Long-term impact of endocrine tumors

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Long-term impact of endocrine tumors

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Chapter 1

General introduction and outline of this thesis

General introduction

The classical endocrine system comprises a number of endocrine glands (Figure 1). Endocrine glands produce hormones – signalling molecules that are transported by the circulatory system to target distant organs to regulate physiology and behaviour. Hormones have diverse chemical structures; steroids and amino acid/protein derivates (amines, peptides and proteins). Normally, the secretion of hormones by endocrine glands is carefully regulated to meet the physiological needs. However, sometimes, abnormal hormonal secretion or enlargement of an endocrine gland occurs due to benign or even malignant growth. In this thesis consequences of abnormal hormonal secretion and/or neoplasia in three of these glands -the pituitary gland, the adrenal glands and the thyroid gland- are discussed.

The pituitary gland

The pituitary gland (Figure 2) is about the size of a pea and weighs around 0.5g in humans. It is situated at the base of the brain [1]. The anterior pituitary arises from an invagination of the oral ectoderm forming Rathke's pouch. Endocrine cells of the anterior pituitary are controlled by regulatory hormones released by hypothalamic cells into capillaries leading to venous blood vessels, which in turn lead to a second capillary bed in the anterior pituitary. This network constitutes the hypothalamo-pituitary portal system. After diffusing out of the second capillary bed, the hypothalamic releasing hormones then influence the function of anterior pituitary endocrine cells, stimulating or inhibiting the release of their hormones [2]. The anterior pituitary synthesizes and secretes multiple hormones with specific actions on their target tissues: prolactin, growth hormone (GH), adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid stimulating hormone (TSH). The peripheral hormones of the target glands have feedback control on the pituitary in order to balance hormonal production. Two of these hormonal axes are disturbed in two of the diseases that are subject of this thesis: the somatotropic axis (acromegaly) and the hypothalamic-pituitary-adrenal (HPA) axis (Cushing's syndrome (CS) or Cushing's disease (CD) in case of pituitary origin). The somatotroph cells account for almost 50% of the total anterior pituitary cell population [3]. GH is mainly secreted under hypothalamic control of growth hormone releasing hormone (GHRH) and its secretion is suppressed by somatostatin. GH induces the production of insulin like growth factor type 1 (IGF-1) in the liver. IGF-1 induces proliferation and inhibition of apoptosis in nearly all cell types of the body. ACTH producing cells account for 20% of the total anterior pituitary cell population. Its production is stimulated by hypothalamic corticotropin releasing hormone (CRH) secretion. ACTH stimulates the adrenal glands to produce cortisol. The major function of the HPA-axis is to mediate the neuroendocrine stress response and maintain metabolic homeostasis.

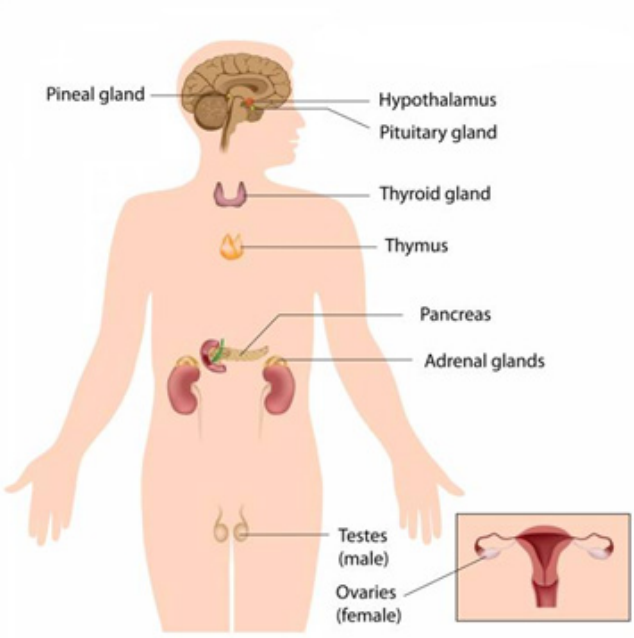

Figure 1 The endocrine system

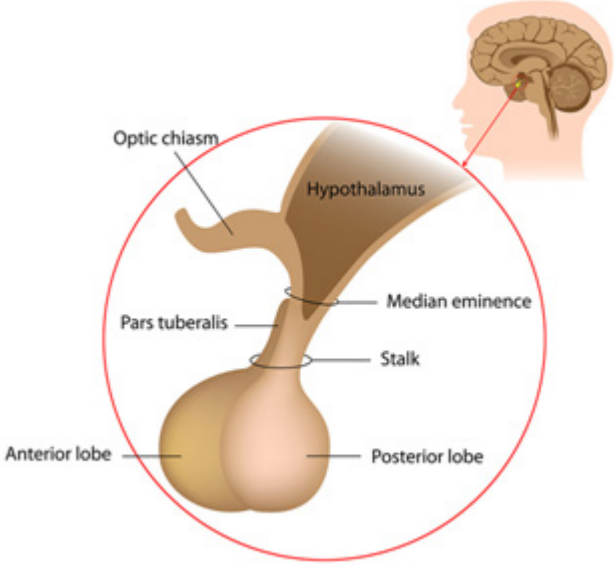

Figure 2 Pituitary gland

The adrenal glands

The adrenal glands are located on top of the kidneys (Figure 3) [1]. They produce a variety of hormones, e.g. adrenalin, aldosterone and, of special interest in this thesis, cortisol. Cortisol is mainly produced by the zona fasciculata of the adrenal cortex. The adrenal cortex is the outermost layer of the adrenal gland. The effects of cortisol are widespread. Major actions of the hormone are that it stimulates gluconeogenesis, glycogen deposition, protein catabolism, fat deposition, sodium retention, potassium loss, free water clearance and uric acid production. Cortisol also recruits neutrophils from the bone marrow and has inhibitory effects on protein synthesis, host response to infection, lymphocyte transformation, delayed hypersensitivity, circulating lymphocytes and circulating eosinophils [4].

The thyroid gland

The thyroid gland consists of two connected lobes. It is found ventral in the neck below the laryngeal prominence (Figure 4) [1]. It secretes thyroid hormones that influence metabolic rate, protein synthesis, and have a wide range of other effects, including on development. The thyroid hormones (triiodothyronine and thyroxine,T_3 and T_4) are synthesized from iodine and tyrosine. The thyroid also produces calcitonin, which plays a role in calcium homeostasis [5]. Hormonal output from the thyroid is regulated by thyroid stimulating hormone (TSH) secreted from the anterior pituitary, which itself is regulated by thyrotropin releasing hormone (TRH) produced by the hypothalamus [2]. In the thyroid gland, nodules, either benign or malignant (thyroid carcinoma) can develop, the latter being of special interest in this thesis.

Cushing's syndrome

Cushing's syndrome was first described by Harvey Cushing in 1932 [6]. Cushing's syndrome is a combination of signs and symptoms caused by prolonged exposure to cortisol. These may include reddish purple striae, plethora, a "buffalo hump", proximal muscle weakness, osteoporosis and a fragile skin that heals poorly (Figure 5) [7]. Patients with Cushing's syndrome frequently have comorbid features caused by cortisol excess, such as abdominal obesity, hypertension and diabetes, that are also common in the general population. Women may experience more hair growth and irregular menstruation. Frequent symptoms are also changes in mood, and a chronic fatigue [8]. Cushing's syndrome is caused either by excessive cortisol-like medication (iatrogenic), or by endogenous causes like mostly benign but sometimes malignant adrenal tumors that produce excess cortisol or by an adrenocorticotropic hormone (ACTH) producing pituitary adenoma or ectopic tumor that results in the excessive production of cortisol by the adrenal glands. Some of these tumors are associated with inherited disorders such as multiple endocrine neoplasia type 1 or Carney complex. About two to three people per million are affected each year by

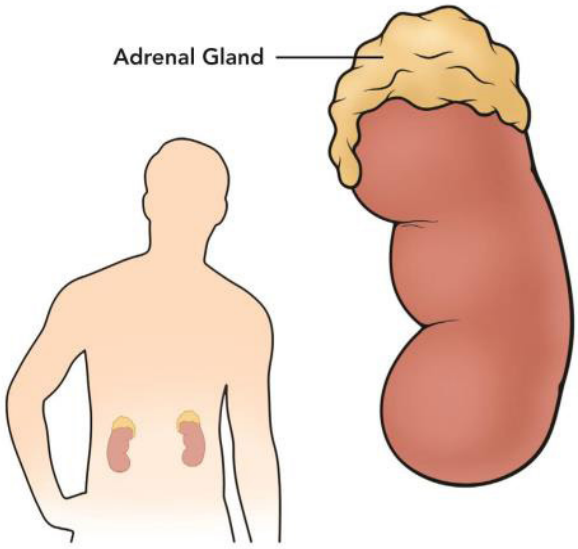

Figure 3 Adrenal gland (www.hormone.org, the endocrine society)

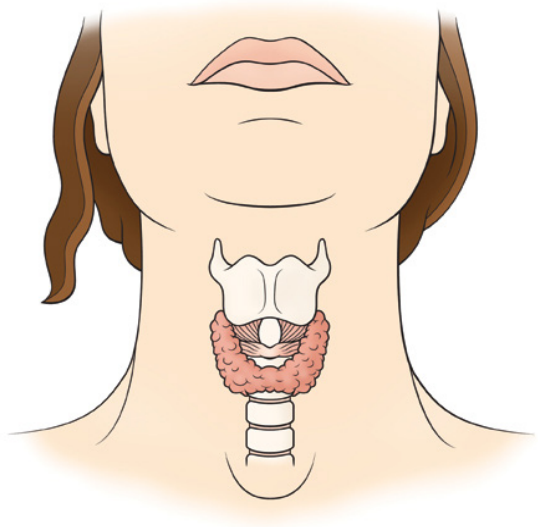

Figure 4 Thyroid gland (www.hormone.org, the endocrine society)

endogenous Cushing's syndrome. It most commonly affects people who are 20 to 50 years of age [9]. Women are affected three times more often than men.

For the initial testing of patients suspected of having Cushing's syndrome it is recommended to use one or more of the following tests: urine free cortisol (at least two measurements), late-night salivary cortisol (two measurements), 1-mg overnight dexamethasone suppression test or the low-dose dexamethasone suppression test (2mg/d for 48 hours) [8]. These four tests have acceptable diagnostic accuracy when the suggested cutoff points are used [10]. After the diagnosis has been established biochemically, imaging studies and/or sampling studies are required to localize the cause.

The goal of treatment of patients with Cushing's syndrome is to eliminate its primary cause and to eliminate its associated signs, symptoms, and comorbidities and to improve quality of life. The recommended first-line treatment option is resection of the primary lesion(s) by means of unilateral adrenalectomy, resection of ectopic ACTH-secreting tumors or transsphenoidal selective adenomectomy, unless surgery is not possible or unlikely to significantly reduce cortisol excess [8]. Additional treatments are recommended in patients with persistent hypercortisolism after surgery. There are multiple second-line treatment options: repeat transsphenoidal surgery, radiotherapy, medical therapy or bilateral adrenalectomy. Repeat transsphenoidal surgery is particularly recommended in patients with evidence of incomplete resection or a pituitary lesion on imaging. Radiotherapy is a possible therapy in patients who have failed transsphenoidal selective adenomectomy or have recurrent Cushing's disease. Medical treatment with steroidogenesis inhibitors (ketoconazole, metyrapone) is recommended as a second-line treatment after transsphenoidal selective adenomectomy in patients with Cushing's disease, as primary treatment of ectopic ACTH secretion in patients with occult or metastatic disease and as adjunctive treatment to reduce cortisol levels in adrenocortical carcinoma (mitotane). Pituitary directed medical treatments (cabergoline, pasireotide) are suggested in patients with Cushing's disease who are not surgical candidates or who have persistent disease after transsphenoidal selective adenomectomy. These treatments are not effective in adrenal causes of Cushing's syndrome, and their role in the treatment of ectopic ACTH production remains to be determined. Glucocorticoid antagonists (mifepristone) are another option in patients who are not surgical candidates or who have persistent disease after transsphenoidal selective adenomectomy. Furthermore, targeted therapies (octreotide) nowadays can be used to treat ectopic ACTH production [11]. In a number of recent reports, tyrosine kinase inhibitors (vandetanib, sorafenib) have shown to rapidly and fully control hypercortisolism caused by ACTH secretion from medullary thyroid carcinomas [12-14]. The lack of tumor reduction in these reports suggests a direct antisecretory effect of these medications.

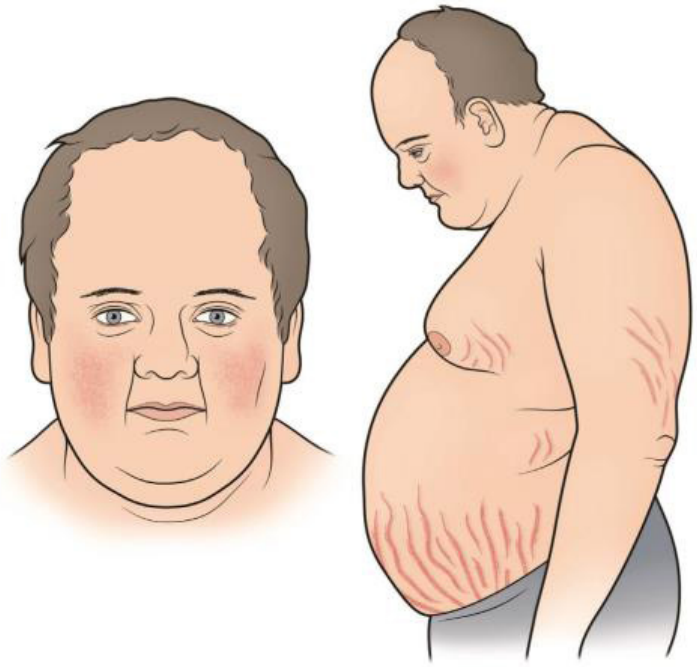

Figure 5 Cushing's syndrome (www.hormone.org, the endocrine society)

The earliest reports on Cushing's syndrome reported a median survival of 4.6 years, and in 1952 a 5-year survival of only 50%. Most deaths were caused by vascular or infectious complications [6, 15]. In modern times, the standard mortality ratio after successful normalization of cortisol was similar of that of an age-matched population during 1-20 years of follow up in one study [16]. However, whether the standardized mortality ratio is similar to the general population remains debatable [16-19]. Successful treatment reverses, but may not normalize all features of Cushing's syndrome. Multiple studies have already shown that for example bone mineral density, cognitive function, cardiovascular morbidity and hypertension improve after treatment but do not normalize in all patients [20-23]. Excess weight and hypertension may persist in 25% of patients [19, 22-32]. The persistent hypertension has been attributed to microvessel remodeling, abdominal obesity and insulin resistance [23, 33]. Similarly, glucose and lipid metabolism improves but does not fully normalize to levels of BMI matched controls several years after remission [19, 24, 27, 31, 33]. In addition, quality of life improves after treatment but remains below that of normal subjects [34, 35]. The persistence of central obesity may play a role in these findings [22, 27, 31, 33, 36]. Evaluating and treating the long-term adverse effects of Cushing's

syndrome may therefore be important to reduce morbidity, improve quality of life, and reduce long-term excess mortality. Furthermore, investigating the effects of chronic hypercortisolism on body tissues and physiology is crucial in order to tailor follow up and treatment of comorbidities. For these reasons, this thesis focuses on a number of long-term physical consequences of Cushing's syndrome.

Acromegaly

The first medical description of acromegaly goes back to 1772 when Nicolas Saucerotte reported "Accroissement singulier en grosseur des os d'un homme âgé de 39 ans" (The unique growth in size of the bones of a 39-year-old man) [37]. The term is from Greek ἄκρον akron meaning "extremity" and μέγα mega meaning "large" and was first coined by French neurologist Pierre Marie in 1886. Acromegaly is a condition that results from excessive growth hormone (GH)- and subsequently insulin like growth factor type 1 (IGF-1) production after the growth plates have closed. The initial symptom is typically acral enlargement. There may also be enlargement of the forehead, jaw, and nose leading to clear physical disfigurement (Figure 6). Other symptoms may include joint pain, thicker skin, deepening of the voice, headaches, and visual disturbances. Complications of the disease may include type 2 diabetes, sleep apnea, debilitating arthritis, carpal tunnel syndrome, hyperhidrosis and hypertension [38]. Acromegaly is typically caused by a GH producing pituitary adenoma [39]. Rarely acromegaly is caused by ectopic hormone production by tumors in other parts of the body. If excessive GH production occurs during childhood it results in gigantism which is beyond the scope of this thesis. Acromegaly affects about 60 per 1000,000 people [40]. It is most commonly diagnosed in middle aged men and women, both genders being equally affected.

The diagnosis of acromegaly is made by measuring increased age-normalized serum IGF-1 levels [41]. In patients with elevated or equivocal serum IGF-1 levels, confirmation of the diagnosis is recommended by finding lack of suppression of GH to <1μg/L following documented hyperglycemia during an oral glucose load [38]. Following biochemical diagnosis, magnetic resonance imaging is recommended to visualize tumor size and appearance as well as parasellar extent [42]. Performance of formal visual field testing is mandatory when the tumor is found to abut the optic chiasm [38].

Transsphenoidal surgery is the primary therapy in most patients [43]. For patients with severe pharyngeal thickness and sleep apnea, or high-output heart failure, preoperative medical therapy with somatostatin receptor ligands (SRLs) like octreotide long-acting release, lanreotide depot/autogel or pasireotide is advised to reduce surgical risk from severe comorbidities [44, 45]. In patients with parasellar disease, which makes total surgical resection unlikely, surgical debulking is advised to improve subsequent response to medical

therapy [46, 47]. When acromegaly persists after surgery, medical therapy is recommended. SRLs or pegvisomant, a human GH receptor antagonist, are the initial adjuvant medical therapy of choice [38]. In mild cases, a dopamine agonist, usually cabergoline, can be used [38]. In patients with inadequate response to these medications, they can be combined [38]. The final therapeutical option is radiotherapy/stereotactic radiotherapy which is recommended in patients with residual tumor mass after surgery and if medical therapy is unavailable, unsuccessful, or not tolerated [39, 40, 48].

The mortality rate of patients with acromegaly appears to be increased. Death is primarily from cardiovascular disease, a risk that may be reduced by strict biochemical control of the disease [49]. The prognosis of acromegaly is generally favorable when the disease is under strict biochemical control [40, 49]. Therefore, long-term psychosocial effects of the disease, such as persistent facial deformities, become more important as will be pointed out in this thesis.

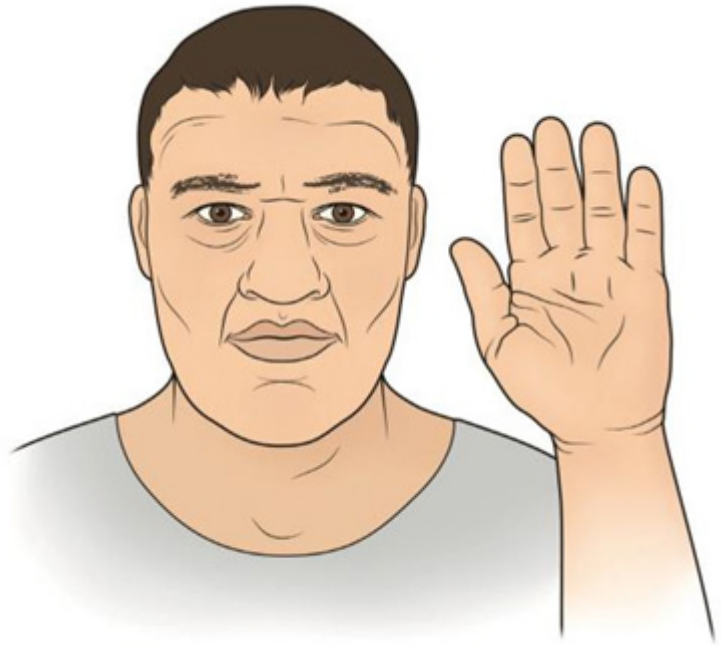

Figure 6 Acromegaly (www.hormone.org**, the endocrine society)**

Thyroid carcinoma

Thyroid carcinoma is a cancer originating from follicular or parafollicular thyroid cells. Follicular cells give rise to the well-differentiated cancers papillary thyroid carcinoma and follicular thyroid carcinoma (80-85% of all thyroid carcinoma patients) and to anaplastic thyroid carcinoma. In the latter case, tumor cells are poorly differentiated (beyond the scope of this thesis). The second cell type, the C-cell or parafollicular cell, produces the hormone calcitonin and is the cell of origin for medullary thyroid carcinoma [50]. Most often the first symptom of thyroid cancer is a nodule in the thyroid region of the neck [51]. However, many adults have small nodules in their thyroids (3-8% of European adults, http://oncoline.nl/schildkliercarcinoom), but typically less than 5% of these nodules are found to be malignant. Sometimes the first sign is an enlarged lymph node. Later symptoms that can be present are discomfort in the anterior region of the neck and changes in voice due to an involvement of the recurrent laryngeal nerve. Thyroid cancer is usually found in a euthyroid patient. The yearly incidence of thyroid carcinoma in the Netherlands is 2/100.000/year in males and 4.5/100.000/year in females (http://oncoline.nl/schildkliercarcinoom).

When thyroid carcinoma is suspected in a thyroid nodule based on clinical and ultrasound findings, ultrasound guided fine needle aspiration cytology (FNAC) is the diagnostic method of choice [52-54]. The aspirated material is then described by the pathologist using the Bethesda-system, which is a valid method for reporting thyroid cytopathology [55, 56].

Based on the Bethesda classification a decision is made on whether or not to perform surgery as an additional diagnostic modality (Bethesda 3/4) or as a treatment (Bethesda 5/6). Depending on the size and subtype of the tumor, a hemi- or total thyroidectomy is performed, followed by radioactive iodine treatment in order to treat any residual disease and lymph node metastases. Clinically significant lymph node metastases are surgically removed by means of selective neck dissections. Removal of all lymph nodes in a certain neck region reduces the chance of locoregional recurrent disease when compared to only removing macroscopic metastases (node picking) [57, 58]. Distant metastases can be surgically resected or treated by radioactive iodine or radiotherapy when resection is not possible. In case of progressive radioactive iodine refractory differentiated thyroid carcinoma, with high tumor volume and/or symptoms, treatment with a tyrosine kinase inhibitor (such as sorafenib or lenvatinib) can lead to partial response or disease stabilization [59, 60]. Thyroid surgery can have a number of complications of which damage of the laryngeal recurrent nerve and hypoparathyroidism are the most important. After surgery and radioactive iodine treatment, TSH suppressive therapy is advised using thyroid hormone depending on risk classification. Suppression of TSH levels has been reported to have a positive effect on disease specific survival but this has been debated [61]. External beam radiation therapy is only recommended in selected patients with macroscopically incomplete resection of

the tumor in whom no other treatments are available [62]. After treatment, follow-up is performed using thyroglobulin measurements (after TSH stimulation). This is a sensitive marker of the presence of (malignant) thyroid cells in the body.

The prognosis of thyroid carcinoma is generally favorable which results in a rising prevalence of thyroid carcinoma worldwide. The 5-year survival reaches 84% nowadays in the Netherlands (www.cijfersoverkanker.nl). This strengthens the importance of the consequences of the disease on the long-term. A recent literature review showed that health related quality of life remains impaired after differentiated thyroid carcinoma treatment especially concerning fatigue, sleeplessness, role functioning and mental health [63]. There are also indications that mental and physical quality of life is impaired after surgery in these patients [63]. There might also be a negative influence on quality of life of long-term TSH suppressive therapy with levothyroxine [63]. A number of factors influencing this health-related quality of life (HRQoL) are the main topic of a number of chapters in this thesis.

The aftermath of cure

For many diseases of the endocrine system there is treatment available. In the diseases that are the subject of this thesis -acromegaly, Cushing's syndrome and thyroid carcinoma- the main symptoms can often be controlled, tumors can be removed and hormonal levels can be normalized. This is what we are often call "cure". Although the primary disease is biochemically cured, patients often remain dependent on long-term hormonal substitution therapy. Furthermore, it becomes more and more clear that these diseases have long-term physical consequences, even after biochemical cure [17, 22, 33, 36, 64-67]. In addition, as survival is nowadays excellent in these patients, HRQoL becomes increasingly important [34, 63, 68-73]. It therefore seems that there exists an aftermath of cure, influenced and caused by different factors, which deserves the attention of all physicians who treat these patients. This is why this thesis focuses on the long-term quality of life and physical consequences of patients treated for acromegaly, Cushing's syndrome and thyroid carcinoma.

Outline

Part 1 Long-term health related quality of life after treatment of thyroid carcinoma and acromegaly

Health related quality of life

The concept of HRQoL and its determinants has evolved since the 1980s to encompass those aspects of overall quality of life that can be clearly shown to affect health—either physical or mental [74]. On the individual level HRQoL includes physical and mental health perceptions (e.g., energy level and mood) and their correlates—including health risks and conditions, functional status, social support, and socioeconomic status. On the basis of a synthesis of the scientific literature and advice from its public health partners, the US center for disease control and prevention (CDC) has defined HRQoL as "an individuals or groups perceived physical and mental health over time". HRQoL questions have become an important component of public health surveillance and are generally considered valid indicators of unmet needs and intervention outcomes. Self-assessed health status is also a more powerful predictor of mortality and morbidity than many objective measures of health [75, 76]. HRQoL measures make it possible to demonstrate scientifically the impact of health on quality of life.

Health related quality of life after treatment of thyroid carcinoma

Patients in remission of thyroid carcinoma for many years still report impaired HRQoL [63, 68]. Patients with differentiated thyroid carcinoma report impaired HRQoL in relation with initial surgery and hypothyroidism preceding treatment with radioactive iodine [63, 77]. One factor that negatively influences QoL in other cancer populations is distress which is defined as an unpleasant experience of emotional, psychological, social or spiritual nature, that interferes with the ability to cope with cancer treatment, which extends along a continuum from common normal feelings of vulnerability, sadness and fear, to disabling problems such as true depression, anxiety, panic and feeling isolated or in a spiritual crisis [78]. Distress is also associated with longer recovery time and worse treatment adherence in cancer patients [79]. Distress is frequently not noticed by physicians and systematic screening may be necessary [80-82]. The level of distress and problems experienced by thyroid carcinoma survivors and its relation to QoL had never been investigated in differentiated thyroid carcinoma patients.

In **chapter 2** we therefore investigated the level of distress and problems experienced by survivors of differentiated, non-medullary, thyroid carcinoma, using the distress thermometer (DT) and problem list and whether this correlates with clinical and demographical variables. In addition we investigated whether reported distress correlated

with clinical, demographical and psychological variables measured by means of the hospital anxiety and depression scale, the illness cognition questionnaire and an ad-hoc questionnaire in order to gain a broader view of the emotional and psychological factors influencing distress in differentiated thyroid carcinoma survivors. The DT in combination with a problem list is a tool validated and currently recommended for screening for distress experienced by oncologic patients [83-85]. This questionnaire is a good starting point for further discussion in a clinical interview and can be completed in just a few minutes, which makes it easy to implement in daily practice. A meta-analysis has shown the DT to be as accurate as the hospital anxiety and depression scale but superior to it with respect to efficiency [86]. Although widely used in other types of cancer, it was never used in patients with differentiated thyroid carcinoma before.

An important factor that could contribute to distress is the fact that surgical treatment of head and neck malignancy, especially when involving neck dissections, is well known to be associated with shoulder complaints, with its subsequent impact on QoL [87, 88]. Shoulder complaints are frequently reported in the outpatient clinic after surgical treatment for thyroid carcinoma. In a recent survey on psychosocial/informational needs in patients with thyroid carcinoma, almost 30% of participants complained of numbness of the neck region and/or restricted shoulder/neck movement more than 1 year after treatment [89]. In other types of cancer, there has been a recent emphasis on quality of life related to spinal accessory nerve damage and shoulder complaints after neck dissection [90, 91]. The few studies on this topic in this disease population were not specifically designed to investigate shoulder complaints in patients with thyroid carcinoma [89].

In **chapter 3** we therefore investigated the prevalence of self-reported long-term shoulder complaints and the associations of shoulder complaints with QoL in a historical cohort of patients who had surgery for thyroid carcinoma compared to a healthy control group using the “disabilities of the arm, shoulder and hand questionnaire” (DASH) and the European Organization for Research and Treatment of Cancer, Quality of Life Questionnaire-C30 (EORTC QLQ-C30). The DASH questionnaire was developed to measure the patients’ perception of the ability to perform different activities and roles and to monitor symptoms associated with any condition in the upper limb and was never used in thyroid carcinoma patients before [92]. In addition, our aim was to identify possible clinical and demographic correlates of shoulder complaints and to evaluate associations between shoulder complaints and QoL.

Health related quality of life after treatment of acromegaly

Psychological changes, including changes in personality due to impaired self-esteem, body image distortion, disturbed interpersonal relationships, social withdrawal, anxiety and

depression are present in acromegaly patients [69, 93]. In patients in biochemical remission of acromegaly, multiple factors have been found to negatively influence quality of life, e.g. prolonged postoperative use of somatostatin analogues [94], radiotherapy treatment [95, 96], persisting joint complaints [97], presence of osteoarthritis [98] and presence of GH deficiency after treatment [99]. It was recently shown by our group that facial appearance in patients in remission of acromegaly does not fully return to normal after biochemical cure [100]. It has been suggested that changes in appearance can lead to self-consciousness about appearance, leading towards self-esteem disintegration, social withdrawal, body image distortion and an impaired quality of life [69]. Despite the fact that the appearance subscale was the most affected subscale in patients in remission of acromegaly in studies that used the AcroQoL questionnaire [70, 101-103], it had never been investigated whether these changes in appearance negatively influence QoL and consequently cause psychosocial dysfunctioning and distress.

