6,2%, respectivamente) un año después de la remisión del hipercortisolismo[23].

Así pues, tanto el exceso de cortisol como la hipertensión arterial contribuyen a alterar la masa cardíaca y aumentan la prevalencia de daño en los órganos diana. Es necesario subrayar la importancia de controlar la hipertensión arterial y otros factores de riesgo cardiovascular preoperatoriamente para mejorar el pronóstico a largo plazo.

Existe mayor riesgo de trombosis venosa (tromboembolismo) en los pacientes con SC, especialmente en el periodo postoperatorio. En pacientes sometidos a cirugía transesfenoidal por EC se ha visto que el riesgo de tromboembolismo es mayor que en pacientes operados por adenoma hipofisario no funcionante, apuntando hacia el papel del cortisol (o la ACTH) en la alteración de los factores hemostáticos[24]. Se han visto concentraciones más elevadas de factor VIII, factor IV y factor Von Willebrand en los pacientes con SC, así como aumento de la síntesis del inhibidor del activador tisular del plasminógeno tipo 1 (PAI-1), el principal inhibidor del sistema fibrinolítico[25]. Probablemente este riesgo aumentado de trombosis está favorecido no solo por esta hipercoagulabilidad inducida por el hipercortisolismo, sino también por la propia cirugía y la obesidad que presentan la mayoría de estos pacientes. Se ha visto mejoría de los parámetros del sistema hemostático un año después de la cirugía exitosa, aunque la hemostasia no se normaliza de forma completa[26]. Algunos trabajos sugieren realizar tromboprofilaxis el primer mes después de la cirugía, aunque hacen falta más estudios para evaluar el tiempo necesario para revertir este estado hipercoagulante después de la cirugía curativa.

Hay que tener en cuenta que tanto las deficiencias hormonales asociadas como el tratamiento sustitutivo, y en algunos casos la curación incompleta del SC, pueden estar implicados en estas complicaciones cardiovasculares. Particularmente los pacientes que no presentan hipocortisolismo tras adenomectomía transesfenoidal podrían tener un hipercortisolismo subclínico parecido a lo observado en algunos adenomas suprarrenales (fundamentalmente incidentalomas) donde este hipercortisolismo subclínico se correlaciona con el síndrome metabólico y con el aumento del riesgo cardiovascular[27-29]

En conclusión, el riesgo cardiovascular puede persistir elevado a pesar del control bioquímico del hipercortisolismo. Estas evidencias justifican que se investigue y trate adecuadamente la enfermedad cardiovascular. Aunque se requieren estudios más amplios para elaborar estrategias de manejo específicas para reducir el impacto cardiovascular negativo de la exposición previa a niveles elevados de cortisol, creemos justificable tratar a estos pacientes como otros de alto riesgo cardiovascular, similar a lo que se hace en la diabetes.

Hueso

La prevalencia de enfermedad ósea, principalmente osteoporosis, es elevada en los pacientes con SC y a menudo infraestimada[30]. Aproximadamente entre el 30 y el 50% de los pacientes con SC presentan fracturas, sobre todo a nivel vertebral[5]. Además de la osteoporosis, también se ha reportado osteoartrosis y osteonecrosis en pacientes con SC iatrogénico, pero raramente en pacientes con hipercortisolismo endógeno[31-33].

Los glucocorticoides afectan de forma importante el metabolismo óseo disminuyendo la densidad mineral ósea (DMO). De forma directa, disminuyen la síntesis de colágeno y aumentan su degradación, dado que inhiben la acción, la replicación y la diferenciación de los osteoblastos. Asimismo, aumentan la acción y la supervivencia de los osteoclastos encargados de la degradación.

Además, in vivo los osteoblastos expresan 11β-HSD1, lo que amplifica localmente los efectos de los glucocorticoides; por lo tanto es una enzima que tiene implicaciones importantes en la acción de los glucocorticoides en el hueso. Esta pérdida de DMO también puede ser debida en parte a un hipogonadismo secundario y/o a una disminución de la GH o factor de crecimiento insulinoide tipo I (IGF-I) inducidas por el exceso de cortisol.

De forma indirecta, los glucocorticoides afectan el metabolismo del calcio, del fosfato, de la vitamina D y de la parathormona (PTH) (disminuyen su absorción intestinal por un mecanismo independiente a la vitamina D y la reabsorción renal de calcio, lo que en ambos casos induce un modesto aumento de las concentraciones de PTH); además inducen una pérdida de fuerza y de masa musculares. Uno de los efectos iniciales de los glucocorticoides a nivel celular es el aumento de la producción de RANKL (*receptor activator of nuclear factor-kappa B-ligand*, que promueve la osteoclastogénesis) y la reducción de la producción de osteoprotegerina (OPG) (un antagonista natural de RANKL)[34], aunque en el hipercortisolismo crónico endógeno se han encontrado concentraciones más altas de OPG comparado con controles, manteniéndose elevados tras la normalización del hipercortisolismo especialmente en los pacientes con un riesgo coronario más elevado[35,36]. El sistema RANKL/OPG actúa como regulador paracrino de la calcificación vascular, dado que también se produce en las células endoteliales, y puede ser un marcador de aterosclerosis subclínica[37]. Por lo tanto, probablemente estas concentraciones elevadas de OPG en el SC podrían estar más relacionados con el daño cardiovascular que con el estatus del hueso. La fisiopatología de la enfermedad ósea en el SC se detalla en la figura 2.

Esta pérdida de masa ósea inducida por exceso de cortisol se ha visto que es más prominente en el hueso trabecular, presente en la columna lumbar o en el cuello femoral[38]. De hecho, los pacientes con SC tienen una predisposición especial a sufrir fracturas vertebrales que se presentan con dolor abdominal o disminución de la talla debido a compresión vertebral. No es raro que la DMO refleje valores inapropiadamente elevados en la columna.

La mayoría de trabajos describen una recuperación parcial de la DMO después del tratamiento del SC, aunque las series son pequeñas y el seguimiento mediano relativamente corto (no más de 2 años). La serie con un seguimiento más largo después de la remisión del hipercortisolismo

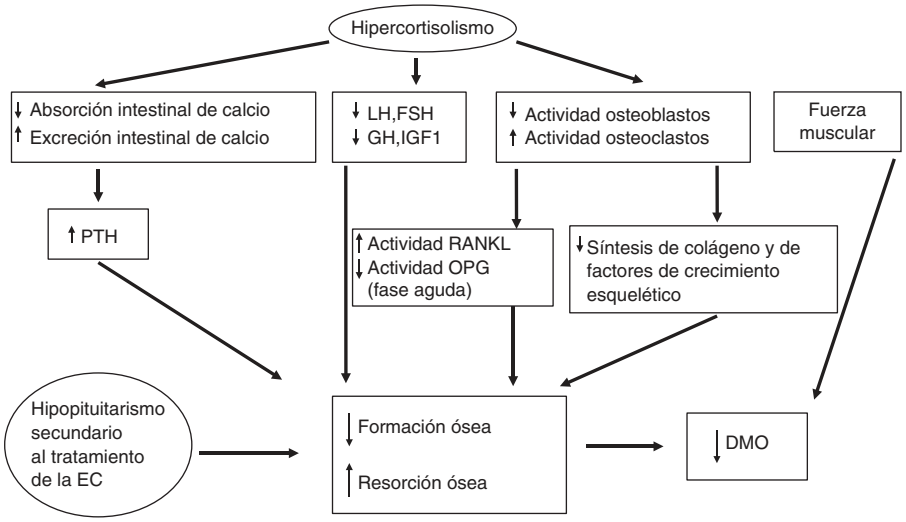

Figura 2 Patogenia de la enfermedad ósea en la EC.DMO: densidad mineral ósea; EC: enfermedad de Cushing; FSH: hormona foliculoestimulante; GH: hormona del crecimiento; IGF-1: factor de crecimiento insulinoide tipo I; LH: hormona luteotropina; OPG: osteoprotegerina; PTH: hormona paratiroidea; RANKL: *receptor activator of nuclear factor-kappa B-ligand*.