In **chapter 4** we investigated the psychological distress and dysfunction related to self-consciousness about appearance and its effect on QoL in patients in long-term remission of acromegaly compared to a gender-, age- and body mass index matched control group. In addition, we aimed to identify the anatomical sources of self-consciousness. In order to do so, patients and controls completed the modified Derriford appearance scale (DAS59) and research and development 36 (RAND-36) questionnaires, and the acromegaly quality of life questionnaire (AcroQoL). The Derriford appearance scale is developed for research in plastic surgery and oncology. It has good validity and reliability and has been recommended for the assessment of feelings about appearance [104, 105]. However, it has never been used in patients with acromegaly before.

Part 2 Long-term physical sequelae of Cushing's syndrome

Long-term physical consequences of previous hypercortisolism in patients in remission of Cushing's syndrome have become a focus of research in the last years. We [36] and others [33, 64] have shown that many adverse metabolic and body compositional changes might persist after treatment, even after long-term remission. Although these adverse metabolic characteristics are common in these patients, their incidence and severity vary among patients. This variation seems not to be explained by differences in cortisol excess or disease duration alone. Therefore, a variable sensitivity to glucocorticoids, due to glucocorticoid receptor (GR) polymorphisms, may play a role in modulating the effect of cortisol excess. The functional role of GR polymorphisms has been extensively studied in the general healthy population. However, only a small number of studies have been performed on the functional role of GR polymorphisms in active Cushing's syndrome and Cushing's syndrome in remission [106-108]. At the start of our project no information was available on whether

the differences between patients in the severity of the adverse metabolic and vascular profile after cure of Cushing's syndrome could be explained by differences in glucocorticoid sensitivity due to GR polymorphisms.

In **chapter 5** we measured the presence of GR polymorphisms (BclI (rs41423247), N363S (rs56149945), ER22/23EK (rs6189/rs6190), and 9β (rs6198)) and investigated their associations with metabolic and vascular alterations in patients in long-term remission of Cushing's syndrome.

Successful treatment is associated with significant decreases of cardiovascular risk and mortality [109]. Only a limited number of studies have investigated vascular health in patients in remission of Cushing's syndrome. However, cardiovascular disease is the main cause of mortality in patients with active Cushing's syndrome[110]. Previous studies in patients in remission of Cushing's syndrome reported contradictory results using different kinds of surrogate markers of vascular health [22, 33, 66, 67, 111-113]. Moreover, these reports used only single markers of vascular function and had small sample sizes.

In **chapter 6** we investigated micro- and macrovascular health in a large group of patients in long-term remission of Cushing's syndrome with adequately treated comorbidity if present, in comparison with a matched healthy control group. We measured serum biomarkers associated with endothelial dysfunction, performed gold standard measurements of endothelial function and investigated the presence of overt atherosclerosis.

Previous studies in Cushing's syndrome in long-term remission have demonstrated that centripetal fat distribution, physical disability and tiredness are frequent self-reported problems [36, 72, 73]. It is suggested that the persistence of these symptoms impairs physical performance in the context of employment and daily life [73]. When physical capacity decreases, for example due to Cushing's syndrome, workload of daily physical activities demands a relatively higher percentage of one's physical capacity, resulting in the earlier onset of experiencing fatigue [114]. No previous study has examined physical fitness in patients in long-term remission of CS. Furthermore, no previous study has examined the relation between physical fitness and daily physical activity levels in these patients nor was investigated whether any long standing direct effects of hypercortisolism on the muscles is present in these patients.

In **chapter 7** we investigated physical fitness, as measured by the gold standard approach of peak oxygen uptake ($VO_{2\ peak}$) during a maximal exercise test, in patients in long-term (> 4 years) remission of CS and in an age-, gender-, body mass index (BMI)-, smoking-, physical activity level- and ethnicity matched control group. Furthermore, we explored the relation

between physical fitness levels and daily physical activity levels in this specific patient category. Skeletal muscle biopsies were taken in order to explore the relation between $VO_{2\ peak}$ and structural or functional abnormalities in muscle tissue.

One of the main physical features in Cushing's syndrome is a centripetal adipose tissue distribution. It was recently shown by our group that this adipose tissue expansion even persists after remission [36]. Adipose tissue expansion in normal obesity is caused by adipocyte hyperplasia, hypertrophy or a combination of both. Centripetal adipose tissue expansion of primarily the visceral adipocytes, as seen in Cushing's syndrome, is more commonly associated with adipocyte hypertrophy, which is enlargement of the existing fat cells. In common obesity this is associated with macrophage infiltration and disturbed adipokine profiles which is related to insulin resistance, endothelial dysfunction and cardiovascular disease [115]. Little is known about these parameters in active Cushing's syndrome. Only one previous study showed enlarged abdominal fat cells in women with Cushing's syndrome compared to non-obese women and obese women with the android and gynoid types of fat distribution [116].

In **chapter 8** we investigated fat cell size, macrophage infiltration and adipokine profiles in different fat compartments of patients with active Cushing's syndrome compared to healthy gender-, age- and BMI matched controls.

Chapter 9 consists of a general summary of the thesis and a general discussion on the previous chapters.

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Part 1

Long-term health related quality of life after treatment of thyroid carcinoma and acromegaly

Chapter 2

High level of distress in long-term survivors of thyroid carcinoma: results of rapid screening using the distress thermometer

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Abstract

Context: Cancer patients are at increased risk for distress. The Distress Thermometer (DT) and problem list (PL) are short-tools validated and recommended for distress screening in cancer patients.

Objective: To investigate the level of distress and problems experienced by survivors of differentiated non-medullary thyroid carcinoma (DTC), using the DT and PL and whether this correlates with clinical and demographical variables.

Participants, design and setting: All 205 DTC patients, under follow-up at the outpatient clinic of our university hospital, were asked to fill in the DT and PL, hospital anxiety and depression scale (HADS), illness cognition questionnaire (ICQ) and an ad hoc questionnaire. Receiver Operator Characteristic analysis (ROC) was used to establish the optimal DT cut-off score according to HADS. Correlations of questionnaires scores with data on diagnosis, treatment and follow-up collected from medical records were analyzed.

Results: Of the 159 respondents, 145 agreed to participate (118 in remission, median follow-up 7,2 years (range 3 months-41 years)). Of these, 34.3% rated their distress score ≥5, indicating clinically relevant distress according to ROC analysis. Patients reported physical (86%) over emotional problems (76%) as sources of distress. DT scores correlated with HADS scores and ICQ subscales. No significant correlations were found between DT scores and clinical or demographical characteristics except for employment status.

Conclusion: Prevalence of distress is high among patients with DTC even after long-term remission and cannot be predicted by clinical and demographical characteristics. DT and PL are useful screening instruments for distress in DTC patients and could easily be incorporated into daily practice.

Introduction

Differentiated non-medullary thyroid carcinoma (DTC) is associated with a favorable prognosis with long-term survival rates that reach 80-95% [1]. Since most patients with DTC become in remission after treatment, the number of disease-free survivors is high. Quality of life (QoL) is an important issue in the care for long-term survivors of DTC [2]. Patients with DTC report impaired QoL in relation with initial surgery and hypothyroidism preceding treatment with radioactive iodine (RAI) [3, 4]. Moreover, several studies have shown that even patients being in remission of DTC for many years report impaired QoL compared to healthy controls [4, 5].

One factor that has negative effects on QoL is the level of distress. There is increasing evidence that cancer patients, including long-term survivors, are at increased risk of distress compared to healthy population controls [6-13], with distress defined as "an unpleasant experience of emotional, psychological, social or spiritual nature, that interferes with the ability to cope with cancer treatment, which extends along a continuum from common normal feelings of vulnerability, sadness and fear, to disabling problems such as true depression, anxiety, panic and feeling isolated or in a spiritual crisis" [10]. Distress has also been shown to negatively influence recovery time and treatment adherence in cancer patients [14]. Distress is often not noticed by medical professionals unless it is explicitly assessed [15, 16]. For these reasons it is nowadays often recommended that all oncologic patients should be systematically screened for distress [9, 17]. In order to meet the need for screening tools that can be used efficiently in an outpatient clinic setting, several short-tools have been developed for detection of distress. However the majority of these have not been robustly validated in cancer settings [18].

The distress thermometer (DT) in combination with a problem list (PL) is a tool validated and currently recommended for screening for distress experienced by oncologic patients [19-21]. In contrast to other well-known QoL questionnaires, such as the hospital anxiety and depression scale (HADS), the self-reported problems on the PL are a starting point for further discussion in the clinical interview. In addition, the DT can be completed in just a few minutes, which makes it easy to implement in everyday outpatient practice. In this respect, a recent meta-analysis found DT to be as accurate as HADS as a short-tool to screen for distress in cancer settings but superior to it with respect to efficiency [22]. Although widely used in other types of cancer, the use of DT has never been evaluated in patients with DTC.

Previous data suggest that patients with DTC perceive their illness as severe on a subjective, emotional basis unrelated to disease severity [23]. Therefore, the aim of this study was to evaluate the level of distress and problems experienced in a cohort of DTC subjects presenting for follow-up at the outpatient clinic of our tertiary referral academic hospital, using the DT and PL as short screening tools. In addition we investigated whether reported distress correlates with clinical, demographical and psychological variables measured by means of the HADS, illness cognition questionnaire (ICQ) and an ad hoc questionnaire in order to gain a broader view of the emotional and psychological factors influencing distress in DTC survivors.

Methods

Patients

All patients (>18 years old) who were treated for DTC and are under follow-up at the Department of Endocrinology of the Radboud University Nijmegen Medical Centre were invited to participate in the present study. The research has been approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre. The patients were requested to fill in a questionnaire package sent by mail. Non-responders received a reminder letter four weeks later. Clinical variables were collected from medical charts and included information on diagnosis, treatment and follow-up. Primary treatment consisted of total thyroidectomy in all but two cases of papillary (PTC) microcarcinoma and one case of minimally invasive follicular thyroid carcinoma (FTC), and modified radical neck dissections in patients with confirmed nodal metastases. This was followed in the majority of patients by ablation with RAI (I^{131}) of residual thyroid tissue 4-6 weeks after surgery. Initial cure was defined as undetectable thyroglobulin (Tg) in absence of anti-Tg antibodies and no evidence of loco-regional disease or distant metastases on whole body iodine scans (WBS) and/or neck ultrasonographic examinations at six months after RAI ablation. Tumor recurrence was defined as evidence of loco-regional disease or distant metastases more than six months after successful primary therapy. Current disease status was defined as cured (in remission) in case of undetectable Tg in the absence of anti-Tg antibodies and no evidence of loco-regional disease or distant metastases at last follow-up visit. Persistent disease was defined as either detectable Tg or evidence of loco-regional disease or distant metastases.

Questionnaires

Distress thermometer and problem list (DT and PL)

The DT is a modified visual analogue scale ranging from 0 (no distress) to 10 (extreme distress) resembling a thermometer [13]. Most of the validation studies in cancer patients

have validated the DT against the HADS and established proper cut-off scores with receiver operating characteristics (ROC) curve analysis [6, 11-13]. In the current study a ROC analysis was performed in order to establish the cut-off score for the DT using a HADS cutoff score ≥15 as the gold standard for detecting cases of severe emotional distress. This cut-off is similar to that used in a validation study performed in a cohort of patients with several other malignancies and having the same cultural background as our cohort [7, 24].

We used the previously validated [7] Dutch version of the DT and problem list (PL) which is currently recommended for use in the clinical practice in the Netherlands. The Dutch version is adapted from the original NCCN version of the DT and PL based on evaluations by the Comprehensive Cancer Center the Netherlands (CCCN) focus groups. In comparison to the original 35 items of the original NCCN version, 15 items were added on the PL in the Dutch version (added items are depicted in italics in Table 2). The items sadness, worry and loss of interest in usual activities were removed from the NCCN problem list, yielding a total of 47 items. The items are grouped in categories: physical problems (25), emotional problems (10), practical problems (7), social problems (3) and spiritual issues and religion (2). Respondents were instructed to indicate whether the items listed had been a problem in the past week by selecting from a fixed yes/no response [25]. In addition respondents were asked to indicate whether they would like to be referred to a professional for additional support [11].

Hospital anxiety and depression scale (HADS)

The HADS is a measure of anxiety and depression for patients with physical illness. It contains 14 items. Each item is scored between 0-3. Half of the items assess anxiety and the other half depression. For the anxiety and depression subscales scores 0-7 indicate normal values, 8-10 mild disorder, 11-14 moderate disorder and 15-21 severe disorder [26]. The 2 subscales can be combined into a single scale, and scores ≥15 on this scale were used to indicate severe emotional distress [7, 24]. The HADS is widely used in oncology and has good reliability and validity [27]. The Dutch version of the HADS was used, which has been validated in several subgroups of Dutch patients [28].

Illness cognition questionnaire (ICQ)

The ICQ was used to measure helplessness, acceptance and perceived disease benefits. This is an 18-item questionnaire that contains three six-item scales, each with a scoring range of 6-24. Each item is answered on a four point scale to the extent to which one agrees with the item [29]. The ICQ was used because illness cognitions are related to psychological distress as theorized in Leventhal's Self-Regulatory Model (SRM) [30] and confirmed by several studies [31-33].

Demographic variables

Demographic information on age, gender, marital status, level of education, religion, age at diagnosis and employment status was collected by means of an ad-hoc questionnaire.

Statistical analysis

Spearman correlations were performed between the demographic characteristics, questionnaire scores and clinical characteristics. Significance was defined as $p<0.05$. ROC analysis was performed to examine the ability of the DT to detect distressed patients, and a HADS cutoff score ≥15 was set as the gold standard for detecting cases of clinically significant emotional distress [7, 24]. Positive (PPV) and negative (NPV) predictive values were calculated for every DT score. All statistical analyses were also performed separately for the subgroup of patients currently being in remission of the disease according to the last follow-up data. All statistical analyses were performed using SPSS 16.0.

Results

Patient characteristics

Two hundred and five patients were eligible for this study. Of these, 159 patients responded (response rate 77.6%). Fourteen patients refused to participate because they had other interests or were unable to answer the questions because of language problems. A total of 145 patients (73.8% female, mean age (SD; range) 51.7 (13.8; 19-83) years) were enrolled in the study. Mean age at diagnosis was 40.1 (SD; range) (13.1; 9-79) years. Median follow-up time (range) since the last treatment was 7.2 years (3 months - 41 years). Regarding current disease status, most patients (118, 81.4%) were in remission and under follow-up, 18 (12.4%) patients had only detectable stable low Tg level without macroscopic evidence of disease and 9 (6.2%) patients had persistent disease detectable on imaging. Serum TSH levels at last follow-up appointment had a mean (SD) value of 0.42 IU/L (1.12). Clinical characteristics are depicted in Table 1. Participants and non-participants did not differ with respect to patient characteristics (p-values >0.2 for all items).

Table 1. Patient characteristics and relation to distress thermometer (DT) score.

			Correlation to DT score	
		%	Spearman's-rho	p-value
Gender (n): male/ female	38/ 107		0.145	0.090
Age: mean (SD) (years)	51.7 (13.8)		-0.054	0.531
Age at diagnosis: mean (SD) (years)	40.1 (13.1)		-0.059	0.497
Duration since diagnosis: median (range) (years)	8.8 (0-41)		0.081	0.349
<1 year: n (%)	8 (5.5)			
1-5 year: n (%)	29 (20.0)			
5-10 year: n (%)	44 (30.3)			
>10 year: n (%)	64 (44.1)			
Follow up since last treatment: median (range) (years)	7.2 (0-41)		0.036	0.676
Histopathological subtype: (n)				
Papillary	106	73.1	-0.123	0.151
Follicular	36	24.8	0.127	0.138
Mixed	3	2.1	0.000	0.994
TNM classification* stage: (n)			-0.094	0.292
I	68	46.9		
II	22	15.2		
III	11	7.6		
IV	33	22.7		
Unknown	11	7.6		
Surgical treatment: (n)				
Hemithyroidectomy	3	2.8	-0.128	0.140
Total thyroidectomy	140	96.6	0.052	0.550
Lymph node dissection	50	34.5	-0.133	0.125
Unknown	2	1.4		
RAI treatment:				
Number of treatments:			0.067	0.437
0	12	8.3		
1	76	52.4		
2	37	25.5		
>2	19	13.1		
Unknown	1	0.7		
Cumulative dose of I-131:			0.055	0.525
0 mCi	12	8.3		
<100 mCi	6	4.1		
100-200 mCi	70	48.3		
>200 mCi	55	37.9		
Unknown	2	1.4		
Complications of surgery: (n)				
Hypoparathyroidism (permanent)	43	29.7	0.134	0.118
Recurrent laryngeal nerve paralysis	8	5.5	0.023	0.786
No complications	95	65.5	-0.141	0.101
Disease status: (n)			0.054	0.533
Remission	118	81.4		
Active disease	27	19.7		
Mean TSH at last follow-up: (IU/L) (SD)	0.4 (1.1)		0.061	0.486

*UICC TNM Classification of malignant tumors, 6th edition [43]

Demographic characteristics

Two of the participants (1.4%) had less than 8 years of education, 42 (29.2%) 8-12 years, 38 (26.4%) 12-16 years, 44 (30.6%) 16-21 years and 18 (12.5%) had a university degree. One participant omitted to fill in the highest education level. One hundred and twenty five (86.2%) participants were married or engaged in a relationship and 20 (13.8%) had no relationship, were divorced or a widow/widower. Eighty-two (56.6%) participants had full- or part time employment, 34 (23.6%) were unemployed, 26 (18.1%) were retired and 3 (2.1%) were students.

Distress thermometer scores and problems

One hundred and thirty seven patients rated their distress level on the DT and showed a mean score (SD) of 3.33 (2.70) with a 0-10 range. Of these, 47 participants (34.3%) rated their distress score ≥5, indicating clinically relevant distress as depicted later in the ROC analysis. 60 participants (43.8%) rated their distress score >3 which is the NCCN distress management guideline cut-off [34]. Eight patients failed to score their distress on the DT because they forgot. Mean DT (SD) scores in the 110 patients being in remission and the 27 patients having persistent disease were 3.40 (2.72) and 3.04 (2.62) respectively and the percentages of patients reporting DT scores $\geq$ 5 in these groups were 36.4% and 25.9%.

There were no significant correlations between DT scores and the clinical characteristics regarding age at diagnosis, severity of the disease, disease status, treatments received or complications (Table 1). In addition there was no correlation between the duration of (disease-free) follow-up or the TSH levels and DT scores. DT scores and level of education did not correlate significantly (r = -0.146, p = 0.089) in the total group. When analyzing the subgroup of patients being in remission of the disease, we found a slight but significant correlation between the DT score and the level of education (r = -0.212, p = 0.027). DT scores did not correlate significantly to having a partner (r = -0.072, p = 0.402). There was a slight but significant correlation between the DT scores and the employment status (r = -0.251, p = 0.003), the participants having a full- or part time employment reporting significantly less distress.

One hundred and forty five respondents completed the PL (Table 2). The most frequently reported problems were fatigue (62.1%), lack of physical fitness (46.9%), sleep problems (41.4%), muscle strength (40.0%) and nervousness (40.0%), weight changes (31.7%) and paresthesia (31.7%). The physical problems category was the most frequently reported category with 86.1% of the patients reporting at least one item. Emotional problems were reported by 76.0%, practical problems by 61.5%, social problems by 48.6% and problems regarding spiritual issues or religion by 39.4% of the patients. In total, 124 patients (89.9%) reported at least one item of concern on the PL. Of the patients reporting DT ≥5, 47

(100%) reported at least one item of concern on the PL. Of the patients with a DT score <5, 69 (76.7%) reported at least one item of concern on the PL.

There was a strong significant correlation between DT scores and the total number of reported problems on the PL (r = 0.827; p < 0.001). Significant correlations were also found between DT scores and the number of reported problems in each specific category. Table 2 shows the frequency distribution for endorsement of the PL and their correlation with the DT score.

Table 2. Problem list (PL) items and correlation to distress thermometer (DT) score (*n represents the number of patients reporting a specific problem in the total group and in the active disease group).

				Correlation of total group PL score to total group DT score	
Problem list	**Mean score (SD)**	**n* (Total / active disease)**	**% (Total / active disease)**	**Spearman's-rho**	***p*-value**
Overall number of problems	9.5 (8.3)			0.827	<0.001
Practical problems	0.9 (1.3)			0.715	<0.001
Child care		13 / 2	9.0 / 7.4		
Housing		8 / 1	5.5 / 3.7		
House holding		41 / 6	28.3 / 22.2		
Transportation		9 / 1	6.2 / 3.7		
Work/school		35 / 9	24.1 / 33.3		
Financial		18 / 4	12.4 / 14.8		
Insurance		8 / 1	5.5 / 3.7		
Social problems	0.4 (0.8)			0.468	<0.001
Dealing with partner		27 / 3	18.6 / 11.1		
Dealing with children		20 / 4	13.8 / 14.8		
Dealing with friends/relative		18 / 3	12.4 / 11.1		
Emotional problems	2.5 (2.8)			0.652	<0.001
Grip on emotions		47 / 4	32.4 / 14.8		
Memory		47 / 11	32.4 / 40.7		
Confidence		35 / 4	24.1 / 14.8		
Fears		31 / 4	21.4 / 14.8		
Sadness		40 / 7	27.6 / 25.9		
Nervousness		58 / 9	40.0 / 33.3		
Loneliness		20 / 2	13.8 / 7.4		
Concentration		51 / 8	35.2 / 29.6		
Feeling of guilt		21 / 2	14.5 / 7.4		
Loss of control		24 / 6	16.6 / 22.2		
Spiritual-religious problems	0.2 (0.5)			0.233	0.006
Meaning of life		16 / 1	11.0 / 3.7		
Faith in god/religion		13 / 5	9.0 / 18.5		

Table 2. Continued

Physical problems	5.3 (4.5)			0.782	<0.001
Appearance		22 / 5	15.2 / 18.5		
Changes in urination		12 / 3	8.3 / 11.1		
Constipation		23 / 2	15.9 / 7.4		
Diarrhea		12 / 3	8.3 / 11.1		
Eating		23 / 5	15.9 / 18.5		
Feeling swollen		35 / 8	24.1 / 29.6		
Fever		1 / 0	0.7 / 0.0		
Mouth sores		18 / 8	12.4 / 29.6		
Nausea		16 / 3	11.0 / 11.1		
Nose dry/congested		38 / 4	26.2 / 14.8		
Pain		35 / 5	24.1 / 18.5		
Sexuality		25 / 4	17.2 / 11.1		
Skin dry/itchy		42 / 5	29.0 / 18.5		
Sleep		60 / 9	41.4 / 33.3		
Breathing		17 / 2	11.7 / 7.4		
Vertigo		28 / 4	19.3 / 14.8		
Speech		17 / 3	11.7 / 11.1		
Taste		14 / 5	9.7 / 18.5		
Weight changes		46 / 11	31.7 / 40.7		
Paresthesia in hands/feet		46 / 14	31.7 / 51.9		
Washing/dressing		3 / 1	2.1 / 3.7		
Getting around		26 / 5	17.9 / 18.5		
Fatigue		90 / 15	62.1 / 55.6		
Physical fitness		68 / 8	46.9 / 29.6		
Muscle strength		58 / 5	40.0 / 18.5		

HADS and ICQ

All participants completed the HADS questionnaire. The mean (SD) total score on the HADS was 9.25 (7.79), mean (SD) scores for anxiety and depression subscales were 5.40 (4.37) and 3.85 (3.93) respectively. Mean score on the HADS, number of patients reaching the subscale score ≥8 (threshold for mild, moderate and severe disorder) and their correlations with DT scores are depicted in Table 3. Thirty-two (22.1%) of the patients had scores ≥ 15 (threshold for severe disorder).

The mean (SD) scores on helplessness, acceptance and disease benefits domains of the ICQ were 9.03 (3.66), 19.08 (4.28) and 16.13 (4.52) respectively. There was a significant correlation between DT score and helplessness and acceptance domains (Table 3). One participant failed to fully complete the ICQ.

Table 3. Hospital anxiety and depression scale (HADS) and illness cognition questionnaire (ICQ) scores and correlation to distress thermometer (DT) scores (*n represents the number of patients with a score above the mentioned threshold).

		Correlation to DT score	
		Spearman's-rho	p-value
HADS			
Total	Mean score = 9.2 SD = 7.7	0.611	<0.001
≥15 n* (%)	32 (22.1%)		
Anxiety	Mean score = 5.4 SD = 4.3	0.547	<0.001
≥8 (n* (%))	36 (24.8%)		
Depression	Mean score = 3.8 SD = 3.9	0.602	<0.001
≥8 (n* (%))	28 (19.3%)		
ICQ			
Helplessness	Mean score = 9.0 SD = 3.6	0.662	<0.001
Acceptance	Mean score = 19.0 SD = 4.2	-0.544	<0.001
Disease benefits	Mean score = 16.1 SD = 4.5	-0.092	0.285

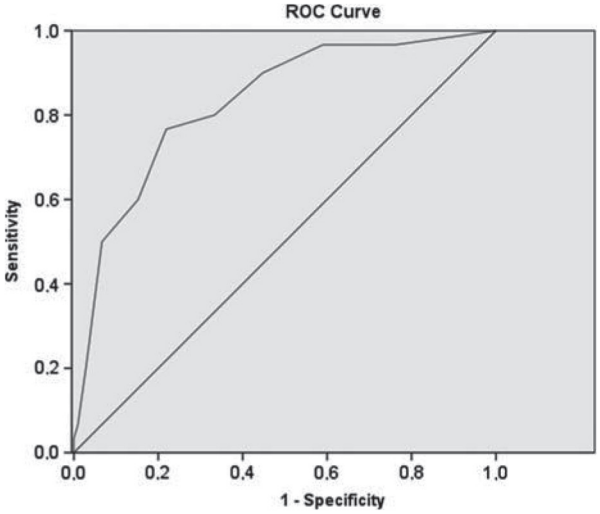
Cut-off scores and predictive values

The ROC curve predicting elevated distress according to the HADS showed an area under the curve of 0.82 (standard error 0.043; 95% confidence interval, 0.739-0.906; *P* <0.001) (Fig. 1). Table 4 lists the sensitivity and specificity values for all DT scores, including frequencies. The cut-off score of 5 correctly identified 77% of HADS cases (sensitivity) and 77% of HADS non-cases (specificity).

Table 4. Frequencies, sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of distress thermometer (DT) scores (*n represents the number of patients with the respective score in the total group and in the group of patients with active disease).

DT score	n* (cum.%) Total group	n*(cum.%) Active disease	Sensitivity	Specificity	PPV	NPV
0	26 (19.1)	6 (22.2)	1.00	0.00	0.22	0.00
1	18 (32.4)	5 (40.7)	0.97	0.24	0.26	0.96
2	17 (44.9)	1 (44.4)	0.97	0.41	0.32	0.98
3	15 (55.9)	4 (59.3)	0.90	0.55	0.36	0.95
4	13 (65.4)	4 (74.1)	0.80	0.66	0.40	0.92
5	12 (74.3)	0 (74.1)	0.77	0.77	0.49	0.92
6	12 (83.1)	3 (85.2)	0.60	0.84	0.51	0.88
7	13 (92.6)	3 (96.3)	0.50	0.92	0.65	0.87
8	7 (97.8)	1 (100.0)	0.20	0.96	0.60	0.81
9	2 (99.3)	0 (100.0)	0.07	0.99	0.67	0.77
10	1(100.0)	0 (100.0)	0.04	1.00	1.00	0.79

Figure 1. Receiver operating characteristics (ROC) curve of distress thermometer (DT) scores versus hospital anxiety and depression scale (HADS) cut-off scores.



AUC 0.82, SD 0.043, 95%CI 0.739-0.906, P < 0,001

Wish for referral

Nineteen patients (13.1%) expressed their wish to discuss with another professional for additional support. Thirty-seven patients (25.5%) answered this question with 'maybe'. Of the patients reporting a DT score ≥5, 19.1% wished to be referred for additional support and 34.0% were considering it (and answered 'maybe'). There was a significant correlation between the DT score and the wish for referral for additional support (r = 0.193, p = 0.025). 'Maybe' willing to be referred for additional support significantly correlated with DT score as well (r = 0.230, p = 0.007). Four respondents omitted to report whether they wished to be referred.

Discussion

Cancer patients experience high levels of distress [6, 10, 20]. Relying on the subjective estimates of distress levels by medical professionals is often inaccurate and misleading [15, 16]. The DT combined with a PL is a tool developed initially by National Comprehensive Cancer Network (NCCN) [34] for screening for distress in patients with breast tumors. It has been validated for use in several types of cancer and in populations with different ethnic backgrounds, including a series of Dutch patients [6, 7, 11, 13, 35]. This easy-to-use instrument helps identify treatable problems and specific issues that may cause distress, allowing physicians to tackle these issues more targeted. In addition, patients are explicitly requested whether they wish to be referred for additional specialized help. This potentially improves communication between patients and healthcare providers as well as patient care and satisfaction and may help to use limited consultation time more effectively [8]. This is the first study evaluating the level of distress using the DT and PL in DTC patients.

The main finding of the present study is that the patients with DTC report experiencing significant levels of distress even years after being cured, with 34.3% of the patients reporting distress scores ≥5 on the DT, which may count for significant distress and possibly requires intervention. Surprisingly, this percentage is comparable to the 28.6-50% reported in series [19, 36-39] with similar response rates, including patients with other types of cancer such as lung-, breast cancer and leukemia, having a more aggressive course and being associated with more physical burden than patients with DTC, the majority of whom were in long-term remission. However, these studies differ with respect to type of cancer, patient characteristics, cultural background, type of treatment, duration of follow-up and DT cut-off level [4-6] used to define distress, and therefore cannot be compared appropriately. In DTC patients, although on the long-term QoL approaches that of the healthy population, previous studies indicate that most long-term survivors report specific persistent problems such as the negative effects of thyroid hormone withdrawal for radioiodine follow up

procedures, the fear and uncertainty related to a cancer diagnosis, feelings of diagnosis being dismissed as not seriously or 'having a benign cancer', confrontation with daily use of thyroid hormone medication and the fluctuating thyroid hormone levels [4]. The nature and intensity of these problems can change during the oncologic trajectory and become sources of distress. As the duration of follow-up increases for instance, the thyroid hormone withdrawal is not required anymore and the thyroid hormone levels become stable and remain within the normal range in the majority of patients. On the other hand other problems such as concerns about fertility can become more relevant after longer follow-up. Therefore, identifying patients who may experience distress and may benefit from additional help and addressing the specific problems should be important objectives in the ongoing care of these long-term DTC survivors. When considering the range of DT scores and the nature of reported problems we conclude that the DT is a useful screening tool for this particular population.

In consistence with findings in other types of cancer [19], no significant correlations were found between level of distress and either clinical or demographic characteristics, with the exception of employment status. The role of employment status in patients with chronic conditions and in oncologic patients is not clear and data in the literature are lacking. It has been suggested that unemployment influences distress levels in patients with cardiovascular disease [40]. Although not completely comparable with DTC survivors, this might support the need for reintegration strategies for patients in the work process after they have been cured. This is particularly important for patients with DTC who are often very young at time of diagnosis. Some specific problems reported by patients in this study slightly correlated with clinical characteristics, such as paresthesia in patients with hypoparathyroidism or mouth sores and impaired speech in patients receiving a higher cumulative dose of RAI (data not shown). These problems are very specific for thyroid carcinoma patients and should be recognized and addressed by the physicians. The majority of reported problems however did not correlate with clinical characteristics. Altogether these results demonstrate that distress is a profound individual reaction which cannot be predicted by objective clinical data and emphasize the need for routine specific screening for distress.

Consistent with previous findings, patients cited physical problems over emotional problems as sources of distress [41]. Nevertheless, the correlation of DT scores with those of HADS and ICQ helplessness domain suggest that physical problems may lead to an increased psychological burden for these patients. In addition, the negative correlation with scores of the ICQ acceptance domain suggests that the increasing burden render the patients less likely to accept these problems.

The high DT score alone does not automatically imply that the patient should be referred for additional support, despite the clear correlation between DT score and the wish to be referred. Many problems that can be important sources of distress can be resolved by discussing them with nurses or physicians, others may require consultation of other professionals such as dieticians, physical therapists or social workers. The PL that accompanies the DT is a very useful starting point when discussing with patients about their specific problems. Previous data suggest that acknowledging and discussing problems alone may already yield clinical benefits and improve patient satisfaction [19]. In addition, the wish for intervention for individual patients must be taken into account, and therefore some patients can explicitly refuse further referral or just 'maybe' consider it. In this series, although majority of patients did not express a wish to be referred for additional support, 13.1% of them addressed wishes for referral and 25.5% 'maybe' considered it. These percentages are lower compared with other forms of cancer such as lung cancer where approximately 22% expressed wishes for referral [20]. Nevertheless, considering that this series consisted mainly of long-term disease-free patients, these numbers are relevant and the needs of these patients should be addressed in a multidisciplinary fashion.

Distress scores correlated with HADS anxiety, depression and total scores. This is in accordance with previous data in other types of cancer [42]. Cut-off score of 5 resulted in optimal sensitivity and specificity relative to the HADS. The sensitivity of the DT in the current study was comparable to the results of a recent meta-analysis (77% vs. 78.3% in the meta-analysis), whereas specificity was higher (77% vs. 66.5% in the meta-analysis) [22]. In addition, NPV in the current study (92%) was higher compared to the meta-analysis (84%) [22], confirming that the DT is excellent for ruling out clinically elevated distress in this patient category, but given its low PPV of 49% it is less useful as diagnostic tool. Therefore a DT score ≥ 5 is only a starting point for further diagnostic evaluation by a physician or specialized nurse, if necessary followed by further intervention.

This study has a number of limitations. The cross sectional retrospective nature of this study does not allow evaluation of the course of distress in time. Some periods during treatment or follow-up might be associated with higher levels of distress, such as the period short after establishing the diagnosis and the period of invasive treatment (surgery, RAI). Also patients with persistent disease might experience more distress than long-term disease-free survivors. This series includes only few patients with persistent disease, as expected given the good prognosis of the disease, and lacks the statistical power required to analyze these correlations. In addition this study was not designed to investigate the clinical benefit of screening these patients.

Nevertheless, this study represents the first evaluation of the level of distress in patients with DTC. It includes a large number of patients with DTC with long-term remission, who represent the majority of patients seen on the oncologic endocrine follow-up clinic. In addition, it is the first study to evaluate the validity of the DT accompanied by the PL against the HADS for screening of distress in this patient category.

In conclusion, the prevalence of distress is high in patients with DTC even after long-term remission. Physical and emotional problems were the main sources of distress. Clinical and socio-demographic data were not correlated with distress in DTC patients, except for the employment status. The DT and PL are useful time efficient screening instruments for psychosocial distress in DTC patients and could easily be incorporated into daily practice. These findings highlight the importance of a routine psychological distress screening even in a later phase after treatment. Future research should focus on the benefits of prospective and systematic screening for distress in patients with DTC, as well as best intervention strategies.

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Chapter 3

High prevalence of self-reported shoulder complaints after thyroid carcinoma surgery

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Abstract

Background: Shoulder complaints are frequently reported after surgical treatment for thyroid carcinoma. However, no specific literature on this topic is available for these patients and hence, its impact on quality of life (QoL) is unknown and there are no known predictors of shoulder complaints in this specific patient population. Therefore the purpose of this study was to assess the prevalence of shoulder-related complaints and its relation to QoL and clinical characteristics after thyroid carcinoma surgery by means of a cross-sectional case control study in a tertiary referral center.

Methods: The prevalence of shoulder complaints and its relation to clinical characteristics and QOL after thyroid carcinoma surgery (n=109) was compared to a healthy control group (n=81). Main outcome measures are prevalence of self-reported shoulder complaints, results of the Disabilities of the Arm, Shoulder and Hand questionnaire (DASH) and the European Organization for Research and Treatment of Cancer, Quality of Life Questionnaire-C30 (EORTC-QLQ-C30).

Results: Patients with thyroid carcinoma, on average 10.2 years after thyroid surgery, reported a 58.7% prevalence of shoulder-related complaints, which was significantly more than the 13.6% reported by healthy controls ($p<0.01$). Patients with thyroid carcinoma scored worse than healthy controls on most of the different subscales of the DASH and EORTC-QLQ-C30. Bivariate association analysis identified level V neck dissection as being associated with the prevalence of shoulder complaints and DASH score, and spinal accessory nerve damage and employment status as being associated with DASH score. Prevalence of shoulder complaints and DASH scores were significantly correlated to several EORTC-QLQ-C30 scores. Only 11.9% of patients with thyroid carcinoma retrospectively reported having received preoperative information on possible shoulder complaints and only 34.9% of patients with thyroid carcinoma retrospectively reported having received additional care for their shoulder complaints.

Conclusions: Shoulder complaints represent an underestimated problem and are reported by many patients who had surgery for thyroid carcinoma. Information provision to the patient should be improved, shoulder complaints should be registered and additional care should be provided after thyroid carcinoma surgery to improve QoL.

Introduction

Surgical treatment of head and neck malignancy, especially when involving neck dissections, is well known to be associated with shoulder complaints [1, 2]. After radical neck dissection, shoulder dysfunction has even been shown to be the most important source of long-term morbidity of the patient with symptoms consisting of shoulder pain, limitation in shoulder mobility and winging of the scapula [3]. This has led to a trend towards more conservative management of the neck. Nevertheless, shoulder complaints are still frequently reported after surgical treatment for thyroid carcinoma.

In a recent multinational patient/survivors initiated survey on the psychosocial/informational needs of patients with thyroid carcinoma/survivors 28.9% of the participants complained of numbness in the neck region and 26.8% reported restricted shoulder/neck movements more than 1 year after treatment [4]. However this study was not specifically designed to investigate shoulder complaints in these patients. Investigation of shoulder function is important because, due to the excellent prognosis of thyroid carcinoma with survival rates exceeding 90%, recovery of functional abilities, which are strongly related to distress in these patients, is essential to improve the quality of life (QoL) [5]. The relation between shoulder complaints and neck dissections is of special interest as, over the last decades, there has been a major emphasis on QoL related to (temporary) spinal accessory nerve (SAN) damage and "shoulder syndrome" after radical neck dissection in head and neck malignancies [6, 7]. However as the majority of patients with thyroid carcinoma do not require extensive neck dissection, the shoulder complaints in these patients are thought to be limited and the majority of patients do not routinely receive information on this topic. Therefore, the purpose of this study was to assess the prevalence of self-reported long-term shoulder complaints in a historical cohort of patients who had surgery for thyroid carcinoma compared to a healthy control group. Exploratory aims were to identify possible clinical or demographic correlates of shoulder complaints, and to evaluate associations between shoulder complaints and QoL.

Patients and methods

Design

A cross-sectional analysis was performed in a historical cohort of adults (18-80 years old) surgically treated for thyroid carcinoma in the past (either in our hospital or in another hospital) and who were currently under routine follow-up at our tertiary referral center. Primary treatment consisted of total thyroidectomy (and neck dissection in patients with preoperative clinically and/or radiologically confirmed nodal metastasis) followed in the

majority of patients by ablation with radioactive iodine (I131) of residual thyroid tissue 4 to 6 weeks after surgery. Patients who had completed treatment as well as those who were still receiving active treatment were included. No minimal time since completion of last treatment was required in order to participate. Subjects were invited to participate in this study by receiving an information letter and questionnaire package by mail. Participating patients were kindly asked to provide their own healthy control subject of the same gender and the same age. The advantage of using acquaintance controls is that they are more likely to be similar to the patients in regard to baseline characteristics such as age, relationship status, social status, education level and community of residence. Patients who could not find a suitable acquaintance control were still included in the analysis. Non-responders received a reminder call 4 weeks later. Subjects with any intercurrent illness or disease such as trauma, inflammatory conditions or cerebrovascular accidents, that could cause shoulder complaints, were excluded. The presence of exclusion criteria in both the patients and the controls was checked using an ad-hoc questionnaire.

This study has been approved by the institutional Ethics Committee and all patients provided written informed consent before participation.

Clinical characteristics

Clinical variables were collected from medical charts and included information on histopathological tumor subtype, tumor stage, time since treatment, radioactive iodine treatment including frequency and cumulative dose, neck dissection type, surgical dissection of the SAN and sternocleidomastoid muscles (SCM) as reported by the surgeon, present disease status, age at diagnosis, thyroid stimulating hormone (TSH) level in the period around completing the questionnaires (<6 months) and comorbidities. Comorbidities at the time of study participation were evaluated using the American Society of Anesthesiologists (ASA) classification [8]. Current disease status was defined as cured (remission) in case of undetectable Tg in the absence of anti-Tg antibodies and no evidence of loco-regional disease or distant metastases at last follow-up visit. Persistent- or recurrent disease was defined as either detectable Tg or evidence of loco-regional disease or distant metastases.

Questionnaires

Disabilities of the Arm, Shoulder and Hand questionnaire

The Disabilities of the Arm, Shoulder and Hand (DASH) questionnaire was developed to measure the patients' perception of the ability to perform different activities and roles and to monitor symptoms associated with any condition in the upper limb [9]. The questionnaire has been validated with a variety of upper limb disorders [10, 11]. The 30-items questionnaire includes 21 physical function items, 6 symptom items, and 3 social or role function items. In this study, optional modules on work (4 items) and sports/performing arts (4 items)

were included. All items refer to the situation in the last week. The responses are scored on a 5-point Likert scale, ranging from *no difficulty* to *unable*, from *none* to *extreme*, from *no impact* to *high impact*. The raw score is transformed to a 0-100 score. A score of 0 reflects no disability, and 100 indicates maximal disability. In this study, a Dutch language version was used which has shown excellent internal consistency in literature (Cronbach's α = 0.95) [12] and an even better internal consistency for the total questionnaire and different subscales in the current study sample (total questionnaire Cronbach's α = 0.98, work module Cronbach's alpha = 0.97 and sports/performing arts Cronbach's alpha = 0.98)

European Organization for Research and Treatment of Cancer, Quality of Life Questionnaire-C30

The European Organization for Research and Treatment of Cancer, Quality of Life Questionnaire-C30 (EORTC QLQ-C30) (version 3.0) is a 30-item questionnaire assessing functional health, symptom experience and quality of life (QoL) of cancer patients, in general. It consists of 5 scales on physical-, role-, cognitive-, emotional- and social functioning, a global health status scale (GHS), 3 symptom scales on fatigue, nausea and vomiting and pain, and 6 single items assessing dyspnea, insomnia, loss of appetite, constipation, diarrhea and financial impact. All items refer to the situation in the last week. Each item is scored on response scale ranging from 1 (*not at all)* to 4 (*very much),* with the exception of the GHS, which is scored on a modified linear analogue scale ranging from 1 (*very poor*) to 7 (*excellent*). All scales and single items are transformed to a 0-100 scale. A higher score on the functional scales and GHS indicates better functioning and QoL, whereas higher scores on the symptom scales indicate more complaints.

Demographical characteristics

Demographic information on age, gender, relationship status, educational level and employment status was collected by means of an ad-hoc questionnaire.

Information provision on shoulder complaints and additional care consumption

An ad-hoc questionnaire consisting of 4 items was used to gather information on whether or not patients had shoulder complaints (yes/no), recalled receiving information preoperatively on the possibility of postoperative shoulder complaints (yes/no) and the amount of extra care/therapy that was received because of shoulder complaints (yes/no) and the time it took after surgery (in months) before extra therapy was started.

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 (SPSS Inc, Chicago, Illinois). Differences in demographical and clinical characteristics and questionnaire scores between the subgroups of participants were analyzed using independent samples t-test or χ^2 test

in case of categorical variables. Cohen's d effect size of differences between patients and controls on questionnaire scores was calculated. Within the thyroid carcinoma group, bivariate associations between clinical and demographical variables and prevalence of shoulder complaints and total DASH scores were tested using Mann-Whitney-U test or Kruskal-Wallis ANOVA. For the bivariate associations between two categorically coded characteristics χ^2-test or Fisher's exact test was used as appropriate. To reveal possible predictors of prevalence of shoulder complaints and total DASH scores, multiple linear/logistic regression analysis was performed as appropriate. Variables with a $p \leq 0.25$ in the bivariate analysis were included in multiple linear/logistic regression analysis as appropriate. On an exploratory basis, correlations between prevalence of shoulder complaints, DASH scores and EORTC-QLQ30 subscale scores in the patients with thyroid carcinoma were examined using Spearman's rank correlation testing in order to examine the relation between shoulder complaints, DASH scores and the diverse aspects of QoL. Statistical significance was defined as a $p<0.05$.

Results

Subject characteristics

One hundred and ninety-three patients were invited for this study. Of these, 112 patients responded (response rate 58%). Two patients refused to participate because of recent traumatic injury to the arm/shoulder. One patient was excluded because of a recent cerebrovascular infarction. Twenty-eight patients were not able to find their own control subject. A total of 109 patients with thyroid carcinoma [72.5% female, mean age (SD) 53.3 (13.3) years] with a median (range) follow-up time since last treatment of 97.3 (0.5-565.1) months and 81 healthy controls [72.8% female, mean age (SD) 51.3 (12.7) years] were enrolled in the study. Clinical- and demographical characteristics of all subjects are depicted in Table 1. Participating and non-participating patients with thyroid carcinoma did not differ with respect to clinical characteristics (*P*-value >0.2 for all items).

DASH

The scaled DASH scores are depicted in Table 2. Patients with thyroid carcinoma had significantly worse full scale DASH scores compared to healthy controls ($P<0.01$). Furthermore, the patients with thyroid carcinoma scored worse on the optional modules on work and sports/performing arts compared to healthy controls ($P<0.05$).

EORTC-QLQ-C30

The scaled EORTC-QLQ-C30 scores are depicted in Table 2. Patients with thyroid carcinoma scored significantly worse, compared to healthy controls, on all subscales (P<0.05) except for the symptom subscales of nausea and vomiting and constipation.

Table 1. Demographic and clinical variables

Variable	Patients with thyroid carcinoma (n=109)	Healthy controls (n=81)	*P*- value
Gender			
Male: n (%)	30 (27.5)	22 (27.2)	0.57
Female: n (%)	79 (72.5)	59 (72.8)	
Age: mean (SD) (years)	53.3 (13.3)	51.3 (12.7)	0.31
Age at diagnosis: mean (SD) (years)	40.8 (13.3)	N/A	N/A
≤45: n (%)	62 (56.9)		
>45: n (%)	47 (43.1)		
Follow up time since last treatment: median (range) (months)	97.3 (0.5-565.1)	N/A	N/A
≤12: n (%)	15 (13.8)		
>12: n (%)	94 (86.2)		
Histopathological subtype: n (%)		N/A	N/A
Papillary	75 (68.8)		
Follicular	21 (19.3)		
Medullary	3 (2.8)		
Mixed	7 (6.4)		
TNM stage*: n (%)		N/A	N/A
I	68 (62.4)		
II	10 (9.2)		
III	15 (13.8)		
IV	16 (14.7)		
Surgical treatment: n (%)		N/A	N/A
Hemithyroidectomy	4 (3.7)		
Total thyroidectomy	105 (96.3)		
Neck dissection[1]	51 (46.8)		
RAI frequency: mean (SD)	1.55 (1.11)	N/A	N/A
RAI cumulative dose: mean (SD)		N/A	N/A
None	9 (8.3)		
≤100 mCi: n (%)	9 (8.3)		
100-200 mCi: n (%)	51 (46.8)		
>200 mCi: n (%)	40 (36.7)		
Neck dissection level: n (%)		N/A	N/A
None	58 (53.2)		
VI	31 (28.4)		
II-IV	43 (39.4)		
V	29 (26.6)		
Additional structures damaged in surgery: n (%)		N/A	N/A
None reported	103 (94.5)		
Sternocleidomastoid muscle	4 (3.7)		
Spinal accessory nerve	3 (2.8)		

ASA classification: n (%)			<0.01
I	0 (0.0)	65 (80.2)	
II	100 (91.7)	13 (16.0)	
III	7 (6.4)	3 (3.7)	
IV	2 (1.8)	0 (0.0)	
Disease status: n (%)		N/A	N/A
Remission	85 (78.0)		
Active disease	24 (22.0)		
Biochemical active disease	17 (15.6)		
Anatomical active disease	7 (6.4)		
TSH around the time of participation: n (%)		N/A	N/A
<0.01 mE/l (suppressed)	26 (23.9)		
0.01-0.40 mE/l	42 (38.5)		
0.40-4.00 mE/l (normal range)	39 (35.8)		
>4.00 mE/l	2 (1.8)		
Relationship status: n (%)			0.66
Married/cohabiting	94 (86.2)	58 (71.6)	
Divorced	5 (4.6)	8 (9.9)	
Widow(er)	3 (2.8)	6 (7.4)	
Single	7 (6.4)	9 (11.1)	
Education level: n (%)			0.30
Primary school	5 (4.6)	1 (1.2)	
Secondary education	21 (19.3)	8 (9.9)	
Higher secondary education	45 (41.3)	36 (44.4)	
University	38 (34.9)	36 (44.4)	
Employment status: n (%)			<0.05
Employed	56 (51.4)	56 (69.1)	
Unemployed	53 (48.6)	25 (30.9)	

TNM, Tumor Node Metastasis; RAI, Radioactive Iodine ; ND, Neck Dissection; ASA, American society of Anesthesiologists; N/A, not applicable; TSH, thyroid stimulating hormone

* Union for International Cancer Control Classification of malignant tumors, 6th edition [27]

Note[1]: Not all neck dissections were performed synchronously with the thyroidectomy.

Note[2]: p-values were derived from independent samples t-test or χ^2-test as appropriate.

Prevalence of shoulder complaints, information supply and additional care consumption

Significantly more patients with thyroid carcinoma (58.7%) reported having shoulder complaints compared to the healthy controls (13.6%) (P<0.01). Only 11.9% of the patients with thyroid carcinoma recalled receiving pre-operative information about possible postoperative shoulder complaints. Of the patients with thyroid carcinomas, 34.9% received additional care for their shoulder complaints. All data about prevalence of shoulder complaints, information supply and additional care consumption are provided in Table 3.

Bivariate associations and regression analysis

Bivariate associations in the patients with thyroid carcinoma between subject characteristics, prevalence of shoulder complaints and DASH scores are depicted in Table 4. Level V ND was significantly associated with higher prevalence of shoulder

complaints (*P*=0.03) and higher total DASH score (*P*=0.02). Reported SAN damage during surgery was significantly associated with higher total DASH score (*P*=0.03) and being unemployed was significantly associated with higher total DASH score (*P*=0.01). The remaining clinical and demographical variables were not significantly associated with shoulder complaints or DASH scores.

Results of the multiple logistic/linear regression analysis are reported in table 5. TNM stage turned out to be significantly associated with prevalence of shoulder complaints (*P*<0.05) and total DASH score (*P*<0.05) with lower TNM stage predicting more shoulder complaints and worse total DASH score. Furthermore, reported SAN damage during surgery was significantly associated with total DASH score (*P*<0.05). Employment status was significantly associated with total DASH score (*P*<0.01).

Table 2. Questionnaire scores and between group differences

Variable	Patients with thyroid carcinoma (n=109)	Healthy controls (n=81)	*P*-value	Cohen's d
DASH				
DASH Total: mean (SD)	21.5 (20.1)	7.2 (13.4)	<0.01	0.84
DASH work: mean (SD)	16.9 (17.8)	6.6 (13.5)	<0.01	0.65
DASH sports/arts: mean (SD)	24.8 (18.9)	6.0 (11.3)	<0.01	1.21
EORTC-QLQ-C30				
Physical functioning: mean (SD)	82.1 (17.5)	95.0 (7.6)	<0.01	-0.96
Role functioning: mean (SD)	77.1 (27.1)	91.6 (17.5)	<0.05	-0.64
Emotional functioning: mean (SD)	82.4 (19.9)	89.5 (13.7)	<0.01	-0.42
Cognitive functioning: mean (SD)	79.8 (25.2)	93.2 (16.0)	<0.01	-0.63
Social functioning: mean (SD)	82.7 (23.6)	96.5 (12.3)	<0.01	-0.73
GHS: mean (SD)	73.4 (20.7)	84.6 (15.4)	<0.01	-0.61
Fatigue: mean (SD)	29.0 (27.1)	12.5 (13.7)	<0.01	0.77
Nausea and vomiting: mean (SD)	2.4 (7.5)	1.0 (4.0)	0.11	N/A
Pain: mean (SD)	18.5 (20.0)	11.9 (19.8)	<0.05	0.33
Dyspnea: mean (SD)	16.2 (23.4)	6.6 (15.3)	<0.01	0.49
Insomnia: mean (SD)	27.5 (30.7)	12.8 (22.1)	<0.01	0.55
Loss of appetite: mean (SD)	5.8 (15.6)	0.8 (5.2)	<0.01	0.43
Constipation: mean (SD)	8.9 (20.1)	4.9 (15.0)	0.13	N/A
Diarrhea: mean (SD)	6.4 (16.7)	1.6 (7.3)	<0.01	0.37
Financial impact: mean (SD)	9.8 (21.4)	2.0 (9.6)	<0.05	0.47

DASH, Disabilities of the Arm, Shoulder and Hand questionnaire; GHS, Global Health Score; N/A, not applicable; EORTC-QLQ-C30, European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire-Core-30-questions.

Note[1]: p-values were derived from independent samples t-test. Cohen's d values depict effect sizes (d=0.2, small effect; d=0.5, medium effect; d=0.8, large effect)

Note[2]: higher scores indicate worse functioning on the DASH, and higher scores indicate better functioning on the EORTC-QLQ-C30 functioning scales but worse status on the symptom scales.

Table 3. Patient's retrospective reports about shoulder complaints and the information and care they received

Variable	Patients with thyroid carcinoma (n=109)	Healthy controls (n=81)	*P*-value
Shoulder complaints: n (%)			<0.01
Yes	64 (58.7)	11 (13.6)	
No	45 (41.3)	70 (86.4)	
Received preoperative information on possible postoperative shoulder complaints: n (%)		N/A	N/A
Yes	13 (11.9)		
No	96 (88.1)		
Received additional care for shoulder complaints: n (%)		N/A	N/A
Yes	38 (34.9)		
No	71 (65.1)		
Time it took before additional care was provided after surgery: mean (SD)		N/A	N/A
Directly: n (%)	1 (0.9)		
1-3 months: n (%)	15 (13.8)		
3-12 months: n (%)	12 (11.0)		
>12 months: n (%)	10 (9.2)		

N/A, not applicable.

Note: p-values were derived from independent samples t-test or χ^2-test as appropriate.

Correlations between prevalence of shoulder complaints, DASH scores and EORTC-QLQ-C30 scores in patients with thyroid carcinoma

The prevalence of shoulder complaints was positively correlated with total DASH score (r = 0.617, P<0.01) and DASH work (r = 0.568, P<0.01) and sports/performing arts module scores (r = 0.670, P<0.01). Furthermore, prevalence of shoulder complaints and total DASH score were negatively correlated with all EORTC-QLQ-C30 functional- (physical-, role-, cognitive-, emotional- and social functioning) and GHS subscale scores and positively correlated with all symptom subscale (fatigue and pain) and single item (dyspnea, insomnia, constipation and financial impact) scores except for nausea and vomiting, loss of appetite, and diarrhea (data shown in Table 6).

Table 4. Bivariate associations between demographic and clinical variables and DASH scores and shoulder complaints for patients with thyroid carcinoma only

Variable	DASH total score (mean (SD))	Shoulder complaints (n (%))
Gender:		
Male	19.6 (17.9)	16 (53.3)
Female	22.2 (20.9)	48 (60.8)
P-value	0.58	0.48
Age: (years)		
≤45	20.8 (18.3)	38 (61.3)
>45	22.4 (22.4)	26 (55.3)
P-value	0.84	0.53
TNM stage:		
I	21.4 (19.3)	43 (63.2)
II	32.8 (15.6)	7 (70.0)
III	14.7 (21.4)	4 (26.7)
IV	20.6 (23.0)	9 (56.3)
P-value	0.08	0.23
RAI cumulative dose:		
None	12.1 (16.4)	6 (66.7)
≤100 mCi	22.6 (24.2)	6 (66.7)
100-200 mCi	23.5 (20.5)	30 (58.8)
>200 mCi	20.7 (19.4)	22 (55.0)
P-value	0.56	0.88
Neck dissection:		
Yes	22.4 (20.1)	34 (66.7)
No	20.7 (20.2)	30 (51.7)
P-value	0.58	0.11
Neck dissection level II-IV:		
Yes	24.2 (20.5)	30 (69.8)
No	19.7 (19.7)	34 (51.5)
P-value	0.22	0.06
Neck dissection level V:		
Yes	28.2 (20.5)	22 (75.9)
No	19.1 (19.5)	42 (52.5)
P-value	0.02*	0.03*
Neck dissection level VI:		
Yes	18.8 (17.9)	19 (61.3)
No	22.6 (20.9)	45 (57.7)
P-value	0.54	0.73
SCM damaged during surgery:		
Yes	27.1 (29.0)	3 (75.0)
No	21.3 (19.8)	61 (58.1)
P-value	0.60	0.64
SAN damaged during surgery:		
Yes	48.9 (19.6)	3 (100.0)
No	20.7 (19.6)	61 (57.5)
P-value	0.03*	0.27

Table 4. Continued

Follow up since last treatment: (months)		
≤12	19.3 (16.0)	10 (66.7)
>12	21.8 (20.7)	54 (57.4)
P-value	0.89	0.30
ASA classification:		
II	20.9 (19.8)	57 (57.0)
III	22.0 (22.1)	5 (71.4)
IV	50.4 (13.6)	2 (100.0)
P-value	0.17	0.37
Disease status:		
Remission	22.4 (20.7)	48 (56.5)
Active disease	18.2 (17.6)	16 (66.7)
P-value	0.40	0.37
Relationship status:		
Married/cohabiting	21.0 (20.2)	56 (59.6)
Single	24.3 (20.0)	8 (53.3)
P-value	0.46	0.65
Education level:		
Primary school	29.3 (22.9)	3 (60.0)
Secondary education	31.9 (22.5)	14 (66.7)
Higher secondary education	17.2 (17.6)	26 (57.8)
University	19.7 (19.6)	21 (55.3)
P-value	0.07	0.86
Employment status:		
Employed	16.1 (16.6)	30 (53.6)
Unemployed	27.2 (22.0)	34 (64.2)
P-value	0.01*	0.26

TNM, Tumor Node Metastasis; RAI, Radioactive Iodine; ND, Neck Dissection; ASA, American Society of Anesthesiologists; DASH, Disabilities of the Arm, Shoulder and Hand Questionnaire; SCM, sternocleidomastoid; SAN, spinal accessory nerve.