(seguimiento medio de 11 años) observó disminución de la DMO en mujeres estrógeno-suficientes comparado con controles apareados por edad, sexo e IMC, pero no en las estrógeno-deficientes (por menopausia o hipogonadismo). Esto sugiere que el efecto protector de los estrógenos en la masa ósea se pierde con el hipercortisolismo. El tiempo de exposición previa al exceso de cortisol endógeno y la duración del tratamiento sustitutivo postoperatorio con glucocorticoides fueron predictores de la baja DMO[39]. Asimismo, la persistencia de una disminución de osteocalcina, tras la remisión del hipercortisolismo, sugiere que la actividad osteoblástica está disminuida, lo que favorecería la no recuperación completa de la DMO.

Además, se ha visto que el hipercortisolismo puede frenar el pico normal de masa ósea en un paciente en crecimiento, contribuyendo a un aumento de fracturas osteoporóticas incluso a largo plazo después de la remisión en jóvenes «curados» de un SC[40]. Probablemente son necesarias terapias adicionales para intentar maximizar el pico de masa ósea en pacientes que han presentado su SC en la infancia o adolescencia para minimizar los efectos secundarios a largo plazo.

Otras evidencias sugieren que las alteraciones en la masa ósea pueden ser reversibles después de remitir el hipercortisolismo. Es probable que en parte esto sea debido a que el tiempo de exposición previo a hipercortisolismo endógeno fuera inferior que en otros trabajos[38,41]. Un estudio prospectivo a largo plazo reporta que la DMO está incluso más aumentada después de la remisión del hipercortisolismo comparado con la situación basal de enfermedad activa, y que esta mejoría se mantiene con los años de la remisión del hipercortisolismo (seguimiento medio de 71 meses)[38]. La recuperación de la DMO se correlaciona de forma positiva con el aumento de los niveles de osteocalcina y del telopéptido C terminal del colágeno tipo 1 (CTX-1), marcadores de recambio óseo ($r = 0{,}92$; $p < 0{,}001$). Se especula sobre los mecanismos que determinan la recuperación de la DMO. Algunos lo atribuyen a un aumento de las concentraciones de osteocalcina al normalizar los glucocorticoides, y a la preservación de la arquitectura trabecular, a pesar del adelgazamiento inducido por los corticoides, de manera que los osteoblastos puedan seguir sintetizando hueso nuevo; esto no ocurre en la pérdida de hueso trabecular por otras causas de osteoporosis[42].

En resumen, la recuperación de la DMO en la mayoría de los casos parece ser solo parcial en los pacientes con SC «curados»; especialmente en pacientes jóvenes que aún no han completado el crecimiento, la hipercortisolemia ejerce un efecto muy negativo sobre el hueso. Se carece de estudios amplios que evalúen la aparición de fracturas a largo plazo en pacientes diagnosticados de SC.

Riñón

Se ha descrito la presencia de nefrolitiasis en la mitad de los pacientes con SC activo y en casi el 30% de los pacientes curados, una prevalencia muy superior a la población general[43]. La patogenia de la nefrolitiasis en el SC no está del todo clara. En parte podría ser consecuencia de la hipercalciuria. Probablemente exista un efecto sinérgico de diferentes alteraciones metabólicas y hemodinámicas producidas por el hipercortisolismo. De hecho, en los pacientes con litiasis renal se ha objetivado una mayor prevalencia de obesidad, hipertensión arterial y diabetes mellitus, condiciones muy frecuentes en el SC. Además, la excreción urinaria aumentada de ácido úrico y cistina son factores que favorecen la nefrolitiasis, y ambas pueden ser consecuencia del exceso de glucocorticoides[44]. En una serie larga donde investigan el papel de los diferentes factores litogénicos conocidos en pacientes con hipercortisolismo, observan que la hipertensión arterial y la excesiva excreción urinaria de ácido úrico son factores de riesgo independientes para presentar nefrolitiasis[43].

Función cognitiva y comportamiento

El hipercortisolismo crónico se ha relacionado con alteraciones de la memoria, del comportamiento, del aprendizaje verbal y del lenguaje, de la actividad neuronal, y con otros procesos del sistema nervioso central. Además, estados psicopatológicos como la ansiedad, la depresión y la manía son muy prevalentes en los pacientes con SC activo, siendo la depresión mayor el trastorno más frecuente, con una prevalencia de entre el 54 y el 81% según las series[45].

A pesar de que la literatura que evalúa la psicopatología después del tratamiento quirúrgico efectivo es escasa, se observa que la mayoría de las alteraciones mejoran al año después de la remisión del hipercortisolismo, aunque no parecen del todo reversibles a largo plazo[46,47].

El hipocampo, la amígdala y la corteza cerebral, importantes estructuras implicadas en la función cognitiva y emocional, son muy ricos en receptores de glucocorticoides. Por lo tanto, son regiones particularmente vulnerables al exceso de cortisol. La patogenia de la pérdida de volumen cerebral inducida por concentraciones elevadas de glucocorticoides probablemente es multifactorial, desde la muerte celular inducida por los glucocorticoides, la interferencia con procesos de transmisión y metabolismo neuronal, hasta la disminución del contenido acuoso cerebral[2]. Los glucocorticoides pueden ocupar receptores tanto de mineralocorticoides como de glucocorticoides. El 11β-HSD2 (que convierte el cortisol en una molécula de cortisona inactiva) no se expresa en el hipocampo ni en otras estructuras límbicas, permitiendo por tanto, una activación sostenida de los receptores de mineralocorticoides por los glucocorticoides. En situaciones de niveles suprafisiológicos de glucocorticoides (donde ambos receptores están ocupados) se produce una disminución de la excitabilidad celular y una atrofia reversible de las dendritas apicales de las neuronas piramidales. Pero si el hipercortisolismo persiste, como ocurre en el hipercortisolismo endógeno, puede conducir a la muerte celular[46].

En estudios con RM cerebrales de alto campo (3 Teslas) se ha objetivado mayor atrofia cerebral comparada con controles normales de la misma edad, que no es totalmente reversible a pesar de la normalización del cortisol[48]. Se ha visto disminución del volumen del hipocampo en el 27% de los pacientes con SC activo comparado con los controles, con una correlación negativa con los niveles de cortisol en plasma. Este menor volumen del hipocampo se asoció a disfunciones en la memoria. De forma interesante, el volumen del hipocampo mejoró al año después de la cirugía comparado con la situación basal, y en algunos de estos pacientes se objetivó mejoría en los tests de función cognitiva[49]. Por otra parte, los glucocorticoides aumentan la acumulación sináptica de glutamato, confiriendo un estado de mayor susceptibilidad a las agresiones y muerte celular. Teniendo en cuenta que la dexametasona es muy potente para el tratamiento del edema cerebral, podría ser que esta pérdida de volumen en el SC fuera en parte secundaria a una disminución del contenido acuoso del tejido cerebral por el exceso de glucocorticoides[46].

Recientemente, una serie larga de 74 pacientes con EC con un seguimiento medio después de la remisión de 13 años presentó peores resultados en términos de memoria y función ejecutiva comparado con controles apareados por edad, sexo y nivel educativo; también tuvieron peor rendimiento que pacientes intervenidos por macroadenoma hipofisario no funcionante. Los resultados mejoraron a mayor tiempo de remisión del hipercortisolismo. El hipopituitarismo y el tratamiento sustitutivo con hidrocortisona fueron predictores independientes de peores resultados en los tests cognitivos[50]. En la misma línea, otro trabajo reciente ha objetivado afectación de la memoria visual y verbal y atrofia cerebral (disminución del volumen de la materia gris total y cortical) en pacientes con SC en remisión comparado con controles. El subgrupo de pacientes con peores resultados en los tests de memoria también tenían una reducción significativa del volumen del hipocampo[48].

El exceso de cortisol se ha asociado a alteraciones en algunos neurotransmisores, como disminución de la síntesis cerebral de serotonina, aumento de la actividad noradrenérgica y concentraciones bajas de ácido 5-hidroxiindolacético en líquido cefalorraquídeo, todos ellos relacionados con la patogenia de la depresión[51]. Doce meses después de la corrección del hipercortisolismo se observa persistencia de psicopatía (principalmente depresión atípica) en el 24% de los pacientes, aunque durante la fase activa de la enfermedad se registró psicopatía en el 66% de los casos. El pánico y las ideas suicidas aumentaron durante el seguimiento. También se han reportado trastornos adaptativos de la personalidad después del tratamiento de la SC, aunque no en todas las series[52].

Todo ello sugiere que la exposición previa y crónica a concentraciones altas de glucocorticoides y la persistencia de alteraciones en el eje HHA después del restablecimiento del eucortisolismo pueden aumentar la vulnerabilidad individual a agentes estresantes[53]. No obstante, para tener conclusiones definitivas hacen falta más estudios con más pacientes, dado que la mayoría son series limitadas y características clínicas heterogéneas.

Podemos concluir que el SC se asocia a elevada prevalencia de psicopatología, principalmente depresión atípica. La exposición previa y crónica a niveles excesivamente elevados de cortisol puede tener efectos irreversibles en las estructuras del sistema nervioso central (principalmente en las áreas de función cognitiva y comportamiento).

Enfermedades autoinmunitarias

Los glucocorticoides ejercen una acción inhibidora sobre el sistema inmunitario. De hecho, en la fase activa del SC existe una involución del tejido linfoide y una linfopenia con un aumento de la susceptibilidad a las infecciones.

Se ha descrito una situación opuesta después de la remisión del hipercortisolismo, donde se objetivada una exacerbación de enfermedades autoinmunitarias previamente existentes. La enfermedad celíaca, la artritis reumatoide, el desarrollo de sarcoidosis o el lupus eritematoso han sido descritos en distintas formas de SC tras de la corrección del hipercortisolismo[54]. De todas formas, la enfermedad autoinmunitaria más frecuentemente descrita es la tiroidits autoinmunitaria (enfermedad de Graves o tiroiditis de Hashimoto). Se objetivó una mayor positividad en la autoinmunidad tiroidea en el 35% de los pacientes «curados» de una EC comparado con los controles (10%).

Parece ser que el desarrollo de enfermedad autoinmunitaria es más frecuente en los pacientes que presentan bocio multinodular o anticuerpos antitiroideos positivos durante la fase activa de la enfermedad, sugiriendo que las anomalías tiroideas preexistentes y una predisposición genética a la autoinmunidad son factores para el futuro desarrollo de trastornos autoinmunitarios de la tiroides después de la normalización del cortisol[55].

La exacerbación de enfermedades autoinmunitarias parece estar relacionada con una mejoría de la actividad inmunitaria, suprimida por el hipercortisolismo endógeno durante la fase activa de la enfermedad; por ello conviene recordar que tras la remisión del SC puede «reaparecer» una enfermedad inmunitaria, silente durante la fase activa del SC.

Calidad de vida relacionada con la salud

La calidad de vida en los pacientes con SC está reducida en la enfermedad activa si se los compara con controles sanos y con pacientes con otros adenomas hipofisarios sin hipercortisolismo. Aunque tras la remisión bioquímica mejora la calidad de vida, no se normaliza del todo, incluso a largo plazo.

En una revisión reciente sobre epidemiología, tratamiento y consecuencias de haber sufrido una EC se describe que la CVRS está comprometida a pesar de la remisión de la enfermedad[4]. En otro estudio con 58 pacientes curados de SC, con un promedio de remisión de 13 años, se objetivó con cuestionarios genéricos peor CVRS comparado con el grupo control, en términos de fatiga, aspectos físicos, ansiedad, depresión y percepción de bienestar, especialmente en aquellos con hipopituitarismo asociado[56,57]. No objetivaron relación con el grado inicial de hipercortisolismo[56].

Recientemente se ha elaborado una CVRS específica para el SC (CushingQoL), que incorpora los aspectos que más afectan y preocupan a los pacientes con SC. El marco temporal se refiere a las 4 semanas previas. En 125 pacientes con SC, algunos con enfermedad activa, otros en remisión o insuficiencia adrenal secundaria al tratamiento, se observó que el hipercortisolismo activo (con cortisoluria elevada) y el sexo femenino fueron los mayores predictores de baja CVRS. No observaron relación entre la CVRS y el tiempo transcurrido desde la cirugía curativa, ni con la presencia de hipopituitarismo, sugiriendo que probablemente las dimensiones evaluadas con el CushingQoL están más relacionadas con el hipercortisolismo que con las otras alteraciones hormonales. Estas alteraciones fueron independientes del origen del SC (adrenal o hipofisario)[58]. Recientemente se ha confirmado que el cuestionario CushingQoL tiene buenas propiedades psicométricas y sensibilidad al cambio para detectar cambios en condiciones de práctica clínica diaria[59].

En pacientes con déficit de GH en tratamiento sustitutivo con GH recombinante humana y EC en remisión se ha observado no solo mejoría de los parámetros metabólicos, sino también de la CVRS a los 3 años de haber iniciado tratamiento con GH[17]. La afectación de la CVRS en pacientes con EC y déficit de GH es mayor que en aquellos con déficit de GH de otras etiologías. Esto sugiere que la exposición previa a hipercortisolismo es un factor de gran impacto sobre la CVRS, más que otras disfunciones hormonales[60].

Esta afectación de la CVRS, incluso años después del tratamiento, tiene consecuencias sociales y económicas. En una entrevista con 74 pacientes con SC tratados, únicamente el 46% se sentían completamente recuperados, el 81% fueron capaces de volver al trabajo, pero al 11% se les concedió la invalidez permanente[61].

Se concluye que a pesar de la reversibilidad del hipercortisolismo, la calidad de vida permanece alterada en pacientes tratados de SC, persistiendo alteraciones físicas y psicológicas, así como cambios metabólicos y cognitivos.