Note: p-values were derived from Mann-Whitney-U test or Kruskal-Wallis ANOVA as appropriate. For the bivariate associations between two categorically coded characteristics χ^2-test or Fisher's exact test was used as appropriate .

Table 5. Multiple logistic/linear regression analyses for patients with thyroid carcinoma only

Variable	DASH total score β (*P*-value)	Shoulder complaints OR (95%-CI) (*P*-value)
TNM stage(I/ II vs. III/IV)	-.239 (0.013*)	.31 (0.12-0.81) (0.017*)
Neck dissection level II-IV (Yes vs.No)	-.015 (0.913)	1.90 (0.53-6.74) (0.323)
Neck dissection level V (Yes vs.No)	.190 (0.155)	1.96 (0.47-8.12) (0.355)
SAN damaged during surgery (Yes vs.No)	.198 (0.031*)	0.00 (0.00) (0.99)
ASA classification (II vs.III/IV)	.101 (0.251)	N/A
Education level (Primary school/ Secondary education vs.Higher secondary education/University)	-.179 (0.054)	N/A
Employment status (Employed vs.Unemployed)	.281 (0.004*)	N/A

DASH, Disabilities of the Arm, Shoulder and Hand Questionnaire; 95% CI, 95% confidence interval; SAN, spinal accessory nerve; ASA, American Society of Anesthesiologists.

Note[1]: Neck dissection (yes/no) was deleted from the regression analysis because of multicollinearity with the predictors "Neck dissection level II-IV" and "Neck dissection level V".

Note[2]: R^2 for the multiple linear regression =.25

Note[3]: R^2 for the multiple logistic regression =.15 (Nagelkerke)

N/A, Not applicable; β, standardized beta; OR, odds ratio.

* p <0.05

Table 6. Correlations between prevalence of shoulder complaints, DASH scores and EORTC-QLQ-C30 scores in patients with thyroid carcinoma

EORTC-QLQ-C30 subscales	DASH total score		Shoulder complaints	
	r	*P*-value	*r*	*P*-value
Physical functioning	-.741	<0.01	-.404	<0.01
Role functioning	-.784	<0.01	-.517	<0.01
Emotional functioning	-.377	<0.01	-.203	0.03
Cognitive functioning	-.362	<0.01	-.242	0.01
Social functioning	-.495	<0.01	-.273	<0.01
GHS	-.551	<0.01	-.306	<0.01
Fatigue	.564	<0.01	.263	0.01
Nausea and vomiting	.181	0.06	.114	0.24
Pain	.793	<0.01	.566	<0.01
Dyspnea	.327	<0.01	.263	<0.01
Insomnia	.529	<0.01	.349	<0.01
Loss of appetite	.135	0.16	.126	0.19
Constipation	.201	0.04	.216	0.02
Diarrhea	.059	0.54	.012	0.90
Financial impact	.303	<0.01	.210	0.03

GHS, Global Health Score; DASH, Disabilities of the Arm, Shoulder and Hand Questionnaire; EORTC-QLQ-C30, European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire-Core-30-questions.

Note: p-values were derived from Spearman's rank correlation testing. *r* = Spearman's-rho.

Discussion

In this study, the prevalence of self reported long-term shoulder complaints and its relation to QoL and clinical characteristics was investigated in a large historical cohort of patients who have been treated for thyroid carcinoma compared to a gender- and age matched control group consisting of healthy controls. The most important finding is that patients treated for thyroid carcinoma suffer from increased shoulder-related symptoms compared to healthy controls. Only a small proportion of patients recalled being informed preoperatively about the possibility of postoperative shoulder complaints. Furthermore, only a small proportion of patients received additional treatment for their problems. Correlation analysis showed that DASH scores and prevalence of shoulder complaints were significantly related to several EORTC-QLQ-C30 subscale scores, which implies that having shoulder complaints is significantly associated with a diverse range of aspects of QoL in these patients.

As expected, SAN damage during surgery was significantly associated with worse DASH scores . The shoulder complaints resulting from neck dissections are characterized by shoulder droop, inability to shrug, limited forward flexion, limited active lateral abduction, constant dull ache and aberrant scapular rotation. The results of the current study in patients with thyroid carcinoma are comparable to the results of studies in patients undergoing surgical treatment for other head- and neck cancers that also used questionnaires on self-reported QoL and complaints. One study in patients with thyroid carcinoma showed that especially extensive neck dissections are associated with shoulder complaints which is an important source of long-term morbidity and QoL impairment [3, 13-15]. Dissections extending into level V are known to cause a higher incidence of shoulder complaints [16]. This is consistent with the finding of an association between level V neck dissection and shoulder complaints/worse DASH scores in the bivariate analysis in the current study. This is, most likely, caused by the fact that the SAN is encountered in level V where it lies superficial and may suffer local trauma or unintentional division [17]. The previous study in patients with thyroid carcinoma did not take this specific relation with level V dissections and SAN damage into account [15].

However, we were surprised to find that a large proportion of patients in our series who did not have extensive neck dissection reported shoulder-related complaints still many years after the primary treatment. Moreover, we were surprised that a lower TNM classification was associated with reporting more shoulder complains. This has led us to consider that apart from the obvious possible causes of shoulder complaints after head and neck surgery such as SAN damage (sometimes intentionally sacrificed in patients with extensive disease) other factors may play a role as well. One can envisage that, even in the absence of a

reported SAN damage, traction, devascularisation or transsection of the fine branches may contribute to a temporary or a persistent shoulder discomfort.

Part of the shoulder complaints in patients with thyroid carcinoma might be attributable to the cutaneous sensory distribution of the four major cervical roots. Especially patients undergoing more limited procedures might experience more radicular pain and abnormal motor activity in this cervical distribution as these nerves are irritated but not sacrificed. Furthermore, SCM preservation will also add to the postoperative discomfort, as the muscle usually retains its sensory capacity. In addition, some authors consider these nerves to provide a contribution to upper trapezius muscle motor innervations [18].

In this study, the shoulder complaints in patients with thyroid carcinoma might also be related to adhesive capsulitis of the glenohumeral joint, associated with non-use of the upper limb during the post-operative period. Adhesive capsulitis could explain the late onset of pain after several weeks frequently seen in these patients and could also provide an explanation on the persistence of shoulder disability when there are no signs of SAN injury.

Surprisingly, the patients with a lower TNM classification reported more shoulder discomfort. We hypothesize that this may be the case, in part, because in patients with high stage, with locally extensive disease and not completely removable tumor, one might prefer performing a more limited surgery aimed to preserve functionality and QoL. On the other hand we cannot exclude that psychological factors might induce a reporting bias as well. Patients with more extensive disease may be more burdened by the possible worse prognosis of the disease and pay less attention to less severe shoulder discomfort. In contrast, patients with less extensive disease with a better long-term prognosis may focus more on optimal QoL and functionality required, for instance for work rehabilitation. Not surprisingly, the regression analysis identified employment status to be significantly associated with shoulder complaints and DASH scores in the patients with thyroid carcinoma. Because this is a cross-sectional study it is difficult to make causal inferences about the relation between employment status and DASH scores. It is however most logical to assume that patients with worse DASH scores are more often unemployed due to their complaints especially as a previous review article found that 46% of patients recovering from head and neck cancer surgery gave up work solely due to shoulder disability [19].

Postoperatively, clinicians should identify shoulder complaints, using questionnaires, for example the DASH, and clinical tests [20], and provide additional physiotherapy treatment. This treatment should not be delayed as appears to be the case in this study, where most patients only commenced additional therapy after more than a month postoperatively. We believe this treatment should continue following discharge from the hospital as these patients

can proceed to lose function and develop adhesive capsulitis/reduced shoulder stability due to loss of trapezius muscle function, especially when the SAN has been damaged. Maintenance of normal active movement and passive stretch are beneficial to prevent adhesive capsulitis and help to gain muscle strength in order to regain full range of motion and avoid the occurrence of joint fibrosis [21, 22]. These interventions have already proven their worth in decreasing shoulder complaints and improving QoL after neck dissections [23]. In addition, the use of botulinum toxin type A has been reported to give significant reduction of shoulder pain with only 1 session of injections in trigger points of painful muscles [24]. Further research is needed to prove the utility of such interventions in patients with thyroid carcinoma, even when no ND is performed.

This study has some limitations. Because of the cross-sectional design, no baseline questionnaire scores or follow-up across time are available and therefore it is impossible to draw causal inferences. However, to compensate for this limitation we have included the control group. The prevalence of complaints of 13,6% in the control group was according to our expectations based on the point prevalence in the general Dutch population [25, 26]. Therefore we conclude that the control population included in our study is representative of the normal Dutch population. The complaints in the general Dutch population are based on a wide variety of etiologies such as impingement, bursitis, tendinitis, rotator cuff lesion, frozen shoulder, capsulitis, arthritis, glenohumeral instability. Recall bias in the patients might have caused underreporting of preoperatively provided information about shoulder complaints and the ad-hoc questionnaire on shoulder complaints, information provision and additional care could not be validated. Our future research is aimed at the prospective assessment of shoulder complaints and analysis of the specific aetiology of the shoulder complaints, by means of physical examination, in order to be able to tailor the treatment and prevention strategies. As the subjects were assessed an average of 10 years after treatment, with a wide range of time since last treatment, it is important to notice that findings may not be generalized to other groups of patients evaluated earlier in recovery, despite the fact that time since last treatment was not associated with shoulder complaints and DASH scores in this study. Furthermore, it was decided not to correct for multiple testing in this explorative study to reduce the incidence of type-2 errors. This implies that we cannot exclude that some of the significant differences/associations may be a finding by chance (i.e., a type-1 error).

In conclusion, the results of this study suggest that the prevalence of self-reported shoulder complaints after thyroid carcinoma surgery is high. It represents an important issue in the management of these patients and is associated with QoL. The patients should be informed about this preoperatively, postoperative physical examination of the shoulder is warranted and physiotherapy should be started as soon as possible when complaints are present or can be expected.

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Chapter 4

Persistent self-consciousness about facial appearance, measured with the Derriford appearance scale 59, in patients after long-term biochemical remission of acromegaly

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Abstract

Context: Acromegaly is associated with impaired quality of life (QoL) and causes anatomical disproportions, which may contribute to the decreased QoL after successful treatment. The Derriford appearance scale 59 (DAS59) is a questionnaire measuring psychological distress and disruptions to everyday life associated with self-consciousness of appearance.

Objective: Investigate the psychological distress and dysfunction related to self-consciousness about appearance and its effect on QoL in patients in long-term remission of acromegaly.

Patients, design and methods: Patients (>18 years old) treated for acromegaly at the Department of Endocrinology of the Radboud University Medical Center Nijmegen were invited to participate. A gender-, age- and body mass index (BMI) matched control group was provided by the patients themselves. Participants were asked to complete the modified DAS59-, research and development 36- (RAND-36), acromegaly quality of life questionnaire (AcroQoL) and a sociodemographic questionnaire. Differences between patient- and control groups and correlations between questionnaire scores and clinical characteristics collected from medical records were analyzed.

Main outcome measures: Questionnaire scores.

Results: Of the 120 respondents, 73 agreed to participate [all cured or under biochemical control, median remission time 10.5 years (range 2.3-43.6 years)]. Of these, 34 (46.6%) reported self-consciousness about their appearance. Twenty-nine of these patients (85.3%) pointed out their face to be a prominent source of self-consciousness. Fifty-seven matched control subjects were included as well. Significant correlations were found between the scores of the DAS59 and the AcroQoL, RAND-36 and VAS in patients.

Conclusions: Even after long-term remission of acromegaly, a large number of patients are self-conscious about their appearance, leading to psychological distress and disruptions to everyday life and decreased QoL. Facial features were the most important source of self-consciousness. This stresses the importance of addressing self-consciousness of appearance and the need for additional support in this regard during follow-up in these patients.

Introduction

Craniofacial disproportions due to soft tissue swelling and new bone formation are highly prevalent in patients with active acromegaly [1]. Although clinically there is a slight improvement in facial appearance after biochemical control of growth hormone (GH) hypersecretion, we have recently shown that significant differences in craniofacial characteristics persist even after long-term remission [2]. It has been suggested that these changes in appearance can lead to self-consciousness about appearance, leading towards self-esteem disintegration, social withdrawal, body image distortion and an impaired quality of life (QoL) [3].

Previous studies, which used the disease specific AcroQol questionnaire, have shown that patients with both active and controlled acromegaly have an impaired QoL. Remarkably, the appearance subscale of the AcroQol was the most affected subscale in these studies [4-7]. Moreover, the patients in remission of acromegaly had only slightly better scores on the appearance subscale than patients with active disease [4, 6, 8]. However the appearance subscale of the AcroQol is limited and does not investigate which aspect of appearance specifically causes the self-consciousness related distress.

The Derriford appearance scale 59 (DAS59) is a psychological questionnaire, developed for research in plastic surgery and oncology. It measures self-consciousness about one's appearance and identifies the anatomical source of this self-consciousness. It is validated for both clinical and research settings. It has an excellent validity and reliability, and has been independently recommended as a measure of choice for the assessment of feelings about appearance [9, 10]. However, the DAS59 had never been used in patients with acromegaly.

The primary aim of the present study was to investigate, by using the DAS59 questionnaire , whether patients in long-term remission of acromegaly suffer from more psychological distress and psychological- and social dysfunction related to self-consciousness of appearance than a gender-, age- and BMI matched control group and whether this affects QoL. In addition we aimed to identify the anatomical sources of self-consciousness in these patients. We hypothesised that (irreversible) changes in craniofacial characteristics caused by the previous period of GH hypersecretion are related to the distress related to appearance in patients in long-term remission of acromegaly.

Subjects and methods

Patients

All patients (18-80 years old) treated at the Department of Medicine, Division of Endocrinology of the Radboud University Medical Center Nijmegen who had been in remission of acromegaly for more than 2 years were invited to participate in this study by letter.

Exclusion criteria were active malignancy, cardiovascular disease (unstable coronary artery disease, heart failure NYHA III-IV), (recent) pregnancy (within 1 year prior to the study), depression, psychosis and personality disorder.

Because the assessment of one's own appearance differs in relation to gender, age and BMI, participants were asked to find their own control subject with the same gender and roughly the same age and BMI [9, 11, 12].

The study was approved by our institutional ethics committee and conformed to the declaration of Helsinki. All participants gave written informed consent for participation in the study.

Questionnaires

DAS59

The DAS-59 is a self-reporting questionnaire that generates a series of valid and reliable measures of the specific psychological distress and disruption to everyday life that are associated with self-consciousness of appearance. It is intended for use in an adult population (> 16 years) [9]. An introductory section identifies whether a subject is self-conscious about their appearance and the aspect of appearance that is of the greatest concern to the respondent. This is referred to as the respondent's 'feature' in the rest of the questionnaire. The DAS59 contains 59 statements and questions with response categories in a Likert format to measure frequency of symptomatology ('almost never'… 'almost always') and levels of associated distress ('not at all distressed'…'extremely distressed'). Fifty-seven items assess relevant psychological distress and dysfunction, and two items assess physical distress and physical dysfunction. The following subscales were examined as described by the questionnaire manual [13]: general self-consciousness (GSC), social self-consciousness (SSC), facial self-consciousness (FSC), Sexual and bodily self-consciousness (SBSC) and negative self-concept (NSC). A higher score on the DAS59 is associated with a greater degree of self image related distress and dysfunction. The format of the introductory

section and a 'not applicable' response category for most items make the scale acceptable for respondents who are not concerned about appearance at all.

In this study a linguistic and cultural translation of the DAS59 from English to Dutch was performed following internationally accepted guidelines [14, 15] and used with permission of the original authors [9]. The translation into Dutch was successful. The backward-translation was compared to the original English version and did not show conceptual discrepancies. During piloting the items were assessed as conceptually and linguistically meaningful and appropriate, and in line with the original (English) items.

The DAS59 questionnaire was modified for the purpose of this study by including a self-rated Visual Analogue Scale (VAS) for the participants to score the level of satisfaction with their facial appearance in general. The VAS is a 10 cm 'ruler' scaled 0 (not at all satisfied) to 10 (very satisfied) [16].

RAND – 36

For the assessment of general QoL the Dutch translation of the RAND-36 was used [18]. It comprises 36 items regarding general well being during the past 30 days. The items are formulated as statements or questions with Likert scale or yes/no response options to assess 9 health concepts: 1) physical functioning; 2) social functioning; 3) role limitations because of physical health problems; 4) role limitations because of emotional problems; 5) mental health; 6) vitality; 7) bodily pain, 8) general health perceptions, 9) health change in the last year. Scores are calculated for each health aspect on a 0-100 scale, in which higher scores represent a better QoL.

AcroQoL

This is a disease-specific questionnaire developed to asses health related QoL in patients with acromegaly [17]. It comprises 22 items. Responses are given as the frequency of occurrence (ranging from always to never) or degree of agreement (ranging from completely agree to completely disagree) on a five-point scale. The questionnaire includes two different scales; a physical performance scale and a psychological well-being scale. The psychological scale is further subdivided into 2 subscales regarding appearance and personal relationships, containing seven items each. The highest achievable score is 110 (100%), which is indicative of an excellent QoL, while the lowest score is 22 (0%) [5].

Sociodemographical and clinical variables

Sociodemographical and clinical characteristics of patients were collected by means of a sociodemographical questionnaire and medical chart review in the patient group.

Definitions

Remission was subdivided into 2 groups and defined as: 1. cure: successful surgical and/or radiotherapeutical treatment of the GH producing adenoma followed by a normalization of the IGF-1 concentration (≤ mean + 2 SDS for age) and suppression of serum GH levels <1.0μg/l during OGTT (after surgical treatment)[18] and 2. disease control: normal IGF1 concentrations (≤ mean +2 S.D.S for age) during the use of somatostatin analogues, dopamine agonists and/or GH receptor antagonists.

Hormonal deficiency was defined as deficiency of one or more pituitary hormones. Hypothyroidism was defined as the use of thyroid hormone substitution therapy. Hypogonadism was defined as use of testosterone substitution in men and use of oestrogen in women. GH deficiency was defined as the need for GH substitution therapy as defined by a maximal GH response <15.3mU/l during an insulin tolerance test (ITT) or a maximal GH response of <12.3 mU/l during an arginine/GHRH test [19]. Glucocorticoid deficiency was defined as the need for glucocorticoid substitution therapy as defined by a plasma morning cortisol <100nmol/l, after withdrawal of glucocorticoids for 24 hours, or a maximal cortisol response <550nmol/l during an ITT [20].

Regarding current disease status, cure or biochemical disease control had been confirmed by a recent (<1 year) normal IGF-1 level in all participating patients.

Statistical analysis

All data are presented as means and standard deviations (SD) or median and range. Data were tested for normality of distribution by means of the Kolmogorov-Smirnov test. T-tests or Mann-Whitney U tests depending on normality of distribution or χ^2 tests (for categorical variables) were performed to compare the variables age, gender and BMI of the patient- and control group. T-tests or Mann-Whitney U tests were performed to compare the scores on the subscales of the DAS59, RAND, AcroQoL and VAS of the patient- and control group. To investigate the presence of correlations between sociodemographic characteristics, clinical characteristics and outcomes of the questionnaires Spearman's rank correlation testing or point-biserial correlation was used as appropriate. Statistical significance was set at the 5% level ($p<0.05$). All statistical analyses were performed using SPSS software (version 20.0; SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

The clinical characteristics of the patients and control subjects are depicted in Table 1. One hundred and thirty-one patients were invited to participate. Of these, 120 subjects responded (response rate 91.6%). Forty-seven of the patients who responded decided not to participate because of a lack of time (n=6), a lack of interest (n=14), physical or emotional problems (n=19) or because they did not have a problem with their appearance (n=5). Three of the patients who responded did not report their reason for refusal to participate. No statistically significant differences were found between the participants and the subjects who refused to participate in this study with respect to clinical parameters, with the exception of age, with the non-participants being slightly older than the participants.

A total of 73 patients (45.2% female, mean age 59.4 ±10.5 years and BMI 28.1 ± 4.7 kg/m^2) agreed to participate. Mean age at diagnosis was 41.6 (11.2) years. Regarding current disease status, cure or biochemical disease control had been confirmed by a recent (<1 year) normal IGF-1 level in all participating patients. Median remission time was 10.5 (2.3-43.6) years. The treatment processes of all patients are depicted in Figure 1. Sixty-three (86.3%) patients had undergone primary pituitary surgery and 3 (4.1%) patients had undergone primary radiotherapy. At the time of the study acromegaly was controlled by medical therapy in 43.8% of the patients: 20 patients (27.4%) used somatostatin analogues (SA) monotherapy, 4 (5.5%) dopamine agonists (DA) monotherapy, 2 (2.7%) GH receptor blockers (GHRB) monotherapy, 6 were using combination therapy. The other 41 (56.2%) patients were in remission of acromegaly without medical therapy. In 41 (56.2%) patients no substitution of pituitary hormones was necessary after surgery or radiotherapy, 32 (43.8%) had some kind of hormonal deficiency, of whom 15 (20.5%) had a hypopituitarism.

Fifty-seven control subjects were included in this study (47.4% female, age 58.6 ± 11.3 years, BMI 26.4 ±3.5 kg/m^2). There was no significant difference between the patient- and the control group regarding age (p=0.510), gender (p=0.860) and BMI (p=0.050), which confirmed the adequate matching.

Derriford appearance scale scores

Table 2 shows the sources of self-consciousness and scores of the two groups on the DAS59. Thirty-four (46,6%) of the patients in long-term remission of acromegaly were self-conscious about their appearance compared to 13 (22.8%) of the control subjects ($p<0.01$). Twenty-nine (85.3%) of the patients indicated that their face was the most prominent source of self-consciousness compared to 3 (23.1%) controls ($p<0.01$). Compared to the control group the patient group had statistically significant higher scores on all subscales ($p<0.01$). No statistically significant correlations were found between the DAS59 scores of the patient group and clinical characteristics like age, gender and BMI (Table 1). However a statistically significant correlation was found between the DAS59 scores of the control group and age ($r=-0.327$, $p<0.05$). Furthermore, in the patient group no correlation was found between the duration of remission and the DAS59 scores ($r=-0.169$, $p=0.153$) and between disease status (cure versus biochemical disease control) and the DAS59 scores ($r=0.021$, $p=0.860$). A slight but significant correlation was found between pituitary function and the DAS59 score ($r=0.279$, $p<0.05$) with patients with a preserved pituitary function scoring lower amounts of self image related distress. Another correlation was found between hypothyroidism and the full scale DAS59 score ($r=-0.249$, $p<0.05$) (Table 1) with patients with hypothyroidism scoring higher. More specifically, a significant correlation was found between hypothyroidism and the GSC-subscale ($r=-0.262$, $p<0.05$). In the patient group, no correlations were found between the DAS59 score in patients and having a partner ($r=0.040$, $p=0.739$), education level ($r=-0.038$, $p=0.749$) or employment status ($r=-0.037$, $p=0.756$). In the control group, these correlations were also not found to be statistically significant; having a partner ($r=0.003$, $p=0.368$), education level ($r=-0.006$, $p=0.963$), employment status ($r=-0.122$. $p=0.368$).

The scores of the AcroQoL and the RAND-36 are shown in Table 3. The DAS59 score correlates inversely with all subscales of the AcroQoL ($p<0.01$). The DAS59 scores were also inversely correlated with most of the subscales of the RAND-36 ($p<0.01$ or $p<0.05$).

Figure 2 shows the visual analogue scale scores. Scores of the patients were significantly lower than the scores of the control-subjects ($p<0.01$). The total satisfaction score ranged from 0 to 10 in the patient group (mean score = 6.2 ± 2.4). Twenty-five patients (34.2%) scored the satisfaction with their facial appearance below 6. Scores in the control subject group ranged from 6-10 (mean score 7.8 ± 0.8). In the patient group the scores on the VAS-scale also showed an inverse correlation with the DAS59 score ($r=-0.567$, $p<0.01$).

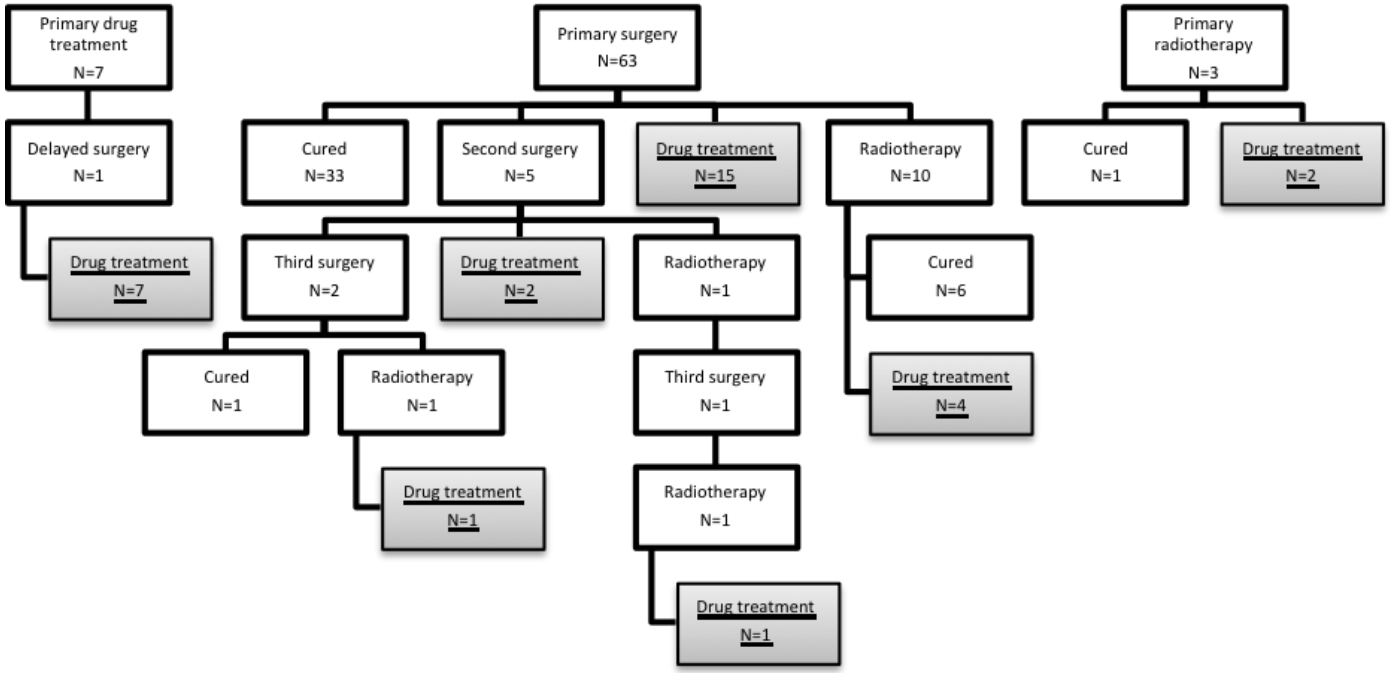

Figure 1. Treatment process of patients. Patients biochemically controlled using drug treatment are depicted in grey.

Table 1. Subject characteristics and correlation with patient's full scale DAS59 score

				Correlation to DAS59 score	
	Patients treated for acromegaly (N=73)	**Control subjects (N=57)**	**p-value**	**Spearman's-rho**	**p-value**
Gender (n): male/ female	40/ 33	30/27	0.860	0.139	0.240
Age: mean (SD) (years)	59.4(10.5)	58.6(11.3)	0.510	- 0.085	0.504
BMI (kg/m^2): mean (SD)	28.1(4.7)	26.4(3.5)	0.050	0.087	0.466
Age at diagnosis: mean (SD) (years)	41.6(11.2)			- 0.095	0.425
Duration since cure: median (range) (years)	10.5 (2.3-43.6)			- 0.169	0.153
2-5 years remission n (%)	8(11.0)				
5-10 years remission n (%)	27(37.0)				
10-15 years remission n (%)	20(27.4)				
> 15 years remission n (%)	18(24.7)				
Treatment modality n (%) (n):					
Surgery	64(87.7)			0.071	0.549
Medical	39(53.4)			- 0.115	0.189
SA	26(35.6)				
DA	8(11.0)				
GHRA	5(6.8)				
Irradiation	14(19.2)			- 0.111	0.351
Disease status: n (%)				0.021	0.860
Cure	41(56.2)				
Biochemical remission	32(43.8)				
Hormonal deficiencies: n (%)					
Preserved pituitary function	41(56.2)			0.279	< 0.05*
Hypothyroidism	26(35.6)			- 0.249	< 0.05*
Hypocortisolism	19(26.0)			- 0.106	0.372
Growth hormone deficiency	17(23.3)			- 0.082	0.493
Hypogonadism	19(26.0)			- 0.150	0.206
Diabetes insipidus	17(23.3)			- 0.068	0.565
Hypopituitarism	15(20.5)			- 0.077	0.516
Co-morbidities: n (%)					
Hypertension	29(39.7)			-0.042	0.725
Diabetes mellitus	14(19.2)			0.007	0.956
Joint related complaints	12(16.4)			-0.109	0.360

*P <0.05 by Spearman's rho

SA, somatostatin analogues; DA, dopamine agonists; GHRB, growth hormone receptor antagonists.