Mortalidad

El SC es una enfermedad potencialmente letal. La EC no tratada se asocia a una supervivencia estimada del 50% a los 5 años[62]. Varios estudios han reportado una mortalidad aumentada entre 2 y 5 veces, comparada con la población de referencia, principalmente debido a causas cardiovasculares[16,63,64]. Un metaanálisis sobre la mortalidad en el SC publicado recientemente observa que la tasa media de mortalidad debida a EC (*Standard Mortality Rate* [SMR]) es de 1,84 (IC 95%: 1,28-2,65). En pacientes con EC persistente después de la cirugía la SMR es de 3,73 (IC 95%: 2,31-6,01), mientras que en pacientes con remisión completa tras la cirugía, su tasa de mortalidad no difiere de la de la población general (SMR: 1,23; IC 95%: 0,51-2,97)[64]. Esta mortalidad está especialmente aumentada el primer año después de la cirugía en los pacientes sin curación inicial posquirúrgica, y en general a mayor tiempo de exposición al hipercortisolismo[65]. Estos resultados han sido confirmados por la mayor serie quirúrgica publicada hasta el momento, en la que se describen 31 muertes en 285 pacientes con EC durante un seguimiento medio de 11,1 años. Comparados con la población normal, los pacientes con enfermedad persistente a pesar del tratamiento mueren con más frecuencia. Además, la supervivencia va disminuyendo a lo largo de los años en aquellos con enfermedad activa. Esto sugiere que debe intentarse conseguir la corrección del hipercortisolismo cuanto antes, para evitar comorbilidades y disminuir la mortalidad[66].

Otro metaanálisis reciente, en el que evalúan únicamente pacientes con EC a lo largo de 50 años, encuentra una mortalidad global que duplica la de la población general (SMR: 2,2; IC 95%: 1,45-3,41), tanto en pacientes con enfermedad activa como en remisión. En los pacientes en remisión no encontraron diferencias en la mortalidad (SMR: 1,2; IC 95%: 0,45-3,2) con respecto a la población general, pero sí en los pacientes con enfermedad activa (SMR: 5,5; IC 95%: 2,7-11,3). En una cohorte de 60 pacientes con un seguimiento medio de 15 años se objetivaron 13 defunciones, 9 de las cuales fueron por causas cardiovasculares, mayor de lo esperado en la población general (SMR 13,8; IC 95%: 7,2-36,5). La presencia de enfermedad activa, mayor edad al diagnóstico, hipertensión y coexistencia de diabetes fueron los mayores determinantes de la mortalidad en esta serie[67].

Desde la utilización de la cirugía transesfenoidal estas tasas de mortalidad han mejorado sustancialmente. De todas formas, la normalización del cortisol puede no ser suficiente para normalizar la mortalidad, ya que pueden coexistir otros factores de riesgo como hipopituitarismo, la

propia cirugía o la persistencia de factores de riesgo cardiovascular.

La edad al diagnóstico y durante el seguimiento de la mayoría de los pacientes incluidos en los estudios oscila entre los 40 y los 60 años. Además, la mayor edad al diagnóstico se relaciona con un peor pronóstico[67]. Por ello, sería recomendable prolongar el seguimiento de estos pacientes más allá de 30 años, para confirmar si la remisión del hipercortisolismo es compatible con la longevidad de la población general. Puesto que la mayoría de estos estudios están realizados con pocos pacientes —dada la rareza del SC—, con un número bajo de muertes y un tiempo de seguimiento variable, deben ser interpretados con cautela, si bien debe hacer reflexionar sobre el peor pronóstico aparente de los pacientes con SC curado.

En resumen, la tasa de mortalidad en los pacientes con EC «curados» podría ser similar a la de la población general, al menos tras 10 a 20 años de seguimiento. Sin embargo, existen evidencias recientes que objetivan mayor riesgo cardiovascular persistente en los pacientes aparentemente «curados» de SC, por lo que parece recomendable tratarlos como pacientes de alto riesgo, implementado medidas profilácticas, como se hace en la diabetes mellitus.

Conclusiones

El hipercortisolismo persistente en el SC se asocia a un elevado número de complicaciones metabólicas, cardiovasculares y cognitivas, solo parcialmente reversibles después de la remisión del exceso de cortisol. Esto conlleva un deterioro en la calidad de vida a pesar de haber pasado años en remisión.

La monitorización crónica a largo plazo es obligatoria para controlar las comorbilidades y aclarar si la persistencia del riesgo metabólico y cardiovascular elevado tiene impacto en la supervivencia de estos pacientes diagnosticados de SC.

Dada la rareza del SC, sería deseable llevar a cabo estudios multicéntricos epidemiológicos exhaustivos que permitan conocer la causa de muerte y la morbilidad en estos pacientes, o estudios prospectivos como el del Registro Europeo de Síndrome de Cushing (ERCUSYN), que actualmente incluye más de 800 pacientes. Solo de esta manera se podrá conocer en detalle el pronóstico a largo plazo y el desenlace final de estos pacientes.

Conflicto de intereses

Los autores declaran no tener ningún conflicto de intereses.

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REVIEW ARTICLE

Telomeres and endocrine dysfunction of the adrenal and GH/IGF-1 axes

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Summary

Telomeres, located at the end of linear chromosomes, are essential to maintain genomic stability. Telomere biology has recently emerged as an important player in the fields of ageing and disease. To maintain telomere length (TL) and reduce its degradation after mitosis, the telomerase enzyme complex is produced. Genetic, epigenetic, hormonal and environmental factors can regulate telomerase function. These include stress hormones such as cortisol and growth factors. The hypothalamic–pituitary–adrenal (HPA) axis has been evaluated in psychiatric diseases where hypercortisolism and oxidative stress are often present. Some researches have linked TL shortening to increases in stress-related cortisol, but others have not. The effects of cortisol on the telomere system are complex and may depend on the intensity and duration of exposure. On the other hand, low levels of IGF-1 are associated with inflammation and ageing-related diseases (ischaemic heart disease, congestive heart failure). Both IGF-1 and TL diminish with age and are positively and strongly correlated with each other. It is not clear whether this positive correlation reflects a single association or a cause–effect relationship. Further research will ideally investigate longitudinal changes in telomeres and both these hormonal axes. To our knowledge, TL dysfunction has not been described in either endogenous hypercortisolism (Cushing's syndrome) or acromegaly where excessive amounts of GH and consequently IGF-1 are produced. This review focuses on the possible relationships between telomere dysfunction and the hypothalamic–pituitary–adrenal (HPA) axis and GH-IGF-1 system.

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Introduction

Telomeres are noncoding repetitive DNA sequences, composed of multiple repetitions of a guanine-rich sequence (TTAGGG), located at the end of linear chromosomes, and protecting them from erosion and end-to-end chromosome fusion. These sequences are covered by a protein complex called Shelterin, which stabilize and protect them. 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.[1] Telomere biology has recently emerged as an important player in the fields of ageing and disease.

To avoid telomere attrition and to maintain telomere length, germ-line cells and a few somatic cells produce telomerase. Telomerase is a specific enzymatic complex involved in telomere repair and elongation. It catalyses telomeric DNA synthesis to reduce chromosomal end degradation after terminal DNA replication and thus maintain telomere length (TL).[2] Telomerase consists of several components, the catalytic component (hTERT) with telomerase reverse transcriptase activity, the telomerase RNA component (TERC) that is used by hTERT as a template to synthesize telomere DNA, dyskerin complex and several proteins which stabilize the whole telomerase machinery.[3]

Telomere length typically decreases with ageing, but the shortening rate is not uniform for all kind of tissues and cells; for example, brain cells and cardiomyocytes show few attritions.[4,5] Undifferentiated stem cells have longer TL, while in more differentiated cells, TL are shorter.[6] Even in 'nondividing' cells, telomeres can be shortened by oxidative stress, which preferentially damages guanine-rich sequences as telomeres, to a greater extent than nontelomeric DNA.[7,8] Stem cell dysfunction provoked by

telomere shortening may be one of the mechanisms responsible for ageing.[9] Moreover, TL is considerably heterogeneous, even in the same cell and for individuals of similar age. Recent studies revealed that TL changes could be dependent on the baseline TL (newborns). In early life, inheritance seems to be an important point, being one of the main determinants of TL. However, the inherited impact decreases with increasing age, due to the effects of environmental factors on TL.[10,11]

Genetic, epigenetic and enviromental factors can regulate telomerase function. These include socio-economic status, lifestyle, autoimmunity, histone methylation and acetylation, stress level, hormones (stress hormones such as cortisol, catecholamines and sex hormones), growth factors, personal habits (smoking, diet, physical exercise) and drugs (such us angiotensin-converting enzyme inhibitors and resveratrol), which can influence and modulate telomerase dynamics and activity.[1] Processes known to modulate telomerase dynamics and to affect telomere length either by shortening or lengthening are summarized in Figure 1. Some behavioural and psychological interventions such as long-term exercise or cognitive behavioural stress management have been shown to increase telomerase activity.[12,13] However, one limitation to most behavioural interventions is poor long-term maintenance of behavioural changes, as these biochemical changes may only last as long as the behavioural and psychological changes are maintained.

Measuring TL may contribute to the understanding of its clinical and biological significance, as it can be used as an indicator of chromosome stability, telomerase activity, proliferative capacity and cellular ageing. Different methods are available to determine TL, each with specific features.[2,14] However, up to now they have mostly been in experimental research rather than in clinical diagnosis and prognosis, for which improvements in cost-effectiveness, sensitivity and availability of large numbers of patients would be necessary.

Therefore, telomere biology can be involved in the pathophysiology of several clinical entities such as cancer,[15] premalignant lesions, aplastic anaemia,[16] fibrosis of the lungs and liver, dyskeratosis congenita, ageing and as a risk factor for cardiovascular disease[17] (poor lipid profile, high systolic blood pressure, fasting glucose, smoking, greater abdominal adiposity).[5,6] Whether a common molecular mechanism is causally involved in the development of these human conditions requires further research with prospective, longitudinal and interventional studies.

Some endocrine diseases like adrenal and GH dysfunction are associated with ageing-like processes and increased cardiovascular risk, but the underlying mechanisms are complex and not always clear. Given the role of telomeres in some of these mechanisms, we decided to review what evidence there was to associate the telomere system with endocrine dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis and the GH/IGF-1 system.

Hypothalamic–pituitary–adrenal axis and the telomere system

To our knowledge, telomere dysfunction has not been described in endogenous hypercortisolism due to Cushing's disease or adrenal adenomas, nor in the more common situation of exogenous hypercortisolism after glucocorticoid (GC) therapy.[18]

In contrast, it has been evaluated in different psychiatric diseases like acute and chronic stress and post-traumatic stress disorder, where hypercortisolism is often present, representing another model of endogenous hypercortisolism. However, these neuropsychiatric conditions are not the best model of hypercortisolism on which to base conclusions about telomere dynamics, due to their complexity with concurrent changes in stress hormones, neurotransmitters, autonomic activity, cytokines, inflammation and oxidative factors.

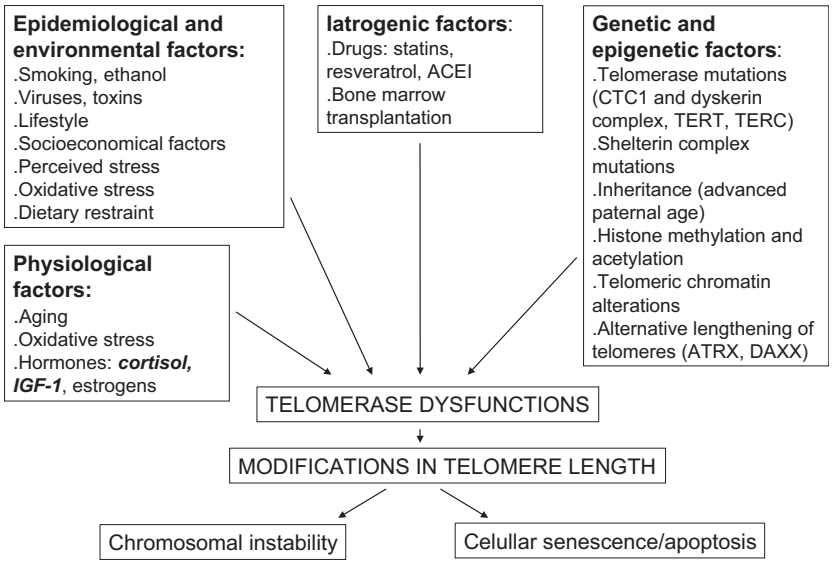

Fig. 1 Processes known to affect telomere length either by shortening or lengthening (CTC1: conserved telomere maintenance complex component 1; IGF-1: insulin-like growth factor type 1; TERT: Telomerase reverse transcriptase; TERC: Telomerase RNA component; ATRX: ATP-dependent helicase; DAXX: death associated protein 6; ACEI: angiotensin-converting enzyme inhibitors).

There is substantial evidence supporting an association between psychiatric disorders and abnormalities in stress-related biological systems, such as the HPA axis and inflammatory responses.[19,20] These abnormalities could provide a basis for investigating a relationship between telomere shortening and accelerated ageing.[21,22]

Chronological ageing impairs an organism's ability to sustain efficient allostasis when responding to different stressors. This is well demonstrated by examining physiological regulation of HPA axis responses. The cortisol response to stressors can be exaggerated in the elderly, with a slow negative feedback, so that cortisol stays elevated for longer.[23] The actual significance of hypercortisolaemia remains unknown, and it is still debatable whether 'hypercortisolaemia' results in net hypercortisolism at the cellular level or rather in net hypocortisolism due to a downregulation of the GC receptor. Furthermore, in some situations, hypercortisolaemia could represent a homeostatic attempt to overcome GC-receptor resistance.[22] Thus, cortisol levels in blood do not necessarily reflect cortisol signalling at the cellular and genomic levels.[24] In fact, the research linking chronic stress, telomere system and HPA function is sometimes contradictory, and some studies reporting increased activation of telomerase activity, while others describe the opposite.[25]

Psychological and oxidative stress-related with increased HPA activity and the telomere system

Recently, a meta-analysis of GC as modulators of oxidative stress showed that they increased oxidative stress with duration of treatment. In addition, GCs cause different levels of oxidative stress among different tissues, with the brain being the most susceptible to damage.[26] It seems that chronic psychological stress is perceived by the cortex of the brain, inducing secretion of hypothalamic corticotrophin-releasing hormone (CRH), leading to increases in ACTH and cortisol levels, which could be used as an index of stress reactivity. Chronic stress is believed to favour disease by activating the HPA axis.[21,27] This stress-related dysregulation of the HPA axis leads to cortisol-induced changes such as reduced availability of intracellular glucose energy stores, neurotoxic effects in certain brain areas (prefrontal cortex and hippocampus), excitotoxicity (increasing glutamate secretion), neuroinflammation (immune alterations) and accelerated cell ageing, via effects on the telomere/telomerase maintenance system. In fact, in patients with major depression, hippocampal atrophy is often reported.[28,29] These lesions are similar to those observed in patients with Cushing's syndrome, suggesting a possible 'pro-ageing' effect of GCs in certain cells of the body.[18,30–32]

Furthermore, altered HPA axis activity together with stress can increase oxidative damage and decrease antioxidant mechanisms. Oxidative stress damage occurs when the production of oxygen free radicals exceeds the capacity of the body's antioxidants to neutralize them. Elevated plasma and/or urine oxidative stress markers have been reported in patients with depression or individuals with chronic psychological stress.[33] As mentioned earlier, the guanine-rich strand of telomeres is more sensitive to oxidative damage compared with other genome sequences.[8] In fact, oxidative stress is inversely correlated with telomerase activity as well as TL.[34–36] Therefore, accelerated telomere shortening may reflect stress-related oxidative damage to cells and accelerated ageing.