Table 2. Derriford appearance scale 59

	Patients (N=73)	Controls (N=57)	p-value
Prevalence of self-consciousness, n(%):	34 (46.6)	13 (22.8)	< 0.01*
Sources of self-consciousness, n (% of self-conscious subjects):			< 0.01*
Face/facial features	29 (85.3)	3 (23.1)	
Large/coarse feet	1 (2.9)	0 (0.0)	
Curved back	1 (2.9)	0 (0.0)	
Unsightly skin	2 (5.9)	4 (30.8)	
Overweight	3 (8.8)	1 (7.7)	
Breasts	0 (0.0)	1 (7.7)	
Scalp hair	0 (0.0)	1 (7.7)	
General aging	0 (0.0)	1 (7.7)	
Abdomen	0 (0.0)	2 (15.4)	
	Mean score (SD) Patients (N=73)	**Mean score (SD) Controls (N=57)**	**p-value**
Full-scale (FS)	66.6 (33.7)	38.3 (22.7)	< 0.01*
General self-consciousness of appearance (GSC)	21.3 (16.3)	8.8 (8.5)	< 0.01*
Social self-consciousness of appearance (SSC)	15.2 (12.3)	8.2 (8.9)	< 0.01*
Sexual and bodily self-consciousness of appearance (SBSC)	7.8 (6.8)	4.2 (5.0)	< 0.01*
Negative self-concept (NSC)	14.4 (3.9)	12.2 (2.6)	< 0.01*
Facial self-consciousness of appearance (FSC)	2.8 (2.3)	1.7 (2.6)	< 0.01*
Not loading on a specific factor			
53. how irritable do you feel?	2.2 (1.0)	1.7 (0.7)	< 0.01*
59. how hostile do you feel?	1.7 (1.0)	1.3 (0.8)	< 0.01*

*P < 0.01, by Mann-Whitney U test

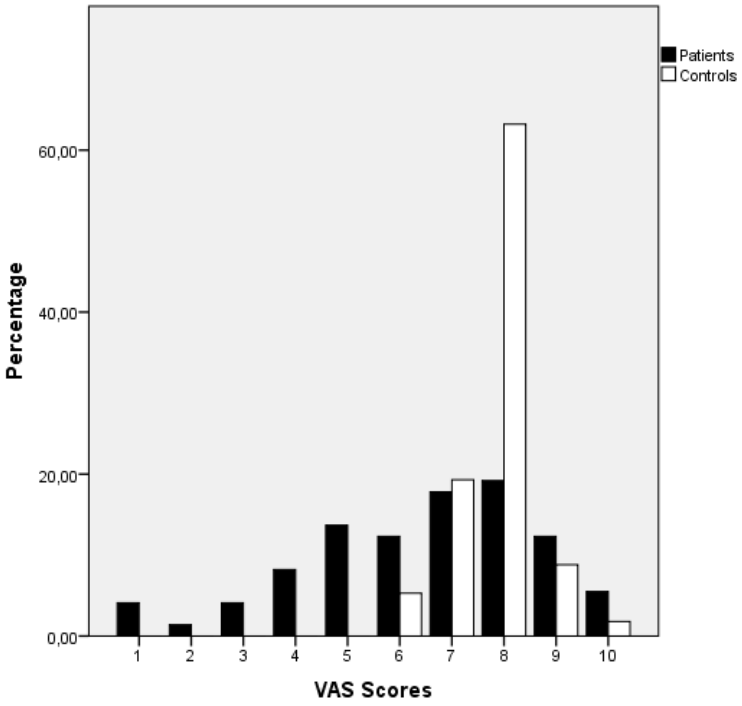

Figure 2. Visual Analogue Scale (VAS) scores

RAND36 scores

Mean scores of the patients in long-term remission of acromegaly were significantly lower than the scores of the control group (Table 3). Subscale scores of patients and controls and comparisons between the groups are depicted in Table 3.

AcroQoL scores

In the patient group, the mean (SD) AcroQoL score was 64.0% (16.6%). The most affected scale was the subscale 'appearance' mean (SD) 57.1% (19.5%). The AcroQol appearance subscale score was significantly correlated to the DAS59 full scale score ($r=-0.597$, $p<0.01$). Other mean subscale scores are further depicted in Table 3.

Table 3. The Derriford Appearance Scale (DAS59) scores and patient group correlations to the scores of the RAND-36 and the scores of AcroQoL

	Mean score (SD)	Mean score (SD)		Correlation to DAS59-score	
Questionnaire	**Patients treated for acromegaly (N=73)**	**Controls (N=57)**	**P-value**	**Spearman's rho**	**P-value**
RAND-36					
Physical functioning	72.8 (23.1)	91.2 (13.3)	< 0.01*	-0.114	0.335
Social functioning	75.7 (23.1)	93.0 (11.1)	< 0.01*	-0.236	< 0.05**
Role limitations due to physical problems	53.8 (44.8)	94.7 (14.7)	< 0.01*	-0.234	< 0.05**
Role limitations due to emotional problems	78.1 (38.2)	94.7 (18.7)	< 0.01*	-0.262	< 0.05**
Vitality	57.5 (20.2)	77.1 (12.5)	< 0.01*	-0.225	0.055
Mental health	74.9 (17.5)	84.4 (10.4)	< 0.01*	-0.322	< 0.01*
Bodily Pain	71.5 (21.7)	89.0 (14.9)	< 0.01*	-0.159	0.179
General Health Perception	54.8 (20.8)	73.9 (16.6)	< 0.01*	-0.189	0.109
Change in health	50.0 (17.2)	56.1 (17.2)	0.064	-0.086	0.470
AcroQol					
Total	64.0 (16.6)			-0.570	< 0.01*
Physical performance	61.3 (20.0)			-0.365	< 0.01*
Psychological well- being	65.5 (17.4)			-0.638	< 0.01*
Appearance	57.1 (19.5)			-0.597	< 0.01*
Personal relations	73.9 (18.2)			-0.570	< 0.01*

*P < 0.01, **P <0.05 by Mann-Whitney U test or Spearman's rho

Sociodemographic characteristics

Of the patients in remission 55 (75.3%) were married or engaged into a relationship versus 47 (82.5%) of the controls (p=0.528), 18 (24.7%) of the patients in remission had no relationship, were divorced or a widow/widower versus 10 (17.5%) of the controls. Two of the patients in remission (2.7%) had less than eight years of education versus 1 (1.8%) of the controls, 6 (8.2%) of the patients in remission versus 6 (10.5%) of the controls had 8-12 years of education, 45 (61.6%) of the patients in remission versus 21 (36.8%) of the controls had 12-16 years of education, 10 (13.7%) of the patients in remission versus 19 (33.3%) of the controls had 16-21 years of education and 10 (13.7%) of the patients in remission versus 9 (15.8%) of the controls had a university degree. Twenty-two of the patients in remission (30.1%) had full- or part-time employment versus 30 (52.6%) of the controls (p<0.05), 29 (39.7%) of the patients in remission versus 18 (31.6%) of the controls were retired, and 22 (30.1%) of the patients in remission versus 9 (15.8%) of the controls were unemployed.

Discussion

In this study we evaluated self-consciousness about appearance and its relation with QoI in 73 patients in long-term remission of acromegaly, and determined which physical aspects have the most influence on the self-consciousness about appearance.

The main finding of the present study is that compared to a matched control group a significantly larger proportion of the patients, are still self-conscious about their appearance (46.6% vs. 22.8%), even after more than 12 years of remission. This is regardless of most clinical and sociodemographic characteristics. The significant correlations between the DAS59 scores and the AcroQol and RAND-36 scores indicate that the concerns about appearance are clearly related to an impaired quality of life and general well-being. In addition, this is the first study that investigates the association between specific "features" and self-consciousness about appearance in patients in long-term remission of acromegaly. An impressing 85.3% of the patients who reported self-consciousness of appearance in our cohort indicated that their face is the main source of discontent. In the general population the distribution of 'features' that cause self-consciousness of appearance is much more evenly distributed throughout the body with only 25-27% focus on the face [11]. This is in concordance with the results of our control group. Moreover, 34 % of the patients scored their facial appearance below 6 on the 0-10 VAS scale compared to 0% in the control group (Figure 2). Our study therefore clearly demonstrates that patients in long-term remission of acromegaly still have significant problems with their facial appearance.

The findings in this study are in line with the findings of previous studies that found that the most affected dimension of the AcroQol was appearance [4, 5]. Matta *et al.* [6] and Paisley *et al.* [7] showed an improvement in the appearance subdomain of the AcroQoL after treatment-induced improvement in IGF-1 levels. However they did not compare these "after treatment" outcomes with the normal population, because the AcroQol is a disease-generated questionnaire specifically designed for patients. Furthermore, previous studies were not able to point out the specific anatomical features that caused the concern and discontent about appearance, while the DAS-59 clearly identifies the face as the primary source of self-consciousness.

Studies about the prevalence of concern about appearance in the general population showed that concern about physical appearance is twice as common among women as among men. The prevalence of concern about appearance is highest during the late teens and early twenties, while it normally decreases with age [8, 11]. In our series there was no correlation between the DAS59 score and gender or age in the patient group, but a statistically significant correlation between the DAS59 scores and age was found in the control

group (r=-0.327, p=<0.05). This indicates that, in contrast to the general population, the individuals in the patient group remained self-conscious about their appearance despite the fact that they aged and men and women are equally affected.

A positive perception of appearance is related to successful psychological functioning and well-being [11]. Therefore, the self-consciousness about appearance and impaired QoL in patients in long-term remission of acromegaly are even more concerning, as these patients are already in remission and these disturbances seem to persist. The concerns about appearance in patients in remission of acromegaly are probably caused by persisting anatomical alterations as craniofacial disproportions remain present, even after long-term remission [2]. A delay in initial diagnosis, often by several years, probably is the major cause of the irreparable changes and damage [6, 21]. In our study persisting co-morbidities may have also contributed to decreased QoL as previously reported [22] as hypertension was present in 39.7% of our patients, diabetes mellitus in 19.2% and joint related complaints in 16.4% of our patients. However no correlations were found between co-morbidities and DAS59 scores.

When the results of the DAS59 mean subscale score on facial self-consciousness of appearance (FSC) of our patient group are compared to the results of previously described clinical subgroups it is notable that the score of our patients (2.8) is comparable or worse than the scores in males considered suitable for rhinoplasty (2.6) , and other cosmetic surgery (2.5) and females considered suitable for a facelift (2.5) and other cosmetic surgery (2.5) [9]. Surprisingly plastic/craniofacial surgery is not widely performed in (ex)acromegaly patients, especially not in Europe. Only a limited number of case reports about corrections of the acromegalic face have been published in literature. However individualized procedures, for example reduction of the vertical dimension of the body of the mandible, rhinoplasty, posterior displacement of the prominent frontal bone, recession and remodeling of cheeks (zygoma) or correction of mandibular prognathism can greatly improve appearance and potentially reduce self-consciousness and associated distress about appearance [23-26]. Another method to support patients that still have concerns about their appearance after remission of acromegaly could be psychological support or psychotherapeutical interventions to prevent evolution of a maladjusted coping style [27-29].

An additional finding in this study is that patients in remission are more likely to be unemployed. This could be a result of the previous illness, but it is interesting to consider the possibility that their appearance could play a role in their unemployment. Literature shows that faces are key to our perception of others and people widely believe that the character of a person can be recognized from his or her face. This phenomenon is commonly labeled the *halo effect* [30]. In addition the slightly higher education level in the control group could

also in part explain the differences we observed. Furthermore, an interesting correlation was found between hypothyroidism and the DAS59 full scale score. Especially the GSC subscale of the DAS59 turned out to be correlated with hypothyroidism, which could be explained by the fact that hypothyroidism, even when adequately treated, is known to be associated with depressive symptoms [31].

Some limitations of this study should be acknowledged. A cross-sectional design was used, which did not account for variability of QoL and perception of appearance over time. However good test-retest reliability for both the AcroQol and DAS59 has previously been demonstrated [4, 9]. The refusal of 5 patients to participate because they did not have any problems with their appearance might have introduced some bias. However even when these patients would have participated the proportion of patients that reported to be self-conscious about a 'feature' would still be 43.6%. The difference in BMI between both groups was at the limit of significance (p=0.05). However, no correlation was found between BMI and full scale DAS59 score. Therefore, we believe that this cannot explain the difference in DAS59 scores found between patients and controls.

In conclusion, even after long-term remission of acromegaly, a large number of patients are self-conscious about their appearance, leading to psychological distress and disruptions to everyday life and a decreased QoL. The facial features were the most important source of self-consciousness. The findings in this study highlight the importance of the fact that physicians should not ignore self-consciousness about appearance in patients treated for acromegaly but have to address these concerns during follow-up. Patients should be encouraged to discuss their concerns about their appearance and should be offered individualized advice about corrective interventions to improve not only their appearance but also functionality. There is a lot of potential for improvement in the support of people with acromegaly regarding their difficulties concerning their appearance and therefore their QoL as shown by a recent study that detected a significant effect of exercise on body self-perception in patients with acromegaly [32]. To optimize management, not only biochemical and radiological parameters but also dimensions like appearance that reflect Qol should be evaluated. In practice the DAS59 is easy to use by physicians and can be an excellent instrument during follow-up. For patients, the DAS59 provides the opportunity to more easily address problems with their appearance and to discuss possible treatment options like psychological support or plastic/craniofacial surgery.

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Part 2

Long-term physical sequelae of Cushing's syndrome

Chapter 5

Glucocorticoid receptor polymorphisms modulate cardiometabolic risk factors in patients in long-term remission of Cushing's syndrome

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Abstract

Context: Glucocorticoid receptor (GR) polymorphisms modulate glucocorticoid (GC) sensitivity and are associated with altered metabolic profiles.

Objective: To evaluate the presence of GR polymorphisms (*Bcl*I (rs41423247), N363S (rs56149945), ER22/23EK (rs6189/rs6190) and 9*β* (rs6198) and investigate their associations with metabolic alterations in patients in long-term remission of Cushing's syndrome (CS).

Design and setting: Cross-sectional case-control study.

Patients and methods: Sixty patients in long-term remission of CS were genotyped. Associations between GR polymorphisms and multiple vascular, body composition and metabolic parameters were investigated.

Main outcome measures: Allelic frequencies of the polymorphisms and their associations with several cardiometabolic risk factors.

Results: This study shows that carriers of the 9*β* polymorphism have a higher systolic blood pressure and lower resistin levels. The GC sensitizing *Bcl*I polymorphism is associated with an adverse cardiometabolic risk factor profile: higher fat percentages of extremities and legs, higher serum leptin and E-selectin levels and higher intima media thickness in carriers versus non-carriers.

Conclusions: The 9*β* and *Bcl*I polymorphisms of the GR adversely affect the cardiometabolic profile in patients who are in remission after treatment of CS. This suggests that genetically altered GC sensitivity modulates the long-term adverse cardiometabolic effects resulting from (endogenous) hypercortisolism.

Introduction

Cushing's syndrome (CS) is a disorder resulting from chronic exposure to increased levels of glucocorticoids (GC), frequently caused by an ACTH-producing pituitary adenoma (Cushing's disease (CD)) or by primary adrenal overproduction of cortisol (adrenal CS (ACS)) [1]. CS is associated with body composition changes, cardiometabolic abnormalities such as type 2 diabetes mellitus (T2DM), hypertension and dyslipidemia, and ultimately cardiovascular disease [2]. We [3] and others [4, 5] have shown that many adverse metabolic and cardiovascular characteristics and body compositional changes persist after treatment, even after long-term remission. Although these adverse metabolic and cardiovascular characteristics are common in these patients, their incidence and severity vary among patients. This variation seems not to be explained by differences in cortisol excess or disease duration alone. Therefore a variable sensitivity to GC possibly plays a role in modulating the effect of cortisol excess [6].

Several investigations in healthy subjects have shown that GR polymorphisms are associated with altered GC sensitivity and alterations in metabolic profiles and body composition. The *BclI* and N363S polymorphisms of the GR gene have been associated with enhanced sensitivity to GC, increased abdominal obesity, an adverse lipid profile and hyperinsulinemia [7-10]. In contrast, the ER22/23EK GR polymorphism has been associated with GC resistance and a favorable metabolic profile and body composition [11, 12]. The 9*ß* polymorphism is associated with increased expression and stabilization of the dominant-negative splice variant GR-β. Enhanced GR-β expression results in greater inhibition of GR-α transcriptional activity, and, as GR-α is the functional GR isoform, in relative GC resistance [13-15]. This polymorphism is associated with increased serum levels of inflammatory parameters and cardiovascular disease despite a more favorable lipid profile in men and body composition in women [16, 17].

The functional role of GR polymorphisms has been extensively studied in the general healthy population. In contrast, only a small number of studies have been performed on the functional role of GR polymorphisms in active CS and CS in remission. These few studies have found an association of the 9*ß* polymorphism with the risk of developing diabetes mellitus [18] and of the *BclI* polymorphism with increased skeletal GC sensitivity and worse cognitive performance [19, 20].

We hypothesize that differences between patients in the severity of the adverse metabolic and vascular profile after cure of CS are related to differences in GC sensitivity due to GC receptor polymorphisms. Therefore, we investigated the associations of these genetic variants with the presence/persistence of the adverse metabolic and vascular profile and body composition after long-term remission of CS.

Subjects and methods

Sixty adult (>18 years old) patients in long-term remission (>4 years) of CS were recruited from the outpatient clinic of the department of internal medicine. Remission was defined as suppression of plasma cortisol to ≤ 50 nmol/L after 1 mg dexamethasone overnight [21] or, if a patient had received radiotherapy of the pituitary gland, a 24-hour urinary free cortisol excretion of < 240 nmol/24h for men or <150 nmol/24h for women (upper levels of normal free cortisol excretion). Clinical history was collected and a physical examination, biochemical and hormonal evaluation, dual-energy X-ray absorptiometry scanning (DXA) and non-invasive vascular function measurements were performed in all subjects. All subjects were genotyped for the presence of four GR polymorphisms; *Bcl*I, N363S, ER22/23EK and 9*β*.

Patients with untreated hormonal deficiencies, or hormonal deficiencies that had not been treated adequately in the last 4 years according to international standards, were excluded. Furthermore, patients with active malignancy or systemic therapy for malignancy in the past, auto-inflammatory diseases and psychiatric pathology were excluded. Hypothyroidism was defined as free thyroxine (fT4) plasma concentrations < 8 pmol/l (reference range 8-22 pmol/l). Testosterone deficiency in men was defined as early morning testosterone levels < 11 nmol/l (reference range 11-45 nmol/l). In women, estrogen deficiency was defined as secondary hypogonadotropic hypogonadism or a postmenopausal state without the use of chronic estrogen substitution therapy. Growth hormone (GH) deficiency was defined as a maximal GH response of < 15.3 mU/l during an insulin tolerance test (ITT), or as a maximal GH response of < 12.3 mU/l during an arginine/GHRH test [22]. Glucocorticoid deficiency was defined as a maximal cortisol response < 550 nmol/l during an ITT [23]. All patients underwent a new 1 mg dexamethasone suppression test (or a 24-hour urinary free cortisol measurement in case of pituitary RT) before entering the study to confirm remission. All subjects were of Caucasian (Dutch) origin.

Clinical history

Clinical history included the etiology of CS, treatment strategies (surgery, radiotherapy, medication), treatment for coexisting hormonal deficiencies, co-morbidities and smoking habits.

Physical examination

Anthropometric measurements included weight and height, supine systolic and diastolic blood pressure (average of 10 measurements, every 3 minutes with an oscillometric sphygmomanometer (Criticon model 1846; Criticon Inc., Tampa, FL)) measured at 09:00AM.

Biochemical evaluation

Biochemical evaluation included plasma level measurements of fasting glucose, glycated hemoglobin (HbA1c), total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and insulin. Insulin sensitivity was assessed by homeostasis model assessment (HOMA) [24]. Furthermore, serum adipokines adiponectin, leptin and resistin were measured. In addition, a number of markers of vascular health were measured in serum; soluble vascular cell adhesion molecule-1 (VCAM-1), soluble intercellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1) and soluble E-selectin. Increased levels of these parameters are indicators of endothelial dysfunction and worse vascular health.

Vascular evaluation

Carotid intima media thickness (IMT) was determined using an AU5 ultrasound machine (Esaote Biomedical) with a 7.5 MHz linear array transducer. Measurement of IMT was performed off-line by the sonographer at the time of the examination, using semi-automatic edge-detection software (M'Ath® Std version 2.0, Metris). IMT was defined as the mean IMT of the four measured segments of the common carotid artery: far wall left, near wall left, far wall right, near wall right.

Body composition

Total body DXA was performed using a Hologic QDR 4500 densitometer (Hologic, Bedford, MA). Standard procedures supplied by the manufacturer for scanning and analyses (using Hologic software version 12.1) were followed. Calibration procedures were performed every day using the appropriate phantoms provided by the manufacturer. Total body fat mass was determined and fat percentage was calculated as the total body fat mass in percent of body weight. Furthermore, trunk-, leg- and extremities fat percentages were determined in order to discriminate between different body regions. Different regions were determined by manually placing regions of interest as defined by the manufacturers software manual. The trunk fat depot is defined as the region between two horizontal lines placed on the lower border of the head and the upper border of the iliac crest and two vertical lines placed against the outer margins of the chest . These lines exclude the arms from the trunk. The leg fat depot is defined by two vertical lines placed against the outer margins op both legs. The pelvis is excluded from the legs by a line trough the femoral neck. Leg fat percentage is depicted as the average of both legs. Extremity fat percentage is depicted as the average of both arms and both legs.

Laboratory measurements

Serum concentrations of leptin, resistin, PAI-1, sICAM-1 and soluble E-selectin were measured by Multiplex Fluorescent Bead Immunoassays (xMAP technology, Millipore, Billerica, MA) and a Bio-plex microbead analyzer (Luminex, Austin, TX) according to the manufacturers protocol. Serum concentrations of adiponectin and sVCAM-1 were determined by enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN). Fasting plasma glucose, HbA1c, insulin and total serum cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol were measured by standard procedures. According to the manufacturer's information, intra-assay precision coefficients of variation for all laboratory measurements were equal or below 5%.

Genetic analyses

DNA was isolated from whole blood using the Gentra Puregene isolation kit (Qiagen, Valencia, CA, USA), according to the manufacturers protocol. Genotyping for the four selected genetic variants of the glucocorticoid receptor (GR) gene (official gene name *NR3C1*) was performed as follows. The presence of the 9*β* (rs6198) and N363S (rs56149945) genetic variants was assessed by applying the predesigned TaqMan SNP assays C_8951023_10 and C_26841917_40, respectively, on a 7300 ABI Real-Time polymerase chain reaction (PCR) system (all from Life Technologies, Applied Biosystems, Foster City, CA).

For the detection of the GR ER22/23EK (rs6189 / rs6190) genetic variant conventional PCR and Sanger sequencing analysis was performed with forward primer 5'-CTG-CCT-CTT-ACT-AAT-CGG-ATC-A-3' and reverse primer 5'-AGA-GTG-AAA-CTG-CTT-TGG-ACA-G-3'. To determine the GR *Bcl*I genotype (rs41423247), DNA was amplified with forward primer 5'-AAG-CAA-TGC-AGT-GAA-CAG-TGT-AC-3' and reverse primer 5'-AAC-AAT-TTT-GGC-CAT-CAG-TTA-TC-3'. Also these PCR products were subjected to Sanger sequencing analysis.

Statistical analyses

Data are expressed as mean ± 95% confidence intervals unless stated otherwise. Data distributions were analyzed using the Kolmogorov-Smirnov test and logarithmic transformation was performed before statistical testing when appropriate. Based on data distribution, comparison of continuous variables was performed using Student's t-test or Mann-Whitney rank sum test. The associations between genotypes and outcome measurements were evaluated using analysis of covariance (ANCOVA) with genotypes as a factor and age, gender and BMI as covariates. Categorical variables were analyzed using the χ^2 test followed by Fisher's exact test if appropriate. Hardy-Weinberg equilibrium for all polymorphisms was determined using a χ^2 test. Significance was set at a p-value of <0.05. Statistical analysis was performed using SPSS Software version 20.0 (SPSS, Inc., Chicago, IL).

Table 1. Subject characteristics

	(n=60)
Gender (n): male/female	12/48
Age in years: mean (SD)	50.7(12.4)
Duration of remission in years: mean (SD)	13.8 (8.5)
BMI in kg/m^2: mean (SD)	26.9(5.3)
Waist circumference in cm: mean (SD)	91.8 (14.7)
Smoking (n): yes/no	14/46
Treatment modalities: n (%)	
Unilateral adrenalectomy	20 (33.3)
Bilateral adrenalectomy	12 (20.0)
Pituitary surgery	38 (63.3)
Pituitary radiotherapy	13 (21.7)
Hormonal deficiencies: n (%)	
Glucocorticoid deficiency*	22 (36.7)
Growth hormone deficiency*	15 (25.0)
Thyroid hormone deficiency*	26 (43.3)
Testosterone deficiency*	6 (10.0)
Mineralocorticoid deficiency*	11 (18.3)
Estrogen deficiency	26 (54.2)
Cardiometabolic co-morbidities: n (%)	
Hypertension**	19 (31.7)
Diabetes mellitus**	4 (6.7)
Hypercholesterolemia**	12 (20.0)
Cushing type: n (%)	
Pituitary	40 (66.7)
Adrenal	20 (33.3)

BMI, body mass index; CS, Cushing's syndrome.

* Adequately substituted according to international standards

** Actively treated for this co-morbidity

Results

Forty eight (80%) subjects were female. Mean (SD) age was 50.7 (12.4) years with a mean (SD) BMI of 26.9 (5.3) kg/m^2. Mean (SD) duration of remission was 13.8 (8.5) years. Nineteen (31.7%) subjects were treated for hypertension, 4 (6.7%) were treated for diabetes mellitus and 12 (20.0%) were treated for hypercholesterolemia. Complete subject characteristics of all subjects are depicted in Table 1. Allelic frequencies of the four polymorphisms are depicted in Table 2. All polymorphisms were in Hardy-Weinberg equilibrium. Because of low allele frequencies the N363S and ER22/23EK polymorphisms were not included in the association analysis. The minor allele of the *9β* polymorphism showed a statistically significant association with higher systolic blood pressure (P = 0.007) and lower resistin levels (P = 0.027) in carriers (Table 3 and Fig. 1a). No associations were detected for the other measured parameters. The

minor allele of the *BclI* genotype showed statistically significant associations with higher mean IMT (P = 0.048), higher extremities fat percentage (P = 0.007), higher leg fat percentage (P = 0.029), higher leptin level (P = 0.038) and higher soluble E-selectin level (P = 0.037) in carriers (Table 3 and Fig. 1b). No associations with the other measures were detected in our CS patients (data not shown).

Table 2. Genotype distributions and allele frequencies of the GR gene polymorphisms in patients cured of Cushing's syndrome (CS)

	CS (n=60)
BclI	
CC	32 (53%)
CG	20 (33%)
GG	8 (13%)
Allele frequency	0.300
9β (A3669G)	
AA	40 (67%)
AG	19 (32%)
GG	1 (2%)
Allele frequency	0.175
N363S	
AA	55 (92%)
AG	5 (8%)
GG	0 (0%)
Allele frequency	0.042
ER22/23EK	
GG	55 (92%)
GA	5 (8%)
AA	0 (0%)
Allele frequency	0.042

Table 3. Associations between glucocorticoid receptor polymorphisms and outcome measurements. Data are expressed as mean and 95% confidence intervals.