Some studies have linked accelerated leucocyte telomere shortening to several psychosocial stress situations, such as mood disorders and in caregivers, like mothers of chronically ill children or partners of patients with Alzheimer's disease. Mothers who look after a chronically ill child have shorter telomeres in peripheral blood mononuclear cells (PBMCs), relating more years of caregiving to lower telomerase activity, and higher levels of perceived stress and oxidative stress index (isoprostanes per milligram of creatinine/vitamin E) compared with controls (noncaregiving mothers).[37] These findings provide a potential mechanism for stress-associated TL attrition. Studies examining the relationship between TL and the HPA axis are summarized in Table 1.

Supporting the chronic stress model of accelerated ageing, preliminary evidence shows that certain mood disorders are associated with accelerated ageing and could be a novel mechanism for mood disorder-associated morbidity and mortality.[38] Shorter leucocyte telomeres in 44 patients with depressive mood or bipolar disorders were observed when compared to 44 gender and sex-matched control subjects, corresponding to 10 years of accelerated cell ageing.[38]

This telomere shortening, at least in part, could be related to increases in stress-related cortisol and catecholamine output. Average leucocyte telomere length was evaluated in 647 women who had a sister with breast cancer, in relation to perceived stress and urinary catecholamines and cortisol. They observed accelerated telomere shortening in the groups with higher perceived stress and with higher levels of urinary catecholamines. A trend towards telomere shortening in those with higher levels of urinary free cortisol was also observed without reaching statistical significance.[39] These results suggest that the effect of stress on TL may vary depending on neuroendocrine responsiveness and external stressors as well as on age.

Similarly, shorter buccal cell TL in children was observed in 6-year-old children exposed to laboratory stressors, with higher levels of salivary cortisol and higher autonomic reactivity. These authors suggest that buccal cell TL may be a useful marker of early biological ageing.[40]

Some preliminary data suggest that telomere shortening depends on the duration of exposure to depression or a stressor.[33] In 18 patients with major depressive disorders and 17 sex- and age-matched controls, average leucocyte telomere length was significantly inversely correlated with lifetime depression exposure, even after controlling for age.[33] This suggests that telomere shortening may progress in proportion to lifetime depression exposure and that a longer exposure to hypercortisolaemia could lead to a greater decrease in telomere length.

Greater cortisol responses and dysregulated patterns of daily cortisol secretion were associated with shorter telomeres in PBMCs in 14 postmenopausal women caring for a partner with dementia, compared with age- and BMI-matched noncaregivers.[41] Specifically, higher overnight urinary free cortisol levels, higher salivary cortisol response to acute stress and flatter daytime cortisol slopes were associated with shorter TL. However, when they evaluated TL in whole blood (mostly of short-life granulocytes),

Table 1. Studies examining the relationship between telomere system and the HPA axis

Diagnosis or type of stress	References	Total *n*	Findings
Perceived stress (Breast cancer sisters)	36	647	Accelerated telomere shortening with higher levels of urinary catecholamines and urinary free cortisol
Laboratory stressors	37	78	Shorter buccal cell TL in children with higher levels of salivary cortisol and higher autonomic reactivity
High caregiver stress (dementia caregiver)	38	14	Shorter telomeres in PBMCs were associated with greater cortisol responses and dysregulated patterns of daily cortisol secretion
	39	22	Telomerase activity increased during acute stress associated with greater salivary cortisol increases in response to stressor
Acute mental stress	48	62	Lower leucocyte telomerase activity was associated with exaggerated autonomic reactivity and with increased excretion of stress hormones (catecholamines and cortisol) in response to acute mental stress
Dietary restraint	40	56	Premature telomere shortening was observed in women with dietary restraints linked to greater perceived stress and elevated salivary and urinary cortisol
High hydrocortisone levels *in vitro*	45		50% reduction of telomerase activity of T lymphocytes was observed with exposure to high hydrocortisone levels *in vitro*
Embryonic exposure to corticosterone	47	60 (eggs)	Shorter telomeres were observed with exposure to exogenous corticosterone during embryonic development (domestic chickens)
Mindfulness-based intervention for stress eating	49	47	Changes in telomerase activity were negatively associated with changes in serum morning cortisol levels (after intervention)
Dexamethasone administration in mice thymocytes	49		Rapid and dynamic loss of telomeric sequences in dexamethasone-treated thymocytes
Major Depressive Disorder and hypocortisolism	52	91	Shorter TL was associated with depression and hypocortisolaemic state (low post-DST cortisol and high percentage of cortisol reduction after the DST)

DST, dexamethasone; PBMCs, peripheral blood mononuclear cells.

they found no relationship between TL and HPA axis dysregulation. This may be explained by the fact that these cells are not exposed to blood cortisol as much as the more long-lived circulating PBMCs, which play an active role in the early acute stress response. Future studies examining different leucocyte cell types and their relationship to TL in specific subpopulations of leucocytes may contribute to clarify these phenomena further.

Another group observed similar findings when 22 high stress dementia caregivers were exposed to a brief laboratory psychological stressor compared with 22 matched low stress controls. At baseline, caregivers had lower telomerase activity, but during acute stress telomerase activity increased similarly in both groups independently of leucocyte cell type and associated with greater salivary cortisol increases. These findings suggest novel relationships of dynamic telomerase activity with exposure to an acute stressor.[42]

Similarly, leucocyte telomere length was evaluated in a group of pre- and postmenopausal women with self-reported dietary restraints (defined as chronic worry about weight and attempts at restricting food intake), which are often linked to greater perceived stress as well as to physiological factors known to be related to long-term stress, such as elevated salivary and urinary cortisol.[43] Dietary restraint, independently of body mass index, was a risk factor for premature telomere shortening, in which HPA dysfunction could be implied.[44]

Moreover, chronic stress is related with a low health index, with an increase of cardiovascular risks factors and alterations in immunological systems, similar to what is observed in patients with Cushing's syndrome. However, the exact mechanisms involved are still unknown. Chronic stress can lead to a state of metabolic stress (overeating, co-elevations of cortisol and insulin levels and suppression of certain anabolic hormones such as androgens or GH), which in turn promotes abdominal adiposity. Both metabolic stress and abdominal adiposity can facilitate systemic inflammation and oxidative stress, which appear to mediate several cell ageing mechanisms such as leucocyte telomere length shortening and cell senescence.[45] Hence, HPA dysregulation could provide a common biological link, inducing changes in the telomere system, impairing health status and increasing cellular damage both in Cushing's syndrome and chronic psychosocial stress. Hypercortisolaemia probably contributes to premature ageing by inducing accelerated telomere shortening, which could be implied in the persistent morbidity and clinical consequences associated with Cushing's disease, even years after being biochemically cured of hypercortisolism.[18,46]