	Carrier minor allele	Non-carrier minor allele	P-value
9β			
Fasting glucose (mmol/l)	5.1 (4.7-5.4)	5.0 (4.7-5.2)	0.578
HbA1c (mmol/mol)	40.9 (38.4-43.6)	38.4 (36.7-40.1)	0.117
Total cholesterol (mmol/l)	5.3 (5.0-5.7)	5.2 (4.8-5.5)	0.596
Triglycerides (mmol/l)	1.4 (1.2-1.8)	1.5 (1.3-1.7)	0.850
HDL-cholesterol (mmol/l)	1.3 (1.2-1.5)	1.4 (1.3-1.4)	0.919
LDL-cholesterol (mmol/l)	3.2 (2.9-3.6)	3.1 (2.9-3.3)	0.498
Insulin(mE/l)	6.0 (4.6-7.8)	7.2 (6.0-8.7)	0.266

Table 3. Continued

HOMA-IR	1.4 (1.1-1.9)	1.6 (1.3-2.0)	0.574
Mean IMT(mm)	0.73 (0.70-0.77)	0.76 (0.73-0.78)	0.254
Systolic blood pressure (mmHg)	138.7 (131.2-146.5)	126.2 (121.5-131.1)	0.007*
Total body fat (%)	32.6 (30.8-34.3)	34.3(33.1-35.2)	0.114
Trunk fat (%)	32.3 (30.4-34.3)	33.2(31.8-34.5)	0.471
Extremities fat (%)	35.8 (34.2-37.5)	38.1(36.9-39.2)	0.340
Leg fat (%)	34.0 (32.1-36.1)	35.4(34.0-36.9)	0.271
Leptin (pg/ml)	3374.5(2289.3-4974.1)	4213.3(3206.7-5535.9)	0.356
Resistin (pg/ml)	2248.5 (1618.1-3124.4)	3558.2 (2824.3-4482.8)	0.020*
Adiponectin (pg/ml)	706.3 (458.1-1089.0)	1087.9 (802.7-1474.4)	0.108
PAI-1 (pg/ml)	1884.7 (1377.2-2392.3)	2384.8 (2027.4-2742.2)	0.114
VCAM-1 (pg/ml)	666.5 (576.5-770.5)	674.5 (609.7-746.2)	0.888
ICAM-1 (pg/ml)	253.4 (177.0-362.9)	304.3 (236.3-391.9)	0.410
E-selectin (pg/ml)	46.2 (37.9-54.5)	42.2 (36.3-48.1)	0.436
BclI			
Fasting glucose (mmol/l)	5.2 (4.9-5.5)	4.8 (4.6-5.1)	0.097
HbA1c (mmol/mol)	40.6 (38.5-42.8)	38.1 (36.2-40.0)	0.089
Total cholesterol (mmol/l)	5.2 (4.9-5.6)	5.2 (4.9-5.5)	0.837
Triglycerides (mmol/l)	1.6 (1.4-1.9)	1.3 (1.1-1.6)	0.198
HDL-cholesterol (mmol/l)	1.4 (1.2-1.5)	1.4 (1.3-1.4)	0.969
LDL-cholesterol (mmol/l)	3.1 (2.9-3.4)	3.2 (2.9-3.4)	0.879
Insulin (mE/l)	7.4 (5.9-9.2)	6.3 (5.1-7.8)	0.313
HOMA-IR	1.8 (1.4-2.3)	1.4 (1.1-1.7)	0.125
Mean IMT (mm)	0.77 (0.74-0.80)	0.73 (0.70-0.76)	0.048*
Systolic blood pressure (mmHg)	126.0 (120.2-132.0)	134.0 (128.3-140.1)	0.060
Total body fat (%)	34.5 (33.0-36.0)	33.1 (31.7-34.4)	0.167
Trunk fat (%)	33.3 (31.7-34.9)	32.5 (31.0-34.1)	0.488
Extremities fat (%)	38.7 (37.3-40.1)	36.1 (34.8-37.4)	0.007*
Leg fat (%)	37.5 (35.8-39.1)	35.0 (33.5-36.5)	0.029*
Leptin (pg/ml)	4999.0 (3644.6-6856.8)	3155.8 (2869.8-3470.3)	0.038*
Resistin (pg/ml)	3630.0 (2738.1-4812.6)	2625.4 (2016.3-3418.7)	0.099
Adiponectin (pg/ml)	1166.8 (811.6-1677.4)	781.3 (556.1-1097.7)	0.112
PAI-1 (pg/ml)	2301.6 (1866.2-2737.1)	2145.0 (1737.8-2552.2)	0.602
VCAM-1 (pg/ml)	726.3 (645.5-817.3)	627.7 (562.3-700.6)	0.075
ICAM-1 (pg/ml)	282.3 (208.5-382.2)	289.5 (218.1-384.1)	0.904
E-selectin (ng/ml)	45.2 (38.8-52.7)	36.1 (31.3-41.7)	0.037*

IMT, intima media thickness; HbA1c, glycated hemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-IR, homeostatic model assessment-insulin resistance; PAI, plasminogen activator inhibitor; VCAM, vascular cell adhesion molecule; ICAM, intracellular adhesion molecule. P*: ANCOVA was used to assess the statistical differences between genotype groups.

All data are adjusted for age, gender and BMI.

* P <0.05

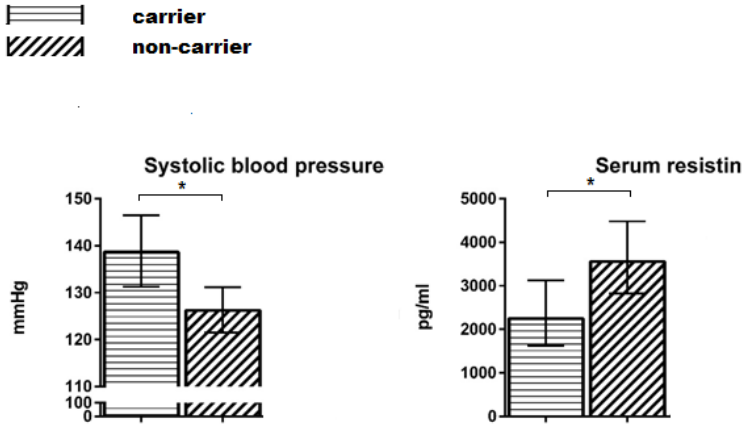

Figure 1a. Associations of the 9β genotype and clinical outcome
* (*P*<0.05)

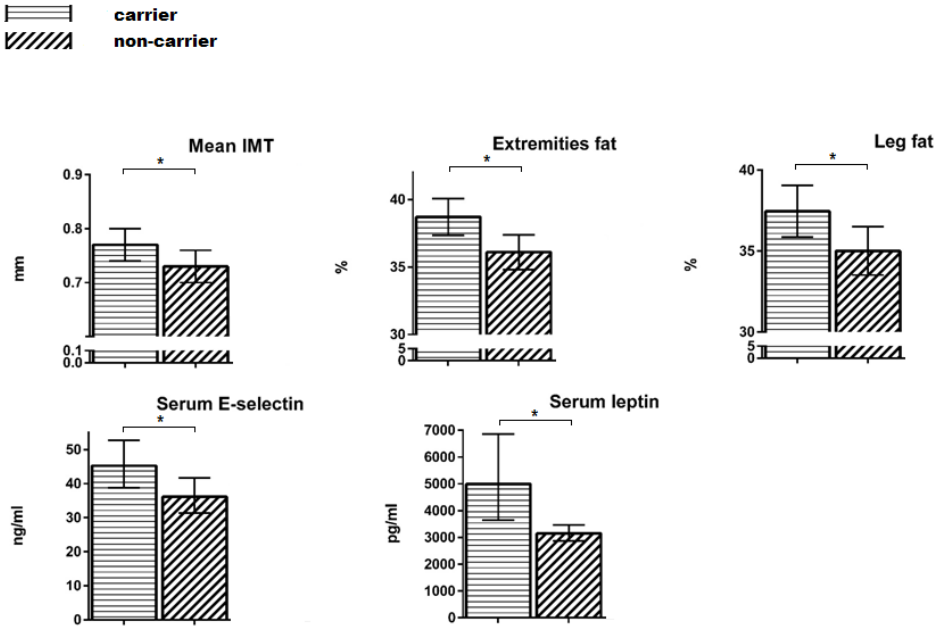

Figure 1b. Associations of the BclI genotype and clinical outcome
* (*P*<0.05)

Discussion

This study investigated the prevalence of GR polymorphisms in patients in long-term remission of CS and the associations of these polymorphisms with metabolic, vascular and body compositional characteristics. In this study we found that carriers of the 9*ß* polymorphism have a higher systolic blood pressure and lower resistin levels and that the GC sensitizing *Bcl*I polymorphism is associated with a number of metabolic and vascular adverse effects: higher fat percentages of extremities and legs, higher serum leptin and E-selectin levels and higher intima media thickness in carriers versus non-carriers.

It has been shown that in patients in long-term remission of CS, cardiovascular and metabolic risk remains increased [3, 5]. In the general population it has been demonstrated that cardiovascular and metabolic risk and body composition are affected by lifelong overactivation or relative inactivation of GC signaling due to GR polymorphisms [25]. Our observation that altered glucocorticoid sensitivity due to GR polymorphisms modulates cardiometabolic risk factors in cured CS patients is in line with these well-known findings in the general population.

Remitted CS patients carrying the minor allele of the 9β polymorphism had a higher systolic blood pressure, which is concordant with previous findings of an increase in carotid atherosclerosis, and a higher incidence of coronary heart disease in carriers of this polymorphism [17]. Systolic hypertension is normally especially seen in the elderly population due to increased arterial stiffening. Arterial stiffness is central to the pathogenesis of isolated systolic hypertension and directly impacts left ventricular afterload, pressure pulsatility in the arterial tree, and its penetration into the microvasculature of target organs such as the brain and kidney. This means that systolic hypertension in carriers of the polymorphism may lead to increased ageing of the vascular tree, but further studies are needed to elucidate whether the polymorphism increases cardiovascular risk. Furthermore, an association was found between the 9*ß* polymorphism and resistin levels, with carriers of the minor allele of the 9*ß* polymorphism having lower resistin levels. It can be hypothesized that this is caused by the fact that subjects with a relative decrease in glucocorticoid sensitivity have less central adiposity. Central adiposity causes local ischemia of the adipose tissue which leads to infiltration of M1-macrophages, which are the main producers of resistin. This hypothesis is however speculative and not supported by the data, since no association between truncal fat and the 9β polymorphism was observed.

The minor allele of the *Bcl*I polymorphism was associated with an increased fat percentage of the extremities and legs in the remitted CS patients. These body areas only contain subcutaneous adipose tissue. As leptin is preferentially produced in subcutaneous adipose

tissue (SAT), carriers also had higher leptin levels [26]. High leptin levels have been widely recognized as an independent cardiovascular risk factor associated with insulin resistance. It also has a pathogenic role in atherothrombosis and endothelial dysfunction. Furthermore, a higher level of E-selectin was found in carriers of the minor allele of the *BclI* polymorphism. This may reflect increased endothelial activation and progressing atherosclerosis in the *BclI* carriers. This is supported by our observation that the IMT is higher in *BclI* carriers.

In women with CS in remission, the *BclI* polymorphism was previously shown to be associated with altered GC sensitivity as the polymorphism was associated with reduced total and femoral neck bone mineral density [27]. Furthermore, in patients in remission of CS, the *BclI* polymorphism was also independently associated with increased fatigue and worse performance on cognitive testing [20]. The findings in literature combined with the findings in the current study suggest that hypercortisolism in patients carrying the *BclI* polymorphism may have more pronounced effects on patient wellbeing in the long-term.

This study has some limitations. Because of the low incidence of CS only a relatively small cohort could be studied. This could influence genetic associations and that is why only polymorphisms with a relatively high frequency were included in the association analysis. In genetic association studies inhomogeneity of study population with regard to ethnicity, gender, age and environmental factors is a frequent limiting factor. This study included Caucasians only and associations were corrected for gender and age. Duration of hypercortisolism likely represents another factor in long-term cardiometabolic effects in CS patients, which however is rather difficult to estimate since diagnosis of CS is generally delayed. Furthermore, it was decided not to correct for multiple testing because of the hypothesis-driven nature of this study. Indeed, from a biological perspective our findings are in line with the expected corticosteroid effects, suggesting that SNPs of the GR gene leading to either hypo- or hypersensitivity to GC, indeed modulate long-term cardiometabolic outcome after treatment of CS. However, because of the limited sample size and, hence, limited statistical power, we cannot exclude that some of the significant differences between carriers and non-carriers may be a finding by chance, i.e., a type-1 error.

In conclusion, this study is one of the first to suggest that glucocorticoid receptor polymorphisms modulate cardiometabolic risk factors in patients in long-term remission of CS [27]. In the context of personalized healthcare, this may implicate that, after treatment of CS, carriers of these polymorphisms are candidates for a more stringent follow-up regarding cardiovascular and metabolic health as our findings suggest that GR polymorphisms may play a role in susceptibility to cardiovascular disease in CS patients. Furthermore, patients treated with glucocorticoids for other diseases and even healthy

subjects, carrying these polymorphisms, are candidates for a more stringent follow-up regarding cardiovascular and metabolic health and the development of metabolic syndrome as was recently debated in literature [28]. However, the results of this study need to be interpreted with caution and further research into these findings in larger CS populations is needed to replicate these findings. In addition, future studies should delineate to what extent the observed associations also apply to prolonged episodes of exposure to physiologically elevated cortisol levels (e.g. severe stress) or exogenous glucocorticoids.

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Chapter 6

Vascular health in patients in remission of Cushing's syndrome is comparable with that in BMI-matched controls

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Context: In active Cushing's syndrome (CS), patients suffer from endothelial dysfunction and premature atherosclerosis. However, it is uncertain to what extent vascular health recovers after long-term remission. This is highly relevant because this topic relates to future development of cardiovascular disease.

Objective: The objective of the study was to investigate whether micro- and macrovascular health is impaired after long-term remission of CS in patients with no or adequately treated comorbidities.

Design and Setting: This was a cross-sectional case-control study in two tertiary referral centers.

Patients and Main Outcome Measures: Sixty-three patients (remission of CS for ≥ 4 y) and 63 healthy, well-matched controls were compared. In group A (58 patients and 58 controls), serum biomarkers associated with endothelial dysfunction, intima media thickness, pulse wave velocity, and pulse wave analysis were studied. In group B (14 patients and 14 controls), endothelium-dependent and -independent vasodilatation was studied in conduit arteries (flow mediated dilation of the brachial artery) and forearm skeletal muscle resistance arteries (vasodilator response to intraarterial acetylcholine, sodium-nitroprusside, and N^G-monomethyl-L-arginine using venous occlusion plethysmography).

Results: There were no significant differences between the outcome measures of vascular health of patients and controls in groups A and B.

Conclusion: The vascular health of patients in long-term remission of CS seems to be comparable with that of healthy gender-, age-, and body mass index-matched controls, provided that the patients have no, or adequately controlled, comorbidities. Therefore, the effects of hypercortisolism per se on the vasculature may be reversible. This accentuates the need for the stringent treatment of metabolic comorbidities in these patients.

Patients with chronic hypercortisolism due to endogenous Cushing's syndrome (CS) have a very high mortality rate, with an estimated 5-year survival of 50% in untreated patients [1]. Cardiovascular disease is the main cause of mortality [1]. Multiple studies have shown that endothelial function is impaired in these patients [2–5], with an increased incidence of atherosclerosis [6, 7]. It has been suggested that this is mainly caused by the fact that most patients with CS have centripetal obesity, impaired glucose tolerance, systemic hypertension, hypercoagulability, and dyslipidemia [8]. All these factors are associated with impaired endothelial function and premature atherosclerosis, especially if they occur simultaneously [9]. In addition, one should realize that the hypercortisolism itself has a direct effect on the vasculature (via both the glucocorticoid and the mineralocorticoid receptor) [10, 11].

Successful surgical treatment of CS, resulting in normalization of cortisol secretion, significantly decreases cardiovascular risk and reduces mortality rate [1, 12]. However, it is unclear to what extent vascular health recovers in patients in long-term remission of CS. Full recovery is not self-evident because centripetal obesity and an adverse adipokine profile (which is known to be associated with endothelial dysfunction and eventually macrovascular disease [13, 14]) persists, even after long-term remission of CS [15, 16]. Furthermore, it is questionable whether the direct effects of hypercortisolism on the vasculature are fully reversible.

A number of studies have previously investigated vascular health in small groups of patients in remission of CS [17–23]. These studies reported inconsistent results, which may partly be explained by the small group size and/or selection of single markers of vascular health that therefore cannot provide a broad insight.

The aim of this study was to investigate micro- and macrovascular health in a large group of patients in long-term remission of CS with adequately treated comorbidity if present, in comparison with a matched healthy control group. We measured serum biomarkers associated with endothelial dysfunction, performed gold standard measurements of endothelial function, and investigated the presence of overt atherosclerosis.

Subjects and Methods

Subjects

All adult patients of Radboud University Medical Center Nijmegen and Leiden University Medical Center, who had been successfully treated for CS (caused by either an ACTH producing pituitary adenoma or a benign adrenal adenoma) and were in remission for at least 4 years, were eligible for inclusion in this multicenter cross-sectional matched case-control

study. Remission was defined as absence of clinical signs and symptoms of hypercortisolism and suppression of plasma cortisol to 50 nmol/L or less after 1 mg dexamethasone overnight or, if a patient had received radiotherapy of the pituitary gland, a 24-hour urinary free cortisol value of less than 240 nmol per 24 hours for men or less than 150 nmol per 24 hours for women. The medical records of all patients were retrospectively reviewed to assess clinical data regarding the etiology of CS, the type of treatments that patients had received, duration of remission, presence of hormonal deficiencies, and comorbidities. Information on the treatment of comorbidities of the patients can be found in Supplemental Table 1.

In our study we investigated 63 patients, divided into two different patient groups. Group A comprised 58 patients and group B 14 patients. Nine patients were included in both groups. Group A was the same group of patients that we previously described in our study on body composition, and extensive information about the patient selection can be found in that article [16]. In short, the following exclusion criteria were applied: untreated (or inadequately treated) hormonal deficiencies, active malignancy or systemic therapy for malignancy in the past, severe inflammatory diseases, and psychiatric pathology. Each patient was matched to a control subject with the same gender, age (±2 y), and body mass index (BMI; ±2 kg/m^2). Control subjects, recruited via advertisements in a local newspaper, had to be healthy and without current use of medication.

For the second group of patients (group B, n = 14), even stricter exclusion criteria were used: all subjects with hormonal deficiencies, except for adequately treated hypothyroidism (free T_4 range 8.0 –22.0 pmol/L), were excluded. Furthermore, in addition to the comorbidities applied for exclusion in group A, all patients with comorbidities that are known to affect vascular function or who used medication that may interfere with the cardiovascular system were excluded. In addition to gender, age, and BMI, the healthy control subjects were also matched for smoking, ethnicity, and physical activity levels (estimated via metabolic equivalent of task scores and measured for 1 week with a SenseWear Pro Armband (Body Media). Female controls were matched for estrogen status and oral contraceptive use.

The medical ethics committees of our institutions approved this study, and all participants provided written informed consent prior to participation.

Methods

All subjects refrained from smoking, alcohol, caffeine, chocolate, and vitamin C for at least 18 hours, and vigorous physical exercise for at least 24 hours before testing. Subjects fasted at least 6 hours before testing.

Biochemical markers associated with endothelial dysfunction (group A)

Serum concentrations of plasminogen activator inhibitor-1 (PAI-1), intracellular adhesion molecule-1 (ICAM-1), and soluble E-selectin were measured by Multiplex fluorescent bead immunoassays (xMAP Technology) and a Bio-plex microbead analyzer (Luminex) according to the manufacturer's protocol. Serum concentrations of vascular cell adhesion molecule-1 (VCAM-1) were determined by an ELISA (R&D Systems).

Noninvasive measurements of atherosclerosis and arterial stiffness (group A)

Measurements of carotid intima media thickness (cIMT), pulse wave velocity (PWV), and pulse wave analysis were performed according to a highly standardized protocol and performed by the same experienced technician (S.H.) in all patients [24]. Mean cIMT was calculated from the mean of four measured segments of the vessel: far wall left, far wall right, near wall left, and near wall right. Subsequently the presence of plaques and size was evaluated at the level of the common, internal, and external carotid arteries. Plaque was defined as any focal protrusion above the surrounding intima of at least 1.5 X mean cIMT.

PWV and pulse wave analysis were measured with applanation tonometry, using SphygmoCor system version 7.1 (Atcor Medical). Central arterial pressure (CAP) and central systolic pressure were derived and central augmentation index (AIx) was calculated. As AIx is influenced by heart rate, an index normalized for a heart rate of 75 beats/min was used. To determine pulse wave velocity, pulse wave forms were recorded at the right carotid artery and left femoral artery sequentially. Wave-transit time was calculated using the R-wave of a simultaneously recorded electrocardiogram as a reference frame. The coefficient of variation (CV) for measuring PWV is 5%–10% [25].

Endothelial function (group B)

Brachial artery flow-mediated dilation (FMD) is widely accepted to reflect endothelium-dependent and largely nitric oxide mediated function of conduit arteries [26]. Measurements were performed by two experienced vascular sonographers (D.H.J.T. and T.H.A.S.). A 10-MHz multifrequency linear array probe attached to a high-resolution ultrasound machine (T3000; Terason) was used for imaging of the brachial artery in the distal third of the upper arm. Subjects rested in a supine position for at least 15 minutes to enable baseline assessment of arterial diameter and blood flow. The arm was extended and positioned at an 80° angle from the torso. A rapid inflation pneumatic cuff (Hokanson) was positioned on the forearm immediately distal to the olecranon to provide the forearm ischemic stimulus. After obtaining an optimal image, the probe was manually stabilized and the ultrasound parameters were set to optimize longitudinal B-mode imaging of the lumen-arterial wall interface. Continuous Doppler velocity was measured using the lowest possible insonation angle (<60°). The forearm cuff was inflated to 220 mm Hg for 5 minutes. Diameter and

flow recordings resumed 30 seconds prior to cuff deflation and continued for 5 minutes thereafter. After a 15-minute resting period, a 1-minute baseline recording of the brachial artery diameter and flow was taken.

Subsequently, brachial artery endothelium-independent vasodilatation was examined after administration of a single spray of sublingual glyceryl trinitrate, which serves as a direct nitric oxide donor, to detect endothelium-independent vasodilator capacity. This was followed by 5 minutes of continuous recording of brachial artery diameter and blood flow. Posttest analysis of brachial artery diameter was performed using customized edge detection and wall-tracking software [27]. Baseline diameter, flow, and shear rate were calculated as the mean of data acquired across the 1-minute preceding the cuff inflation period. Peak diameter after the cuff deflation was automatically detected as previously described [28]. FMD was calculated as the percentage rise of this peak diameter from the preceding baseline diameter.

The time to peak diameter (seconds) was calculated from the point of cuff deflation to the maximum postdeflation diameter. According to a recent study, inadequate scaling for FMD would be present if the upper confidence limit of the regression of the relation between logarithmically transformed base diameter and peak diameter is less than 1.0 [29]. In such an event, the FMD percentage is not an appropriate measure for the estimation of endothelial function. Data were checked for this phenomenon and subsequently allometric modeling was applied [29]. Furthermore, the FMD percentage was corrected for shear rate stimulus by adding this factor as a covariate in our analysis [30]. The CV for measuring FMD with our protocol is 6.7% [30].

Forearm blood flow (FBF) measurements using venous occlusion plethysmography measures changes in blood flow (mainly determined by arteriolar resistance arteries in the muscle bed) in response to the infusion of intraarterial vasoactive medications [25, 31]. It therefore mainly assesses microvascular function. FBF was measured at the forearm using electrocardiogram-triggered bilateral strain-gauge venous occlusion plethysmography [31]. Measurements were performed at 9:00 $_{AM}$ in a quiet, temperature-controlled room (22°C). Mercury in silastic brand strain gauges (Dow Corning) placed around the widest portion of the upper third of both forearms were electrically coupled to a plethysmograph calibrated to measure normalized changes in volume. For each measurement, venous flow was occluded just proximal to the elbow by rapidly inflating a blood pressure (BP) cuff to 60 mm Hg. A wrist cuff was inflated to suprasystolic (220 mm Hg) pressures to exclude the hand circulation from the blood flow during the measurement starting 30 seconds prior to each measurement. A brachial artery catheter (angiocatheter 20 gauge, 1.88 in.; BD Angiocath) was inserted into the nondominant arm after local anesthesia (lidocaine 2%),

which was elevated slightly above the right atrium. Systolic BP, diastolic BP, mean arterial BP, and heart rate were monitored continuously. The other arm was used as a control for systemic changes in vasomotor tone.

Table 1. Group A: Clinical Characteristics of Patients in Long-Term Remission of CS and Healthy Controls

	Patients (n = 58)	Controls (n = 58)	*P* Value
Gender, male/female, n	12/46	12/46	
Age, y, mean (±SD)	50.8 (12.3)	51.2 (12.4)	.863
BMI, kg/m^2, mean (±SD)	26.5 (4.2)	26.3 (4.1)	.793
Duration of remission, y, median (±range)	13.6 ± 8.0		
Smoking, yes/no	14/44	5/53	.024[a]
Pack-years (±SD)	11.5 (15.6)	6.9 (13.9)	
Alcohol consumption, yes/no	10/48	13/45	.485
Treatment modalities, n, %			
Unilateral adrenalectomy	19 (32.8)		
Bilateral adrenalectomy	12 (20.7)		
Pituitary surgery	38 (65.5)		
Pituitary radiotherapy	13 (22.4)		
Hormonal deficiencies, n, %			
Glucocorticoid deficiency	21 (36.2)		
GH deficiency	15 (25.9)		
Thyroid hormone deficiency	25 (43.1)		
Mineralocorticoid deficiency	11 (19.0)		
Testosterone deficiency	6/12 (50.0)		
Estrogen deficiency[b]	25/46 (54.3)	29/46 (63.0)	
Comorbidities, n, %			
Hypertension	18 (31.0)		
Diabetes mellitus	4 (6.9)		
Hypercholesterolemia	12 (20.7)		
Cushing type, n, %			
Pituitary	40 (69.0)		
Adrenal	18 (31.0)		

[a] $P < .05$.

[b] Secondary hypogonadotropic hypogonadism or a postmenopausal state without the use of chronic estrogen replacement.

To establish resting FBF, we administered 0.9% saline for 30 minutes. Vasoactive agent infusions were then started. Between each series of drug infusions, FBF was allowed to return to basal value during a 20-minute resting period, during which solvent (0.9% saline for acetylcholine [Ach] and 5% glucose for sodium nitroprusside [SNP]) was infused to maintain a constant infusion rate. Ach (Miochol-E intraocular solution, 20 mg; Bausch & Lomb; 1–2-4 μg/dL forearm volume/min) was used to explore endothelium-dependent vasodilatation. SNP (25 mg/mL, 2 mL; Sigma-Aldrich; 0.2– 0.4 – 0.8 μg/dL forearm volume/min) was used to explore nonendothelium dependent vasodilatation. Finally, the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine acetate (250 mg, Clinalfa Basic; Bachem; 0.2– 0.4 – 0.8 μmol/dL forearm volume/min) was infused to investigate the contribution of nitric oxide to basal vascular tone. Each substance-dose was infused for 5 minutes. FBF values are reported in milliliters per minute per 100 mL of forearm volume. The baseline value is a mean of all measurements during the baseline measurement period. The values during drug infusion are a mean value of the last six measurements per drug dose during a measurement period. In addition to changes in blood flow, the blood flow ratio between the infusion and control arm was also calculated to correct for possible systemic effects [32]. The CV of FBF has been reported to be 8%–10% during stimulation [31, 33].

Statistical methods

Data were analyzed using SPSS 20.0 statistical package for Windows (SPSS Inc). Data were expressed as mean ± SD, unless mentioned otherwise. Data distributions were analyzed and logarithmic transformation was performed before statistical testing when appropriate. Differences between patients and controls were tested with paired *t* tests. Differences in categorical variables were analyzed using the x^2 test. In group A, stepwise backward multiple linear regression analysis was performed in the patients to detect clinical characteristics (etiology of CS, treatment strategies, presence of hormonal deficiencies, use of alcohol, smoking, and comorbidity) that are predictors of vascular function. A stepwise backward multiple linear regression analysis could not reliably be performed in group B because of the small sample size. $P < .05$ was considered statistically significant.

Results

Subject characteristics

Table 1 shows the clinical characteristics of the patients and control subjects for group A and Table 2 for group B. Intraarterial cannulation was not successful in three patients, and therefore, the vasomotor response to intraarterial drug infusions was investigated in 11 patients and controls (Table 3). Adequate matching was reflected by the fact that no differences between patients and controls were present in both groups in gender, age, and

BMI. In group A patients differed from controls only with respect to smoking habits (more smokers in the patient group, $P < .05$).

Table 2. Group B (FMD): Clinical Characteristics of Patients in Long-Term Remission of CS and Healthy Controls

	Patients (n = 14)	Controls (n = 14)	*P* Value
Gender, male/female, n	2/12	2/12	1.00
Age at time of test, y, mean (SD)	46.8 (11.8)	45.7 (10.9)	.79
Duration of remission, y, median (range)	12.9 (4.8 –29.4)		
BMI, kg/m^2, mean (SD)	25.6 (2.3)	25.6 (2.5)	.98
CS type, n			
Pituitary	7		
Adrenal	7		
Treated hypothyroidism, n	4		
Estrogen status in females, n			1.00
Sufficient	7	7	
Insufficient	5	5	

Table 3. Group B (Venous Occlusion Plethysmography): Clinical Characteristics in Long-Term Remission of CS and Healthy Controls

	Patients (n = 11)	Controls (n = 11)	*P* Value
Gender, male/female, n	2/9	2/9	1.00
Age at time of test, y, mean (SD)	45.6 (13.2)	45.8 (12.1)	.98
Duration of remission, y, median (range)	12.8 (4.8 –28.8)		
BMI, kg/m^2, mean (SD)	25.7 (1.7)	25.3 (2.7)	.62
CS type, n			
Pituitary	5		
Adrenal	6		
Treated hypothyroidism, n	3		
Estrogen status in females, n			1.00
Sufficient	5	5	
Insufficient	4	4	

Biochemical markers associated with endothelial dysfunction (group A)

No statistically significant differences in soluble VCAM-1 (sVCAM-1), soluble ICAM-1, E-selectin, and PAI-1 were detected between patients and controls (Table 4).

Noninvasive measurements of atherosclerosis and arterial stiffness (group A)

cIMT, PWV, and CAP were not different between patients and controls (Table 4). A trend toward a statistically significant difference between the two groups was found for the AIx (P = .056). Atherosclerotic plaques were detected in 10 patients and 10 controls. Plaque thickness was not significantly different between patients and controls.

Table 4. Micro- and Macrovascular Health Parameters in Patients in Long-Term Remission of CS and Matched Controls

	Patients			Controls			
Variable	**Mean**	**95% CI**	**n**	**Mean**	**95% CI**	**n**	***P* Value**
Group A							
Serum biomarkers							
ICAM-1, pg/mL[a]	280.4	226.7–346.7	57	314.9	234.7– 422.4	57	.545
PAI, pg/mL[a]	1810.8	1505.8–2163.1	57	1940.5	1653.9–2276.7	57	.497
VCAM-1, pg/mL[a]	670.0	615.1–729.9	57	682.4	637.3–730.6	57	.721
E-selectin, pg/mL[a]	40.0	35.7– 44.6	57	38.5	34.6 – 43.0	57	.661
Noninvasive measurements of arterial stiffness and atherosclerosis							
CAP, mm Hg (HR75)	10.1	8.8 –11.5	52	9.4	7.6 –11.3	52	.457
Aortic AIx (HR75)	26.0	23.2–28.8	53	23.1	19.6 –26.6	53	.056
PWV, m/sec	8.4	8.0 – 8.9	58	8.3	7.8 – 8.8	58	.648
Mean cIMT, mm	0.75	0.72– 0.78	58	0.75	0.72– 0.77	58	.617
Plaque thickness, mm[b]	2.66	1.94 –3.38	10	1.95	1.71–2.18	10	.092
Group B							
Measurements of FMD							
Baseline diameter, mm	3.60	3.33–3.86	14	3.56	3.30 –3.82	14	.839
FMD, %	5.13	4.10 – 6.15	14	6.22	4.72–7.72	14	.125
GTN, %	18.6	15.5–22.0	14	19.4	15.0 –22.9	14	.691
Time to peak diameter, sec	40.3	33.4 – 47.3	14	54.3	42.1– 66.6	14	.059
SR_{AUC}, sec, 10^3	30 323	25 530–35 115	14	32 164	26 471–37 857	14	.597

Abbreviations: CI, confidence interval; HR75, corrected for a heart rate of 75 beats per minute; GTN, glyceryltrinitrate; SR_{AUC}, shear rate area under the curve.

[a] For ln-transformed data, the geometric means and backtransformed 95% CI were calculated to enable clinical interpretation of the outcomes.

[b] For plaque thickness the comparison between the groups was performed using an unpaired *t* test.

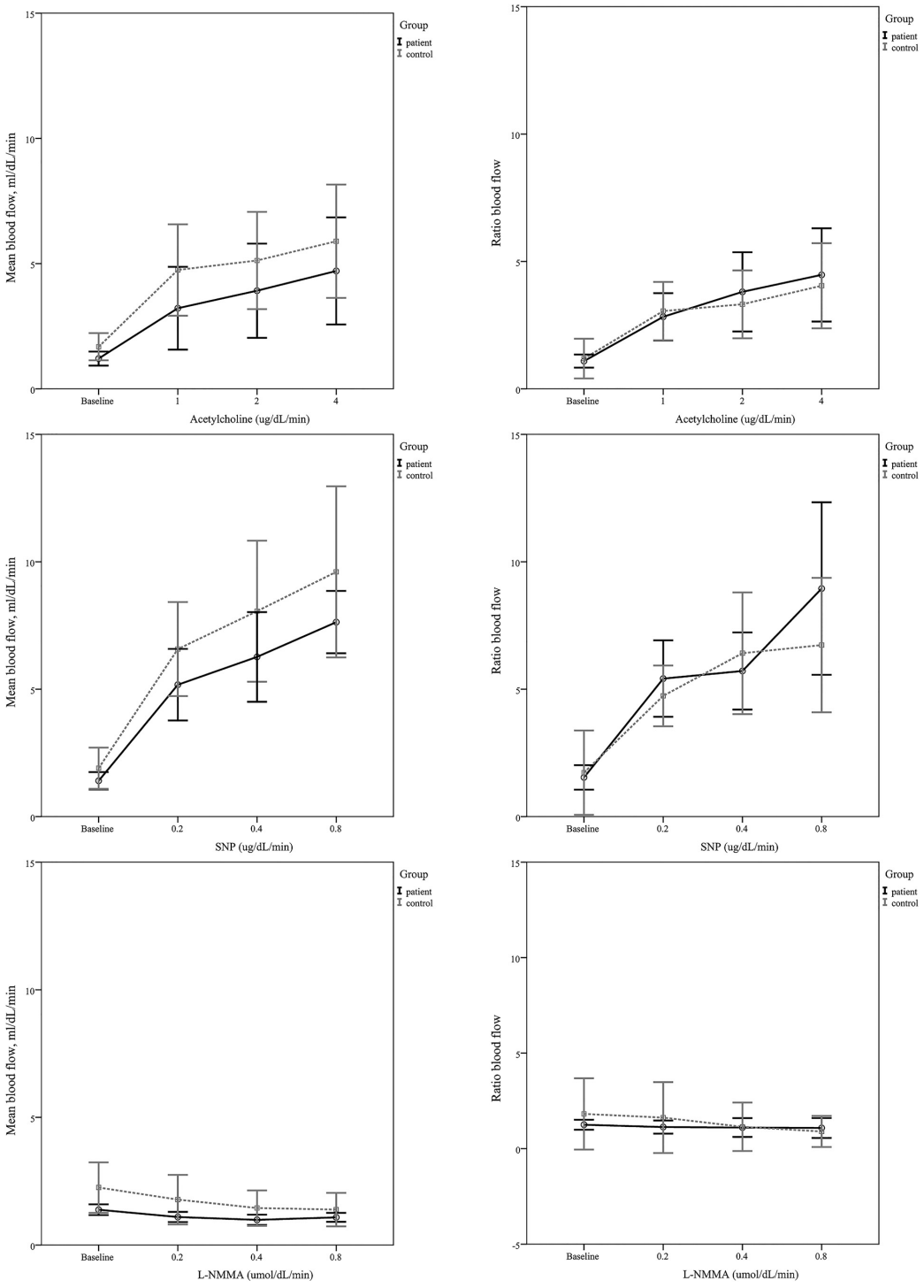

Figure 1. Change in forearm blood flow from baseline in response to infusion of different vasoactive agents in increasing dosages.
Note: On group level, no acute vasomotor responses were observed in the control arm after drug infusions. Error bars, 95% CI.

Endothelial function (group B)

No statistically significant differences were found between patients and controls in all FMD measurements (Table 4). Furthermore, no statistically significant differences were found between patients and controls regarding FBF or blood flow ratio responses at baseline or in response to the incremental doses of Ach, SNP, and N^Gmonomethyl-L-arginine (all $P > .09$) (Figure 1).

Stepwise backward multiple linear regression analysis (group A)

Having diabetes mellitus (DM) predicted both a higher PWV ($P = .01$) and higher sVCAM-1 levels ($P < .01$). Subgroup analysis was performed for these two outcomes after exclusion of all matched patient-control couples containing a patient with DM. This did not lead to significant differences between patients and controls (PWV, $P = .796$; sVCAM-1, $P = .865$). Being a smoker was a predictor for a higher AIx ($P < .01$). Subgroup analysis, after exclusion of all patient-control couples with a smoker, did not lead to a significant difference between patients and controls (AIx; $P = .078$). Mineralocorticoid replacement was a predictor for higher E-selectin levels ($P < .01$). Subgroup analysis, after exclusion of all couples with mineralocorticoid users, did not lead to a significant difference between patients and controls (E-selectin; $P = .913$). Thyroid hormone replacement was a predictor for higher sVCAM-1 levels ($P < .01$). Subgroup analysis after exclusion of couples with thyroid hormone users did not lead to a significant difference between patients and controls (sVCAM-1; $P = .504$).

Discussion

In this study we investigated micro- and macrovascular health in patients in long-term remission of CS who had no, or adequately treated, comorbidities using a combination of state-of-the-art methods that has not been used in any previous study. We compared the patient group to a strictly one-to-one-matched healthy control group. The main finding of our study is that the vascular health of patients in remission of CS is not significantly different from that seen in healthy control subjects matched for age, gender, and BMI. This suggests that the direct effect of the period of hypercortisolism per se on the vasculature during the active disease is potentially reversible.

Our findings that endothelial function recovers after remission of CS are in line with the study of Akaza et al [22], who investigated arterial endothelial function, with FMD, in a group of 12 patients shortly after remission (>3 mo) of CS. They found that the impaired FMD in active CS was reversible after remission. Previous studies have shown that in vitro (cell culture) and in vivo (mouse) exposure of endothelial cells to glucocorticoids reduced

the mRNA and/or protein content of endothelial nitric oxide synthase [34, 35] and reduced acetylcholine-induced vasodilation of mouse resistance arteries [34] and rat aortas [36]. Therefore, Akaza et al [22] proposed that endothelial dysfunction in active CS is largely accounted for by the direct effect of hypercortisolism on vascular endothelium and that this is reversible after treatment.

On the other hand, five other studies observed persistent impaired vascular health after remission of CS [17, 18, 20, 21, 23]. However, in three of these studies, there was either a short period of remission [17] or a pediatric study population [20, 21], so these studies are not comparable with our study. The studies reported by Colao et al [18] and Barahona et al [23] are more comparable. They both found a higher prevalence of atherosclerosis (measured by cIMT and the presence of coronary artery disease detected by computed tomography, respectively) compared with gender-, age-, and BMI-matched controls [18, 23]. However, the patients in these studies had significantly more uncontrolled metabolic comorbidities than their matched controls. In our study population, the comorbidities in group A were adequately treated [16], and the patients in group B had no known comorbidities (except for treated hypothyroidism in four patients).

A more recent publication of Faggiano et al [19] also supports our findings. This study measured differences in cIMT and artery stiffness between active disease and 1 year after remission of CS in 25 patients. There was a significant decrease in both variables between active disease and remission. After 1 year of remission, both variables did not differ from a gender-, age-, and BMI-matched control group as used in our study, but they were still higher than in controls with a lower BMI, matched only for gender and age. Moreover, diastolic BP and low-density lipoprotein- and high-density lipoprotein-cholesterol levels were not different between the patients and the BMI-matched control group, but they were significantly more adverse in the patients compared with the controls with a lower BMI. This emphasizes the importance of strict matching of each patient to a healthy individual of at least the same gender, age, and BMI if one wants to investigate the effect of the previous period of hypercortisolism per se. Taken both our results and the previous findings into account, we conclude that patients in remission of CS, who are equally well controlled for comorbidities as age-, gender-, and BMI-matched healthy subjects, have comparable vascular health. This accentuates the need for stringent treatment of metabolic comorbidities in these patients. Interestingly, the normalized vascular health seems to be irrespective of the fact that these patients have, as we have previously shown, a more centripetal adipose tissue distribution and adverse adipokine profile than their age-, gender-, and BMI-matched controls [16]. However, the patients in our study are relatively young, and vascular problems are more frequent as age increases. So even though we did not find indications for impaired vascular health at approximately 50 years of age, the

fact that persistent central adiposity and an adverse adipokine profile are still present after long-term remission of CS may mean that patients still are at higher vascular risk later in life.

As could be expected in group A, DM was associated with a higher PWV and higher sVCAM-1 levels and smoking predicted a higher AIx, but this did not affect the results of the total group. Moreover, the trend toward a higher AIx in the patient group disappeared after correcting for smoking. Interestingly, except for an association between mineralocorticoid replacement and E-selectin levels and the use of thyroid hormone replacement and VCAM-1 levels, no other patient characteristic (eg, etiology of CS, treatment strategies, hormonal deficiencies) negatively affected vascular health parameters. This is in contrast to previous studies, in which, for example, the use of glucocorticoid replacement therapy was associated with an increased cardiovascular risk [10].

The major strength of our study is the broad spectrum of methodologies we used to investigate vascular health. All techniques are well validated and reproducible [25, 30, 31]. Furthermore, this is the first study that investigates endothelial function in patients in long-term remission of CS both in conduit arteries (FMD) and forearm resistance arteries (FBF, which is considered the gold standard procedure to measure endothelial dysfunction) [25]. Thus, we have investigated both the macrovasculature and the microvasculature.

A possible limitation of this study is the relatively small sample size for group B. For FBF and FMD, a number of about 10 patients was found to be adequate to detect a relevant difference [31, 37] and that, however, the subjects within our patient group (and thus also the control group) were more heterogeneous than in most previous studies, leading to a greater SD. Therefore, it is possible that we missed subtle but relevant differences. For example, there seems to be a nonsignificant trend toward a lower baseline FBF in the patients, which could indicate a reduction in muscle microvascular density. The latter might explain the exercise intolerance experienced by the patients [38]. Because blood flow in the skin and subcutaneous adipose tissue also contribute to FBF [31], future research measuring microvascular density in muscle biopsies will have to confirm whether skeletal muscle microvascular density is indeed lower in patients in remission of CS.

A multitude of epidemiological studies reported an increased cardiovascular risk and standardized mortality in patients in long-term remission of CS compared with an age- and gender- but not BMI-matched reference population [1]. Because patients in remission of CS tend to have an overall higher BMI and waist circumference than the general population, this may negatively affect cardiovascular risk and standardized mortality. Furthermore, these studies did not analyze potential differences between patients with

and without comorbidities. However, it may be possible that cardiovascular risk is still elevated in the healthiest patients in remission of CS because of a persistent effect of the prior hypercortisolism on other organs than the vasculature, eg, the myocardium [10, 11]. However, this was not supported by a small study (39). Therefore, further research is necessary to investigate these issues.

In conclusion, the vascular health of patients in longterm remission of CS seems to be comparable with that of healthy gender-, age-, and BMI-matched controls, provided that the patients have no, or adequately controlled, comorbidities. Therefore, the effects of the previous hypercortisolism per se on the vasculature may be reversible. This accentuates the need for stringent individualized treatment of metabolic comorbidities in these patients.

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Supplemental table 1: Outcomes of other cardiovascular risk factors in patients and controls in Group A.

Variable	Controls (n=58) (mean)	SD	Patients (n=58) (mean)	SD	P-value
Total serum cholesterol (mmol/l)	5.38	0.198	5.16	0.165	0.188
HDL-cholesterol (mmol/l)	1.44	0.242	1.33	0.216	0.061
LDL-cholesterol (mmol/l)	3.38	0.255	3.05	0.236	0.055
Triglycerides (mmol/l)	1.01	0.445	1.43	0.531	<.001***
Creatinin (µmol/l)	68.10	0.144	70.81	0.188	0.194
Insulin (mE/l)	6.51	0.552	6.51	0.722	0.933
Hba1c (mmol/mol)	37.49	0.088	39.10	0.151	0.355
Fasting glucose (mmol/l)	4.98	0.107	4.99	0.172	0.973
HOMA_IR	1.71	0.99	2.36	5.08	0.371
fT4 (pmol/l)	12.28	0.136	15.20	0.202	<.001***
IGF-1 (nmol/l)	16.02	0.353	13.25	0.434	0.011*
Systolic blood pressure (mmHg)	132.37	19.06	126.04	14.55	0.095
Diastolic blood pressure (mmHg)	77.24	9.27	73.85	9.02	0.134
Heart rate (bpm)	64.03	8.42	66.81	9.48	0.151

Differences were tested by means of paired t-tests. For ln-transformed data the geometric means were calculated using back transformation to enable clinical interpretation of the outcomes. HDL, high density lipoprotein; LDL, low density lipoprotein; Hba1c, glycated hemoglobin; HOMA_IR, homeostatic model assessment _ insulin resistance; IGF-1, insulin like growth factor type 1 p<0.05, ** p<0.01, *** p<0.001.*

Note 1: In our hospitals patients continue to visit our outpatient clinic at least once a year after remission of Cushing's syndrome (CS). During that visit patients are screened for the presence of hypertension, diabetes mellitus and hypercholesterolemia. If needed treatment is initiated. (If they already had hypertension, diabetes mellitus or hypercholesterolemia during the active phase of CS, we try to taper medication and if possible to stop medication to see if it is still needed).The choice of which medication is used was dependent on the preferences of the individual physicians and patients, but usually metformin was the first choice for diabetes mellitus type 2, simvastatin was the first choice for hypercholesterolemia and a thiazide diuretic or an ace-inhibitor were the first choice for hypertension. The effect of treatment was monitored regularly (each 3-6 months) and treatment was adjusted till treatment goals (a blood pressure of < 140/90 mmHg, a HbA1c < 53 mmol/mol and a LDL-cholesterol of < 3.5 mmol/l) were reached.
Note 2: In case a patient had CS of pituitary origin biochemical evaluation is carried out on the fourth day postoperatively to evaluate the function of the pituitary gland (after glucocorticoid substitution had been stopped for at least 24 hours), by measurement of fasting (08:00 h) plasma cortisol, ACTH, thyrotropin, free thyroxine, gonadotropins, testosterone or estradiol and insulin-like growth factor type-1. If basal plasma cortisol is lower than 200 nmol/l substitution therapy with hydrocortisone, 30 mg a day, was prescribed. Patients were re-evaluated every 2–4 weeks during the first 3 months after TS and thereafter at 2–3 months intervals during the first year. The fasting plasma cortisol concentration was measured at each visit. If a patient received glucocorticoid substitution therapy postoperatively, the dose was reduced and stopped, if possible, between 3 and 12 months after TS. Thereafter the integrity of the hypothalamic–pituitary–adrenal axis was assessed by an insulin tolerance test. Growth hormone deficiency is tested with a growth hormone stimulation test. If a hormonal deficiency is present substitution is initiated to reach reference values.
Note 3: Hypertension is defined as a blood pressure ≥140/90 mmHg. Diabetes mellitus is defined as a HbA1c ≥ 6.5% (≥ 48 mmol/mol), a fasting glucose of ≥7.0 mmol/L (126 mg/dL) or a non fasting glucose ≥11.1 mmol/l (199 mg/dl). Hypercholesterolemia is defined as a LDL cholesterol of > 3.5 mmol/l or non-HDL cholesterol of > 4.0 mmol/L (in case no other co-morbidities are present; otherwise we use stricter criteria).

Chapter 7

Decreased aerobic exercise capacity after long-term remission from Cushing's syndrome: exploration of mechanisms

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Submitted

Abstract

Background: Although major improvements are achieved after cure of Cushing's syndrome (CS), fatigue and decreased quality of life persist. This is the first study to measure aerobic exercise capacity in patients in remission of CS for >4 years in comparison with matched controls, and investigate whether the reduction in exercise capacity is related to alterations in muscle tissue.

Methods: 17 patients were included. A control subject, matched for gender-, estrogen status-, age-, BMI-, smoking-, ethnicity-, and physical activity level was recruited for each patient. VO_{2peak} was assessed during incremental bicycle exercise to exhaustion. In 8 individually matched patients and controls, a percutaneous muscle biopsy was obtained and measures were made of cross-sectional areas, capillarization, and oxphos complex IV (COXIV) protein content as indicator of mitochondrial content. Furthermore, protein content of eNOS and eNOS phosphorylated on serine^{1177} and of the NAD(P)H-oxidase subunits NOX2, p47phox and p67phox were measured in the microvascular endothelial layer.

Findings: Patients showed a lower mean VO_{2peak} (SD) (28.0 (7.0) vs. 34.8 (7.9) ml O_2/kg bw/min, $P<0.01$), -maximal workload (SD) (176 (49) vs. 212 (67) watt, $P=0.01$) and -oxygen pulse (SD) (12.0 (3.7) vs. 14.8 (4.2) ml/beat, $P<0.01$) at VO_{2peak}. No differences were seen in muscle fiber type specific cross-sectional area, -capillarization measures, -mitochondrial content and -protein content of eNOS, eNOS-P-ser^{1177}, NOX2, p47^{phox} and p67^{phox}.

Interpretation: As differences in muscle fiber and microvascular outcome measures are not statistically significant, we hypothesize that cardiac dysfunction, seen in active CS, persists during remission and limits blood supply to muscles.

Introduction

Cushing' syndrome (CS), in most cases, is of pituitary or adrenal origin. Skilled surgeons supported by expert endocrinologists have high success rates in reducing plasma cortisol levels to the normal range and achieving substantial improvements in phenotype in patients. However, after remission most of the patients, independent of the origin of CS, report subjective feelings of fatigue and limitations in their ability to perform exercise [1-3].

The current study aimed to be the first to measure aerobic exercise capacity during incremental cycling exercise to exhaustion in patients in remission of CS for 4-28 years. The second aim was to investigate whether the patients for a given habitual physical activity level (to include exercise activities) have a lower VO_{2peak} than healthy matched controls. The third aim was to investigate whether the mechanisms limiting VO_{2peak} in the patients reside at the level of the vastus lateralis muscle taken as representative of the major muscles used during walking, running and cycling exercise. The major determinants of VO_{2peak} during incremental exercise at the level of the contracting muscles are mitochondrial content, capillary density and the vasodilatory response of terminal arterioles (TAs).

In this study we measured VO_{2peak} in patients in long-term remission of CS using the gold standard method measuring VO_2 during a stepwise incremental exercise test until exhaustion. A comparison was made with a control group individually matched for age, gender, body mass index (BMI), smoking, physical activity level and ethnicity. These conditions/characteristics were selected as they are known to affect VO_{2peak} independent of previous CS. Therefore, this design, with all other conditions being equal, allowed us to investigate whether previous CS is an independent condition reducing VO_{2peak} and not just the result of e.g. a lower physical activity level or a higher BMI.

To investigate the potential mechanism(s) that lead to a reduction in VO_{2peak} in the patients compared to their controls we measured in percutaneous muscle biopsies 1) mitochondrial content; 2) several measures of capillary density and structure; 3) the cross-sectional area of type 1 and type 2 fibers as measure of potential muscle fiber atrophy.

Previous reviews of research [4, 5] have shown that intra-abdominal (visceral) obesity and hypertriglyceridemia in sedentary obese individuals leads to an impairment in the exercise induced vasodilation of TAs in the muscles. This then leads to a reduction in the exercise induced recruitment of additional capillaries and capillary surface area and, consequently, to a reduction in the transendothelial transport rate of oxygen and nutrients/fuels from the blood in the capillary lumen into the contracting muscle fibers. Previous research [6] suggests that in sedentary obese young men with metabolic syndrome, an imbalance exists

between the protein content of eNOS and the NAD(P)H-oxidase complex. A reduction in protein content and serine^{1177} phosphorylation of eNOS reduces eNOS activity and the production of the vasodilator NO, while increased expression of subunits of the NAD(P)H-oxidase protein complex (NOX2, p47^{phox} and p67^{phox}) increases the production of superoxide anions and subsequent quenching of NO. These measurements were made in the current study to test the hypothesis that the eNOS/NAD(P)H-oxidase protein ratio is lower in the patients than the matched controls and, therefore, may limit the exercise induced vasodilation of TAs and recruitment of additional capillaries during exercise.

Subjects and methods

Subjects

In this cross-sectional matched case-control study, patients who were successfully treated for CS in the period between 1985 and 2009 in the Radboud University Nijmegen Medical Center and Leiden University Medical Center, both in the Netherlands, could be included. Medical records of all patients were reviewed to assess data on demographics, diagnosis of CS, etiology of CS, type and number of treatments received, and follow-up data on remission, recurrences, and hormonal deficiencies. Adult patients (>18 years old) in long-term (>4 years) remission of CS were eligible for this study. Remission was defined as suppression of plasma cortisol to ≤ 50 nmol/l after 1 mg dexamethasone overnight and absence of clinical signs and symptoms of active hypercortisolism, documented no longer than 1 year before the inclusion [7]. Subjects with hormonal deficiencies, except for adequately treated hypothyroidism (free T4 range 8.0-22.0 pmol/l), were excluded from this study. All eligible pituitary CS subjects had been tested, after their last pituitary surgery, for growth hormone (GH) deficiency by means of an insulin tolerance test (ITT), because GH deficiency is known to have a strong influence on $VO_{2\ peak}$ [8]. GH deficiency was defined as a maximal GH response <15.3mU/l during an ITT [9]. Serious co-morbidity (i.e. active malignancy, serious psychiatric pathology and known diabetes mellitus), pregnancy, use of medication interfering with the cardiovascular system (angiotensin converting enzyme inhibitors, calcium antagonists, angiotensin II receptor antagonists, beta blockers), severe cardiopulmonary disease, and orthopedic and/or neurological diseases were exclusion criteria.

For each patient, a gender-, age-, BMI-, smoking- (yes/no), ethnicity- and physical activity level-matched control was recruited from the general population by means of an advertisement in a newspaper. Female patients were matched for estrogen status and oral contraceptive use.

Physical activity was estimated, before exercise testing, using metabolic equivalent of task scores (METS). Subjects reported their weekly physical activities, enabling the estimation of their daily average METS-score using the 2011 compendium of physical activities [10]. Daily energy expenditure (EE) was assessed using an activity monitor (Sensewear Pro3 Armband, SWA, Body Media, Pittsburgh, PA, USA) after inclusion in order to assure adequate matching on physical activity level.

This study was approved by the institutional medical ethics committees and conformed to the declaration of Helsinki. All participants provided written informed consent.

Methods

Aerobic exercise capacity

Aerobic exercise capacity was assessed using an exercise stress test on a stationary bicycle ergometer (Lode, Excalibur Sport, Groningen, the Netherlands) using a progressive incremental exercise protocol. All subjects refrained from alcohol, caffeine, and intensive physical exercise for at least 24 h prior to testing. All tests were performed in laboratory conditions with constant temperature (18-20°C) and humidity (35%). All tests started at the same time of the day (9:00 AM). Participants were instructed to cycle at 60-80 rotations per minute (rpm) to volitional fatigue or until they reached symptom-limited exhaustion. Spiro-ergometric equipment (Oxycon alpha, Jaeger, Breda, the Netherlands) was used to continuously measure breath-by-breath minute ventilation (V_E), respiratory rate, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) with calculations of the respiratory exchange ratios (RER, VCO_2/VO_2). Aerobic exercise capacity was determined as the peak oxygen uptake in ml O_2/min/kg (VO_{2peak}). Oxygen pulse, a noninvasive estimate of cardiac stroke volume, was calculated as the ratio of peak VO_2 (ml/min) to peak heart rate (bpm) [11]. Values were obtained from expired air as 30 seconds averages. A 12-lead electrocardiogram was used to observe heart rate. Blood pressure was measured manually before testing to ensure volunteer safety. Capillary blood lactate (Accutrend plus, Roche, Woerden, the Netherlands) was measured before and 2 minutes after the test. Upon cessation of exercise, participants reported their rating of perceived exertion (RPE) using a 0-10 Borg scale [12]. VO_{2peak} was deemed to have been reached and the test data was included in the analysis when 3 of the following 4 criteria were met: 1) clinical signs of full exhaustion including Borg scale score ≥8, 2) RER ≥ 1.10 at cessation, 3) maximal heart rate within 10 beats of the maximum predicted heart rate (220 – age), and 4) flattening of VO_2 uptake curve (≤150ml increase during the last minute of exercise) [13].

Physical activity levels

Average daily energy expenditure (EE; mean total calories used per day), average active EE (mean total calories used during activities >3 METS), average daily sedentary hours (activity < 1.5 METS) and average daily active hours (activity >3 METS) was assessed using an activity monitor (Sensewear Pro3) around the upper right arm. The activity monitor measured physical activity 24 hour per day for 7 consecutive days close to the exercise stress test. Each 24 hour interval was analyzed from 12:00 PM to 12:00 PM the following day and was included when the monitor recorded at least 90% of the time in each 24 hour cycle. The activity monitor has been validated to examine EE and activity behavior in humans [14].

Muscle biopsy

A muscle biopsy was taken from vastus lateralis muscle using percutaneous needle biopsy technique under local anesthesia (1% lidocaine) as previously described [15]. The vastus lateralis muscle was chosen as it is easy to access by percutaneous biopsy and the fact that this muscle makes a significant contribution to the work load of the upper leg muscles during exercise, especially during cycling. Furthermore, this is the muscle that has previously been investigated in active CS [16]. Samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) and frozen in liquid nitrogen cooled isopentane (Sigma-Aldrich, Dorset, UK). Samples were stored at -80°C.

Skeletal muscle mitochondrial content and capillarization

The method to make a fiber type specific quantitative estimate of mitochondrial content from the fluorescence intensity of oxphos complex IV (COXIV) has been described previously [17]. Briefly, muscle sections were first incubated with primary antibodies targeting oxphos complex IV (COXIV) (Invitrogen, UK) and myosin heavy chain type I (A4.840-c, DSHB, developed by Dr Blau), followed by incubation with appropriate secondary antibodies (Alexa Fluor goat anti-mouse IgG_{2a} 488 and Alexa Fluor goat anti-mouse IgM 546, respectively) and a wheat germ agglutinin (WGA) Alexa Fluor 350 conjugate (to visualize the cell border) (Invitrogen, UK).

The method to assess fiber type specific capillarization has been described previously [6]. Muscle cross-sections were first incubated with the same myosin heavy chain type I primary antibody to identify the type I fibers. This was followed by incubation with a goat anti-mouse IgM 546 secondary antibody in combination with Ulex Europaeus-FITC conjugate (UEA-I-FITC; Sigma-Aldrich, UK) and the WGA-350 conjugate. A Leica DMI600B microscope with a 40x/0.6 NA objective, coupled to a Leica DFC365 FX CCD microscope camera (Leica Microsystems, UK) was used to obtain digital images of cross-

sectionally orientated muscle. Both DAPI UV (340-380 nm) and FITC (465-495 nm) excitation filters were used to view the Alexa Fluor 350 and 488 fluorophores, respectively, and a Texas red (540-580 nm) excitation filter was used to view sections stained with Alexa Fluor 546. Image processing and analysis was undertaken using Image Pro Plus 5.1 software (Media Cybernetics Inc, Bethesda, MD, USA). A total of 50±11 fibers per muscle cross-section were analyzed. Fluorescence staining intensity was used to indicate differences between the patients and their matched control in mitochondrial content of each fiber type. Capillaries were also quantified in a fiber type specific manner manually, using the UEA-I, WGA-350 and myosin heavy chain images [6]. The following indexes of muscle tissue fibers and capillarization were measured: 1) total fiber cross sectional area of type I and type II fibers, 2) number of capillaries around a fiber (capillary contacts), 3) capillary density and 4) capillary-fiber perimeter exchange index.