Consistent with these observations, one study *in vitro* observed that exposure to high hydrocortisone levels comparable with those that might be reached *in vivo* during stress, is related to a significant reduction of telomerase activity in T lymphocytes, by as much as 50% 3 days later.[47] This effect is observed in both CD4 and CD8 T lymphocytes and is associated with reduced transcription of hTERT, the telomerase catalytic component. This could be one of the mechanisms in which hyperstimulation of the HPA could alter the immunological system, inducing immunosenescence and conferring higher infection

susceptibility, as observed in patients with higher levels of stress or with Cushing's syndrome. These data suggest that immunosenescence may be closely related to both psychological distress and stress hormones (cortisol) and partially to telomere dysfunction.[48] Based on the hypothesis that glucocorticoids are a well-known immunosenescense inducers, Ichiyoshi *et al.* investigated the changes in thymocytes after dexamethasone administration in mice. They observed that dexamethasone-treated thymocytes exhibited rapid and dynamic loss of telomeric sequences and upregulation of telomerase RNA as an early event in the apoptotic process. The loss of thymocytes coincided with the appearance of small dense cells with characteristic features of apoptosis (condensed chromatin, internucleosomal DNA cleavage and hypodiploid peak on flow cytometry).[49] Some mechanisms, such as the regulation of shelterins and dyskerin expression or the regulation of genes implicated in the lengthening of telomeres (such as ATRX or DAXX) could be affected by glucocorticoids. Moreover, the methylation pattern of the subtelomeric regions either directly or indirectly by some miRNA families could also be regulated by cortisol levels. However, to our knowledge, the effect of cortisol in these possible mechanisms, which could modify telomere length, has not been evaluated and is unclear.

Recent research has observed that embryonic exposure to corticosteroids in domestic chickens resulted in higher levels of reactive oxygen metabolites and shorter telomeres compared with control birds.[50] Similarly, in 62 healthy women, it was found that lower levels of leucocyte telomerase activity were associated with exaggerated autonomic reactivity to acute mental stress and with increased excretion of stress hormones (catecholamines and cortisol). It was also observed that low telomerase activity was associated with major risk factors for cardiovascular disease (smoking, poor lipid profile, high systolic blood pressure, high fasting glucose, greater abdominal adiposity). However, PBMCs TL was not correlated with cardiovascular disease risk factors, suggesting that telomerase activity may be an earlier marker of cell ageing than TL.[51]

Recently, the first study to show a longitudinal association between coexisting changes in cortisol and telomerase activity in unstimulated PBMCs has been published.[52] The authors examined whether participation in a mindfulness-based intervention and improvements in psychological distress, eating behaviour and metabolic factors (weight, serum cortisol, fasting glucose and insulin, and insulin resistance) were associated with increases in telomerase activity in PBMCs. They observed that changes in chronic stress, anxiety, dietary restraint, cortisol and glucose were negatively correlated with changes in telomerase activity. These results support the model that changes in stress-related cortisol might be one of the signals regulating telomerase levels in humans.[52]

Psychological and oxidative-stress related with decreased HPA activity and the telomere system

Although stress has traditionally been associated with increased cortisol secretion and HPA axis overactivity, some recent literature describes low cortisol levels in certain stress-related disorders, suggesting that chronic stress could lead to an exhaustion of the HPA axis.[25] Hypocortisolaemia, or low CRH, has been related with atypical depression, states of chronic fatigue and post-traumatic stress syndrome, contributing functionally to symptoms of inflammation and fatigue.[53,54] A recent paper reports that shorter leucocyte telomeres were associated with depression and hypocortisolism.[55] To our knowledge, telomere dysfunction has not been described in Addison's disease, the ideal model of primary endogenous hypocortisolism, or in hypopituitarism, where secondary adrenal insufficiency is often present.

Dexamethasone cortisol suppression (the percentage change of cortisol between pre- and postdexamethasone cortisol) was higher in a group of depressive patients compared with a control group. Furthermore, subjects exhibiting a high level of suppression (lower postdexamethasone cortisol levels) had significantly shorter leucocyte TL.[55] Decreased activity of the HPA axis has been shown to develop from long-term chronic stress exposure, where an initial stage of a hyperactive HPA axis eventually evolves into a hypo-active HPA axis. Highly sensitive negative feedback in the HPA axis (low postdexamethasone cortisol, high degree of cortisol suppression) is probably the most common finding in subjects exhibiting hypocortisolism. The observation of shorter TL and hypocortisolism could be the result of independent pathways of chronic stress exposure or due to higher degrees of inflammatory processes, which would lead to increased proliferation of leucocytes and higher levels of oxidative stress, both contributing to accelerated TL shortening.[3] It is difficult to know which is responsible for accelerated telomere shortening when a hypocortisolaemic state is often preceded by a hypercortisolaemic phase. The observation of shorter leucocyte TL in these situations suggests that leucocyte TL could be a good measure of cumulative stress.

To summarize, it should be noted that the effects of cortisol on the telomere system are complex and may depend on the intensity and duration of exposure. Shorter exposure and shorter duration appear stimulatory, rather than suppressive, to the telomerase system. Although acute spikes in cortisol could be associated with a short-term increase in telomerase, they are also associated with a longer term shortening of leucocyte telomere length, suggesting that, over time, stress and cortisol reactivity could promote telomere shortening.

We should consider some important limitations of the available studies that could provide explanations for the differences observed. Different methods to measure cortisol exposure have been used (questionnaires, circadian rhythm disruption, urinary free cortisol, salivary cortisol, response to dexamethasone, hydrocortisone exposure *in vitro*). Moreover, different methods to measure telomere length have been reported, mostly conventional techniques (Southern blot, PCR), while none of the presented studies use novel technologies such us STELA, which seems to show a better relationship with ageing and disease.

Future research will ideally enable further investigations into longitudinal changes in telomeres.

Growth hormone (GH) and Insulin-like Growth Factor 1 (IGF-1) axis and the telomere system

It is well known that leucocyte TL reduces with increasing age. The shortening of telomeres may act as a mitotic clock regulating the number of divisions a cell can undergo, being a biomarker of ageing.[4, 56] However, in elderly men, TL may not decrease further, reaching a plateau, possibly due to selection by mortality, meaning that mortality may increase in men with shorter telomeres and the disappearance of men with shorter telomeres would result in an increase in the mean value in the remaining men. Alternatively, telomerase may be more active in increasing leucocyte TL in men with critically short leucocyte TL.[57]

IGF-1 is an important regulator of cell growth and proliferation. Its serum concentration reduces with increasing age. Also, serum IGF-1 concentration, with increasing age, is positively associated with parameters reflecting general health such as lean mass, physical activity and nutritional intake. Relatively low circulating levels of IGF-1 in humans are associated with age-related diseases and decrease in longevity. Diminished longevity has been observed in pathological situations, which display low levels of IGF-1 such us hypopituitarism due to multifactorial reasons compared with age- and sex-matched controls.[58] In GH resistance syndromes or untreated patients with isolated childhood-onset GH deficiency reduced longevity has also been observed.[59]

Despite these links between GH/IGF-1 and good metabolic health in humans, IGF-1 has been linked to shorter lifespan in lower species and some mammalian models.[60] Therefore, the link between IGF-1 and longevity, in humans, does not fit neatly into a simple paradigm; for these reasons, some groups have examined the association of leucocyte TL with circulating levels of IGF-1. Low levels of IGF-1, and also short leucocyte TL, are associated with age-related diseases, mainly atherosclerosis and diminished longevity. Barbieri *et al.* examined this possible association in healthy individuals free of any major ageing-related diseases. Both variables, leucocyte TL and IGF-1, diminished with age and showed positive and strong correlations between each other.[61] Therefore, short leucocyte TL may be a reflection of the poor general health in these men.

On the other hand, IGF-1 may reduce inflammation, which could have a protective role against telomere attrition. IGF-1 acts as an anti-inflammatory molecule inhibiting IL-6 expression and increasing its clearance. Both higher IL-6 and lower IGF-1 levels confer increased risk of having metabolic syndrome.[62] IGF-1 seems to upregulate nitric oxide synthase in the vascular endothelium, which would cause vasorelaxation, a beneficial phenomenon to the ageing vasculature, which also would decrease oxidative stress/inflammation.[63] This systemic effect of IGF-1 might ultimately explain the link between IGF-1 and TL in humans. Additionally, low serum IGF-1 concentration has been found to be a risk factor for ischaemic heart disease, congestive heart failure and even for increased mortality.[64] It is not clear whether the positive relation between IGF-1 concentration and TL reflects a single association or a cause and effect relationship.

The possible interaction between circulating IGF-1 and TL has been studied in a few series. Table 2 summarizes studies examining the relationship between the telomere system and the GH/IGF-1 axis.

IGF-1 affects cell replication and is involved in growth, proliferation and transformation of many cell types. It plays a critical role in the G1 and S phase of the cell cycle. On its own, it cannot stimulate entry into the G1 phase, but it is thought to be necessary for maintaining G1 and entry into the S phase in many cell types, including mitogen-stimulated human leucocytes. Therefore, IGF-1 could be a tangible candidate involved in telomerase activation in cell growth and proliferation. Upregulation of telomerase activity by IGF-1 has been observed in several cancer cell lines.[65] For the first time, Tu *et al.* in 1999 studied *in vitro* the effect of IGF-1 on telomerase activity and on telomerase component's complex in human cord blood mononuclear cells. Interestingly, IGF-1 alone did not increase the telomerase activity of cord blood mononuclear cells but could enhance the phytohaemagglutinin-induced (T-cell stimulating agent) increase in telomerase activity. The results suggested that IGF-1 may modulate telomerase activity supporting its potential role in increasing replicative potential of cord blood lymphoid cells or haematopoietic stem cells. Nevertheless, little is known about whether these two systems interact *in vivo*[66] The mechanisms of telomerase activation in cancer cells by IGF-1 and the potential effects of IGF-1 on telomerase in normal somatic cells need to be further elucidated.

In a recent study, the relationship between leucocyte TL and IGF-1 in 551 adults older than 65 years was evaluated. No correlation between TL and plasma IGF-1 concentration was observed

Table 2. Studies examining the relationship between TL and the GH/IGF1 axis

Study population	References	Total *n*	Findings
Healthy subjects	58	476	Longer leucocyte TL were associated with higher circulating levels of IGF1
In vitro: cord blood MNC stimulated by PHA	62		IGF1 increased telomerase activity in PHA stimulated cells
Participants among the cardiovascular health study (adult men)	63	551	Higher IGF1 values may be an independent predictor of longer leucocyte TL
Elderly men	64	2744	TL was positively associated with serum IGF1 and negatively associated with age

TL, telomere lenght; MNC, mononuclear cells; PHA, phytohaemagglutinin; IGF1, insulin growth factor 1.

in univariate regression analysis. However, in multivariate regression analysis, a positive association between plasma IGF-1 and TL was observed after adjustment for multiple confounding factors, such as age, sex, race, smoking status, body mass index, hypertension, diabetes and serum lipids.[67] The results of this study suggest that higher IGF-1 values may be an independent predictor of longer leucocyte TL, consistent with prior evidence suggesting a role of IGF-1 in mechanisms related to telomere maintenance in immune cells.[67] They also observed that this association was stronger in men than in women, possibly due to gender differences in the regulation of leucocyte TL.

Another large population-based cross-sectional study with 2744 elderly men (mean age 75·5 years), observed that leucocyte telomere length was positively associated with serum IGF-1 and negatively associated with age.[68] In contrast with other studies, in this last series, leucocyte TL was independently associated with serum C-reactive protein concentrations, where IGF-1 seems to reduce inflammation.[62]

Mechanisms underlying the association between TL, IGF-1 and senescence remain to be determined. It is not fully clear whether measurements of TL in leucocytes are representative of the processes that occur in other somatic cells, as TL may differ by cell type. Nevertheless, there are correlations between TL in different tissues, which suggests that TL in leucocytes could serve as a surrogate for relative TL in other tissues. We must also take into account that telomere shortening and IGF-1 axis are not the only mechanisms that affect cell senescence; environmental stress-mediated accumulations of DNA mutations (reactive oxygen species, ultraviolet irradiation, chemical mutagens or endocrine signals such us IGF-1/insulin signalling) and the intrinsically encoded biological clock that dictates lifespan events of any particular cell type can also affect cell senescence. Some genes implied in the regulation of the mechanism of alternative lengthening of telomeres such as ATRX (ATP-dependent helicase) or DAXX (death domain-associated protein) participate in chromatin remodelling of telomeres and other genomic sites. In ATRX-null embryonic mice, which exhibit telomere dysfunction, reduced growth and shortened lifespan, DNA damage and tissue attrition are found in the anterior pituitary cells, resulting in low circulating levels of IGF-1.[69] On the other hand, a type III protein deacetylase (SIRT1) is considered a novel anti-ageing protein involved in regulation of cellular senescence/ageing and inflammation, being a positive regulator of telomere length *in vivo*. SIRT1 has been shown to modulate the activity of FoxO, a transcriptor factor that is downstream of the IGF signalling system. The loss of SIRT1 in mice results in increased expression of the IGF-binding protein type 1 (IGFBP1), a modulator of IGF-1 function. Whether these alterations are also present in humans, and any potential effects on telomere system, are unclear.[70]

These mechanisms are directly tied to changes in nuclear function and structure and affect both somatic and stem cells, which are responsible for proper tissue rejuvenation.[71]

To our knowledge, the telomere system has not been evaluated in patients with acromegaly, where excessive amounts of GH and consequently IGF1 are produced.

Further investigations are necessary to examine how the interplay between the GH/IGF1 system and telomere regulation affects immune ageing and the risk of age-associated diseases.

To summarize, telomeres are essential to maintain genomic stability. When telomeres are critically short, cell division stops, and senescence and apoptosis are induced. Telomere length can be influenced and modified by genetic, epigenetic, environmental and hormonal factors. The review focuses on the possible relationships between telomere dysfunction and the HPA axis on the one hand and the GH-IGF-1 system on the other.

Most of the evidence linking the telomere system and HPA function has been evaluated in psychiatric diseases (chronic stress, post-traumatic stress disorders and major depression), where hypercortisolism is often present. Some of the findings have been contradictory, with some studies reporting increased activation of telomerase activity, while a few others conclude the opposite. The possible mechanisms by which cortisol could modify telomere length have not been systematically evaluated and are presently unclear.

Both IGF-1 and TL diminish with age and are positively and strongly correlated. Low levels of IGF-1 are associated with inflammation and ageing-related diseases, processes in which TL has been found to be reduced. However, mechanisms underlying the association between TL, IGF-1 and senescence remain to be determined.

TL dysfunctions have not yet been evaluated in either endogenous hypercortisolism due to Cushing's syndrome or in acromegaly where excessive amounts of cortisol or IGF-1, respectively, are present.

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Conflict of interest

The authors declare no conflict of interests.

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