Quantitative immunofluorescence microscopy to estimate skeletal muscle eNOS, eNOS-P- ser^{1177}, NOX2, p47phox and p67phox protein content

Endothelial specific eNOS content and eNOS ser^{1177} phosphorylation were assessed using previously established methods [18, 19], with the modification that the method was adapted such that the eNOS-P-ser^{1177}/eNOS ratio was calculated for individual vessels. Methods to assess endothelial and membrane specific NOX2 content have also been described previously [18, 19]. Assessment of endothelial specific p47phox and p67phox apart from using different primary antibodies use the same method as described for NOX2.

Sections were fixed in acetone and ethanol (3:1). For assessment of eNOS ser^{1177}/eNOS ratio, sections were triple stained with antibodies against eNOS (Transduction Laboratories, Lexington, KY, USA), and p-eNOS ser^{1177} (Cell Signaling Technology, Beverly, MA, USA). For assessment of NOX2, p47phox and p67phox content, sections were double stained with antibodies against either NOX2, p47phox, or p67phox (all kind gifts from prof Mark Quinn, Montana State University). All sections were then incubated with appropriate secondary antibodies (Invitrogen, Paisley, UK) in combination with the endothelial marker UEA-I-FITC (Sigma-Aldrich, UK). A plasma membrane marker, WGA-633 (Invitrogen, Paisley, UK), was also included when staining samples for NOX2.

Images were acquired using an inverted confocal microscope (Zeiss LSM-710, Carl Zeiss, Germany) with a 40x NA oil immersion objective. Alexa Fluor 405 was excited using the 405 nm line of the diode laser and detected with 371-422 nm emission. FITC fluorescence was excited with a 488 nm line of the argon laser and detected with 493–559 nm emission. Alexa Fluor 546 and 633 fluorophores were excited with 543 nm and 633 nm lines of the helium–neon laser and 548–623 nm and 638–747 nm emission, respectively. Identical settings were used for all image capture within each participant.

All image analysis was performed using ImagePro Plus 5.1 (Media Cybernetics Inc, Bethesda, MD, USA). The endothelial (UEA-I-FITC) outline was overlaid onto the corresponding vascular enzyme image. Fluorescence intensity of the vascular enzyme signal was then quantified within the endothelial specific area. As eNOS and eNOS ser^{1177} phosphorylation had been stained on the same sections it was possible to establish eNOS ser^{1177}/eNOS ratio on an individual vessel basis, as the same endothelial outline could be placed over both eNOS and eNOS ser^{1177} images. Cell membrane specific fluorescence for NOX2 was determined using the WGA-633 stain to create an outline of the cell membrane. This mask was then overlaid onto the corresponding image to determine membrane specific fluorescence intensity for NOX2.

Statistical analysis

Statistical analysis was performed using SPSS (version 22.0, Chicago, Ill. USA). Data is expressed as mean and standard deviation (SD) unless stated otherwise. Before analysis, data was checked for normality of distribution using the Shapiro-Wilk test. Differences between the groups were analyzed using paired t-tests after confirmation of adequate group matching using independent t-tests. Correlations between aerobic exercise capacity and clinical parameters were determined using Spearman's correlation coefficient. The level for significance was set at $\alpha \leq 0.05$.

Results

Baseline characteristics

Seventeen patients in long-term remission of CS, and 17 healthy controls matched for gender, age, BMI, estrogen status, ethnicity, physical activity level and smoking habits, were included (Table 1). Ten (58.8%) patients had CS of pituitary origin, and were treated by selective transsphenoidal pituitary adenomectomy. Seven (41.2%) patients had CS of adrenal origin, and were treated by unilateral adrenalectomy.

Aerobic exercise capacity

Mean VO_{2peak} of patients in long-term remission of CS was significantly lower compared to matched controls ($P<0.01$) (Figure 1). A significantly lower maximal workload ($P=0.01$) and shorter test duration ($P=0.02$) was observed in patients in long-term remission of CS compared to the control group. V_E was significantly lower in patients in long-term remission of CS ($P=0.02$). This lower V_E consisted of a lower respiratory rate ($P=0.047$) with comparable tidal volumes in the patients and their controls. Furthermore, oxygen pulse was lower in the patients compared to their controls ($P=0.01$). The peak heart rate, RER, and post-test blood lactate concentrations were not statistically different between the groups (Table 2), but occurred at a lower absolute workload ($P=0.01$) in the former CS patients.

Table 1 Characteristics of patients and controls.

	Patients (n=17)	Controls (n=17)	*P*
Gender (n): female/ male	15/2	15/2	1.00
Age at time of test: (years)	45.7 ± 11.1	45.2 ± 10.1	0.89
Age at diagnosis: (years)	34.0 ± 10.2	-	-
Duration of remission: median (range) (years)	11.3 (4-28)	-	-
Height: (cm)	171.6 ± 6.3	174.1 ± 6.3	0.25
Weight: (kg)	73.9 ± 8.1	76.1 ± 9.8	0.50
BMI: (kg/m^2)	25.1 ± 2.4	25.1 ± 2.6	0.95
Systolic BP: (mmHg)	126 ± 13	130 ± 12	0.32
Diastolic BP: (mmHg)	81 ± 7	82 ± 2	0.70
Cushing's syndrome type: n (%)			
Pituitary	10 (58.8)	-	-
Adrenal	7 (41.2)		
Treated hypothyroidism: n (%)	4 (23.5)	-	-
Estrogen status in females: n (%)			
Sufficient (premenopausal)	10 (66.7)	10 (66.7)	1.00
Insufficient (postmenopausal)	5 (33.3)	5 (33.3)	
Smoking: n (%)			
Yes	1 (5.9)	1 (5.9)	1.00
No	16 (94.1)	16 (94.1)	

Data are presented as means ± SD unless stated otherwise. BMI, body mass index; BP, blood pressure

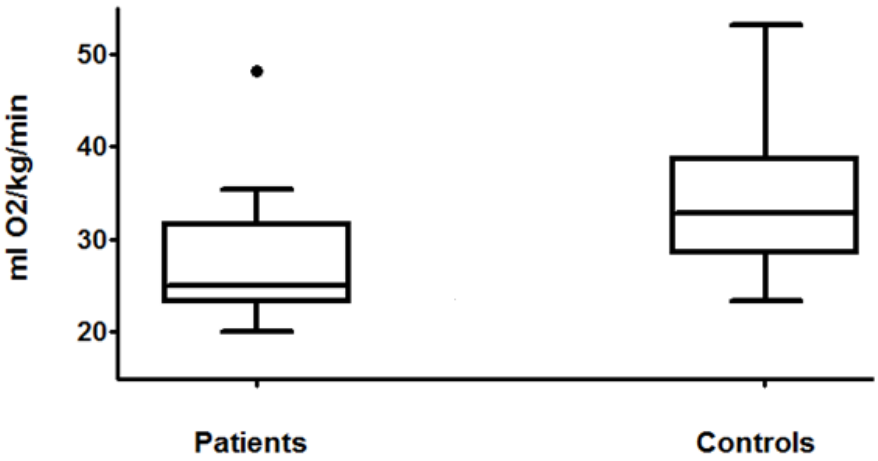

Figure 1 VO_{2peak} of patients in long-term remission of CS compared to matched controls.
Tukey boxplot with whiskers indicating 1.5 interquartile range of the lower and upper quartile and black dot indicating an outlier.

Physical activity levels

No differences were found in the current average daily total EE, active EE, total daily sedentary time and total daily active time (Table 2).

Correlations between aerobic exercise capacity and clinical characteristics

In the patient group, older age (r=-0.62, P<0.01) was significantly associated with a lower VO_{2peak}. Adequately treated hypothyroidism in patients was also associated with a lower VO_{2peak} (r=-0.65, P < 0.01). CS subtype, BMI, smoking, age at diagnosis, estrogen status and duration of remission were not significantly correlated with VO_{2peak}. In the control group, older age (r= -0.55, P=0.02) was significantly associated with a lower VO_{2peak}. After exclusion of the 4 patients with treated hypothyroidism and their controls, VO_{2peak} remained significantly lower in patients (25.2 ± 3.8) vs. controls (32.5 ± 5.5) (P<0.01)) (Table 3).

Table 2 Peak exercise responses and daily energy expenditure of patients and controls (n=17)

	Patients	Controls	P/C ratio	*P*
VO2peak: (ml O2/kg/min)	28.0 ± 7.0	34.8 ± 7.9	0.80	<0.01**
HR: (bpm)	174 ± 16	180 ± 13	0.97	0.22
Work load: (watt)	176 ± 49	212 ± 67	0.83	0.01*
V_E: (l/min)	89.4 ± 27.3	101.0 ± 19.8	0.89	0.02*
Respiratory rate: (b/min)	38 ± 8	42 ± 6	0.90	<0.05*
RER	1.22 ± 0.09	1.17 ± 0.08	1.04	0.11
Lactate: (mmol/l)	9.8 ± 2.8	11.0 ± 3.1	0.89	0.16
Test duration: (minutes)	12.0 ± 2.4	14.3 ± 3.8	0.84	0.02*
VO_2/HR: (ml/beat)	12.0 ± 3.7	14.8 ± 4.2	0.83	0.01*
Daily EE: (cal)	2498 ± 594	2567 ± 570	0.97	0.25
Daily active EE: (cal)	556 ± 527	606 ± 530	0.92	0.45
Daily sedentary hours (<1.5 METS)	10.7 ± 1.7	10.4 ± 1.6	1.03	0.54
Daily active hours (>3 METS)	4.1 ± 1.6	4.9 ± 1.8	0.84	0.12

Data are presented as mean ± SD. VO_{2peak}, maximal aerobic capacity; RER, respiratory exchange ratio; HR, heart rate; cal, calories; EE, energy expenditure; V_E, minute ventilation; P/C ratio, mean of the ratio of the indicated variable measured in the patient and its matched control.
* P<0.05 ** P<0.01

Table 3 Peak exercise responses and daily energy expenditure of patients and controls after exclusion of couples containing a patient with treated hypothyroidism (n=13)

	Patients	Controls	P/C ratio	*P*
VO_{2peak}: (ml O_2/kg/min)	25.2 ± 3.8	32.5 ± 5.5	0.78	<0.01**
HR: (bpm)	173 ± 18	178 ± 15	0.98	0.43
Work load: (watt)	158 ± 33	192 ± 35	0.82	0.01*
V_E: (l/min)	80.8 ± 19.4	95.1 ± 15.9	0.85	0.02*
Respiratory rate: (b/min)	37 ± 8	42 ± 6	0.88	0.06
RER	1.23 ± 0.08	1.17 ± 0.08	1.05	0.10
Lactate: (mmol/l)	9.1 ± 2.6	10.3 ± 2.6	0.88	0.24
Test duration: (minutes)	11.5 ± 2.2	14.0 ± 3.9	0.82	0.03*
VO_2/HR: (ml/beat)	10.6 ± 1.8	13.9 ± 3.4	0.76	<0.01**
Daily EE: (cal)	2288 ± 308	2374 ± 257	0.96	0.24
Daily active EE: (cal)	408 ± 247	459 ± 250	0.89	0.52
Daily sedentary hours (<1.5 METS)	11.1 ± 1.3	10.7 ± 1.4	1.04	0.53
Daily active hours (>3 METS)	3.8 ± 1.4	4.6 ± 1.9	0.83	0.17

Data are presented as mean ± SD. VO_{2peak}, maximal aerobic capacity; RER, respiratory exchange ratio; HR, heart rate; cal, calories; EE, energy expenditure; V_E, minute ventilation; P/C ratio, mean of the ratio of the indicated variable measured in the patient and its matched control.
* *P*<0.05 ** *P*<0.01

Skeletal muscle capillarization and mitochondrial content

Thirteen paired couples provided informed consent to undergo skeletal muscle biopsy. Due to technical problems (frost damage) and/or a small biopsy sample size in one of the 2 members of a paired couple, mitochondrial content and skeletal muscle capillarization could not be determined in five paired couples. No differences were found between the patients and their matched controls with regard to muscle total fiber cross sectional area, capillary contacts, capillary density and capillary-fiber perimeter exchange index. In addition, no differences were found between patients and controls in mitochondrial content (Table 4). The range for the mitochondrial contents and each of the capillary measures was large in both groups, but the mean patient/control ratio for each of these measures in the individually matched pairs was close to 1 (Table 3 and Table 4).

Table 4 Skeletal muscle capillarization and mitochondrial content

	N	Patients	Controls	P/C ratio	*P*
Mitochondrial content type 1 fibers	7	18.9 ± 4.3	19.4 ± 5.3	0.97	0.89
Mitochondrial content type 2 fibers	7	13.8 ± 4.1	14.4 ± 4.2	0.96	0.84
Capillary contacts type 1 fibers	8	4.3 ± 1.1	4.9 ± 1.5	0.88	0.43
Capillary contacts type 2 fibers	8	3.3 ± 0.9	3.5 ± 1.0	0.94	0.58
Average total fiber cross-sectional area (mm^2)	8	4527 ± 897	4846 ± 2364	0.93	0.71
Average type 1 fiber cross-sectional area (mm^2)	8	5220 ± 1205	5251 ± 1836	0.99	0.97
Average type 2 fiber cross-sectional area (mm^2)	8	3834 ± 1221	4439 ± 3033	0.86	0.53
Average total fiber perimeter (mm^2)	8	320 ± 57	310 ± 90	1.03	0.76
Average type 1 fiber perimeter (mm^2)	8	350 ± 84	322 ± 77	1.09	0.48
Average type 2 fiber perimeter (mm^2)	8	290 ± 53	297 ± 108	0.97	0.81
Total capillary-fiber perimeter exchange	8	4.9 ± 0.9	5.6 ± 1.1	0.88	0.20
Type 1 capillary-fiber perimeter exchange	8	5.1 ± 1.3	6.4 ± 1.4	0.80	0.14
Type 2 capillary-fiber perimeter exchange	8	4.6 ± 1.0	4.7 ± 1.0	0.98	0.74
Capillary density (capillaries/mm^2)	8	569 ± 91	596 ± 93	0.95	0.58

Mitochondrial content in type 1 and type 2 fibers was measured as the fluorescence intensity of COXIV. Data are presented as mean ± SD. P/C ratio, mean of the ratio of the indicated variable measured in the patient and its matched control.

Table 5 Skeletal muscle eNOS, eNOS ser^{1177}, NOX2, p47phox and p67phox protein content. The content of these proteins was measured as their fluorescence intensity as described in detail in the methods section.

	N	Patients	Controls	P/C ratio	*P*
eNOS protein content	8	86.0 ± 29.5	75.5 ± 21.7	1.14	0.13
eNOS ser^{1177} phosphorylation	8	81.8 ± 14.5	75.9 ± 17.6	1.08	0.16
eNOS ser^{1177}/ eNOS ratio	8	1.14 ± 0.4	1.20 ± 0.4	0.95	0.59
NOX2 protein content (endothelial specific)	7	86.3 ± 28.5	88.0 ± 36.7	0.98	0.72
NOX2 protein content ratio (membrane specific)	7	66.9 ± 22.1	70.6 ± 23.9	0.95	0.22
p47phox	7	118.9 ± 38.3	120.7 ± 45.6	0.99	0.95
p67phox	7	100.9 ± 24.3	112.3 ± 19.4	0.90	0.23

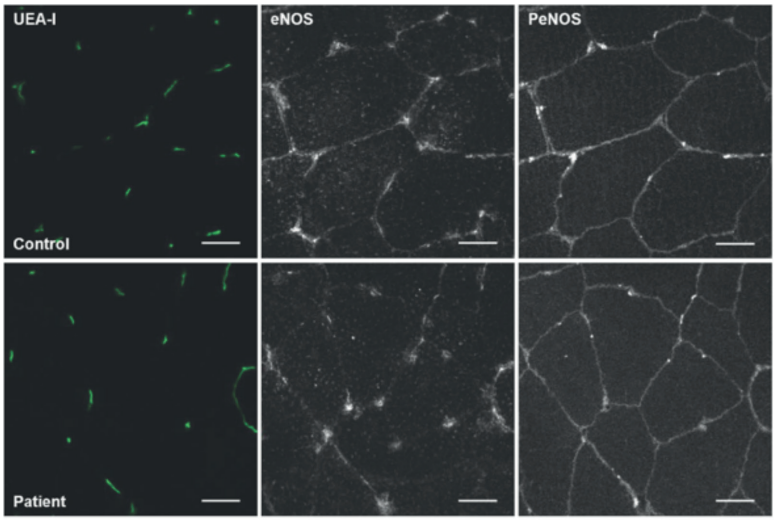

Figure 2 Comparison of the eNOS and eNOS-P-ser^{1177} (eNOS phosphorylated on serine^{1177}) content in skeletal muscle capillaries and arterioles in a former patient and its matched control.

Cross-sectional images of muscle fibers were generated with a confocal immunofluorescence microscope. Capillaries and arterioles were visualized with *Ulex europaeus*-FITC conjugated lectin (UEA-I in green; left panels) creating an endothelial mask for each individual microvessel. eNOS and PeNOS were visualised with specific primary and secondary antibodies on the same section, so that the eNOS/PeNOS fluorescence intensity ratio could be measured in each individual microvessel.

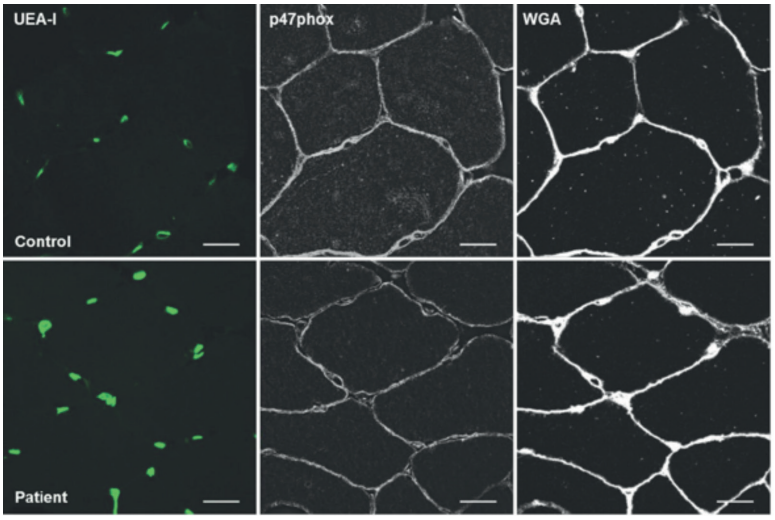

Figure 3 Comparison of the p47phox content (images in the middle) in skeletal muscle capillaries and arterioles (stained with UEA-I in images on the left) and in the plasma membrane of the skeletal muscle fibers (stained with WGA in images on the right) in a former patient and its matched control.

The 3 stains were applied to a single cross-section for each individual. P47phox is present both in the endothelial mask of the capillaries and arterioles and mask of the plasma membrane of the skeletal muscle fibres (both in the former patient and its matched control).

Skeletal muscle eNOS, eNOS-P-ser^{1177}, NOX2, p47phox and p67phox protein content

In 8 paired couples skeletal muscle eNOS and eNOS-P-ser^{1177} protein content and eNOS ser^{1177} phosphorylation (Figure 2), and in 7 paired couples NOX2, p47phox and p67phox protein content were determined specifically in the endothelial layer of muscle capillaries and terminal arterioles (Figure 3). NOX2 was also quantified on the same slides in the plasma layer of the skeletal muscle fibers. No statistically significant differences were detected in the mean protein content of eNOS, eNOS-P-ser^{1177} and the NAD(P)H-oxidase subunits between the patients and controls (Table 5). The range for each of these proteins was large in both groups, but the mean patient/control ratio for each of the quantified proteins in the individually matched pairs was close to 1 (Table 5).

Discussion

In this study, aerobic exercise capacity of 17 patients in long-term remission of CS was compared to aerobic exercise capacity of 17 healthy participants individually matched for gender, age, BMI, smoking behavior, ethnicity and physical activity level. The rationale of matching for these variables was that they are major determinants of VO_{2peak}. The main finding of this study is that the former CS patients have a significantly lower aerobic exercise capacity (VO_{2peak}) than their individually matched control. To investigate whether this lower VO_{2peak} is the result of 1) a reduced mitochondrial content, 2) differences in the structure and density of the muscle capillary network and/or 3) an increased imbalance between the protein content and phosphorylation state of eNOS and of the subunits of the NAD(P)H-oxidase protein complex in the muscle microvasculature, muscle biopsies were collected to make these measurements in 7-8 patient/matched control pairs. As the patient/matched control ratio for each of the measured variables was close to 1.0 (Table 4 and Table 5) this leads to the conclusion that the reduction in VO_{2peak} in the patients must be the result of impairments in the blood supply to the exercising muscle or of a reduction in the efficiency of mitochondrial respiration (lower ATP/O ratio) in the exercising muscle. This conclusion is important as it may explain the persistent complaints of fatigue and lack of energy reported by this patient population [1].

The lower aerobic exercise capacity in patients was independent of gender, age, BMI, and current physical activity level as careful matching for these variables between individual patients and their control was performed. The lower aerobic exercise capacity also did not correlate with CS subtype, age at diagnosis, estrogen status and duration of remission. Aerobic exercise capacity in the CS patients and controls was inversely related to age in accordance with existing literature [13]. The patient group studied was not receiving any

medical treatment at the time of testing, except for thyroid hormone substitution in four patients. None of the patients had other hormonal deficiencies, nor other co-morbidities, like hypertension or impaired glucose tolerance/diabetes. Therefore, the treatment outcome of these specific patients can be considered as 'best case scenario', and any observed difference in outcome is likely to be, at least in part, a persisting effect of pre-exposure to cortisol excess, potentially affecting long-term outcome. Decreased aerobic exercise capacity has also been demonstrated in untreated hypothyroidism. This is (at least partially) reversible when adequately treated [20]. This effect may have negatively influenced VO_{2peak} in the patient group. Indeed, in our patients with treated hypothyroidism VO_{2peak} was lower than in patients without hypothyroidism. However, after excluding patients with treated hypothyroidism, VO_{2peak} in patients in remission of CS remained significantly lower compared to the matched controls (Table 3).

One could argue that an explanation for our findings could be that patients in remission of CS are physically deconditioned during their previous episode of active CS and/or have a more sedentary lifestyle. However, the individual patients were matched for physical activity levels with their controls and they had a similar daily EE, active EE and the same amount of sedentary and active hours as their individual controls. This study does not provide information about the period shortly after surgery and, therefore, prospective studies are needed to determine whether a physical rehabilitation program short-term after surgery may improve long-term aerobic exercise capacity in these patients.

One could also argue that the interpretation of our data is influenced by still improving health status in the patients as the duration of remission is variable. However, there are some facts arguing against this. In one of our previous publications, quality of life was investigated in patients in remission of CS >2 years compared to matched controls [1]. The study used the RAND-36 questionnaire which includes questions regarding changes in general health status. The RAND-36 subdomain "health change" was the only domain that was not different between patients (in remission of CS for > 2 years) and controls. In addition, the patients in the present study have been in remission for > 4 years and did not have comorbidities so it is unlikely that their health status is still improving. Furthermore, in active Cushing's syndrome there is muscle atrophy with a diminished cross-sectional diameter of the muscle fibers [16]. In the current study, muscle fiber cross-sectional area of type I and type II fibers is exactly the same in patients and their individually matched control independent of the length of time that the patients were in remission and no significant correlation between duration of remission and VO_{2peak} was found.

Several mechanisms could explain our finding of the lower aerobic exercise capacity (VO_{2peak}) of the patients in long-term remission of CS. First, it could be the result of a reduced supply of arterial blood and, therefore, of oxygen and blood borne fuels to the skeletal muscle fibers during exercise. With regard to this explanation our group has previously shown that the vasodilator response to acetylcholine, sodium nitroprusside and N^G-monomethyl-L-arginine compared to individually matched controls was normal using venous occlusion plethysmography to measure total blood flow in patients with long-standing remission of CS [21]. This implies that functioning of the larger conductance and resistance vessels in adequately treated patients in remission of CS is comparable to that of healthy controls [21]. However, this does not exclude that impaired exercise-induced vasodilation of the muscle TAs reduces the recruitment of additional capillaries and of additional capillary surface area available for transendothelial transport of oxygen and nutrients into the interstitium of the muscle for uptake and oxidation by the contracting muscle fibers [4].

This study did not show a statistically significant difference in the cross-sectional area of type I and type II fibers, the mitochondrial content of type I and type II fibers and all the capillary measures that were performed in the muscle biopsies of the patients and their individual controls. There was also no difference in the ratio of the protein content of eNOS seen in the patients and their controls and this also applies to the NOX2, $p47^{phox}$ and $p67^{phox}$ protein cluster. This implies that the protein balance between NO production by eNOS and scavenging of NO by superoxide anions generated by NAD(P)H-oxidase in the endothelial layer of TAs and capillaries is not different between the patients and their individually matched controls and that they, with 24 h EE and number of physical activity hours being equal, are receiving the same training stimulus. The underlying assumption, that the protein expression of eNOS increases with training load and that of the subunits of the NAD(P)H-oxidase protein complex decreases with training, is confirmed by previous observations of the authors in exercise training studies in previously sedentary healthy lean men [19] and previously sedentary obese men with and without metabolic syndrome [6]. Exercise training interventions inducing increases in VO_{2peak} of 10-20% led to significant 5-10% increases in eNOS protein content in both studies [6, 19], while the NOX2 protein content remained at the same low expression level in the healthy lean men [19] and was significantly reduced in the obese men by 10% [6]. The absence in the current study of a significant difference in the protein content of eNOS and of the NOX2, $p47^{phox}$ and $p67^{phox}$ protein cluster supports the assumption that the metabolic adaptation of the endothelial layer to an equal physical activity level and 24 h EE was the same in the patients in remission of CS and their individually matched controls. We also matched the patients and their controls for BMI as there is convincing evidence in the literature that the protein expression of $p47^{phox}$ (cytosolic activator of NAD(P)H-oxidase) increases with BMI in vascular endothelial cells obtained from sedentary overweight and obese adults [22].

Although we did not find a lower mitochondrial COXIV content in type I and type II muscle fibers in the patients compared to their controls (Table 4), we cannot exclude that the lower VO_{2peak} is caused by a lower functional capacity (eg the ADP/O ratio) of the mitochondria in the patients. However, the finding of comparable lactate levels immediately after exercise at VO_{2peak} in the patients and their matched controls pleads against this option.

A previous publication from our group provided evidence that the lower leg muscle mass was reduced in patients in long-term remission of CS in comparison to the general population [23]. This theoretically could also explain the reduction in VO_{2peak} that we observe in the patients during incremental exercise. We, however, can exclude that this is the case in the current study as the patients and controls investigated in the present study were matched for the most important factors and conditions that affect VO_{2peak} to include physical activity levels. The observation that the cross-sectional area (CSA) of the type I and type II fibers did not differ between patients and their matched controls also excludes a lower muscle mass in the patients. As explained by previous research [24] a lower muscle mass in sedentary compared to trained men and women (called disuse atrophy) always is the result of muscle fiber atrophy with by far the largest decrease in CSA occurring in type II fibers. Obesity and inflammation leading to skeletal muscle insulin resistance will enhance the severity of disuse atrophy via larger increases in CSA.

In our study we focused on changes at the level of skeletal muscles that might cause a reduced aerobic exercise capacity. In addition, other factors such as cardiac output can also negatively affect aerobic exercise capacity. A lower oxygen pulse was detected in the patients in long-term remission. This finding might be caused by a limitation in exercise cardiac output [25, 26]. There is evidence that, despite long-term remission of CS, these patients have more coronary artery disease [27], subclinical biventricular and left atrium systolic dysfunction [28, 29] and increased left ventricular mass, diastolic dysfunction [29] and increased myocardial fibrosis [30]. It is also known that these structural and functional abnormalities ameliorate already in the first year after remission, but do not fully disappear [28-31]. These persistent abnormalities could in theory reduce cardiac contractility and cardiac output and therefore reduce the supply of oxygen to the active muscles.

In conclusion, this is the first study that demonstrates that patients in long-term remission of CS have a lower aerobic exercise capacity when compared to a well-matched healthy control group. In addition, this study demonstrates that this finding is independent of current daily activity levels. The study is the first to generate evidence that there are no differences between patients and matched controls in the cross-sectional area of muscle fiber types, any of the capillary measures, and mitochondrial content. There were also no significant differences in the protein content of eNOS, eNOS-P-ser^{1177}, NOX2, p47^{phox}

and p67^{phox}. These findings need validation in a prospective study with a larger cohort of patients making multiple measurements over a 6-7 year period. The finding of a decreased oxygen pulse in patients during exercise testing warrants further investigation into cardiac function in this future prospective study. Although CS is a rare disorder, glucocorticoids are frequently used as therapeutic agents in a wide spectrum of diseases. Therefore, our observations are relevant for medicine in general.

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7

Chapter 8

Increased adipocyte size, macrophage infiltration and adverse local adipokine profile in perirenal fat in Cushing's syndrome

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Abstract

Objective: To analyze changes in fat cell size, macrophage infiltration and local adipose tissue adipokine profiles in different fat depots in patients with active Cushing's syndrome.

Methods: Subcutaneous (SC) and perirenal (PR) adipose tissue of 10 patients with Cushing's syndrome was compared to adipose tissue of 10 gender-, age- and BMI-matched controls with regard to adipocyte size determined by digital image analysis on hematoxylin-eosin stainings, macrophage infiltration determined by digital image analysis on CD-68 stainings and adipose tissue leptin and adiponectin levels using fluorescent bead immunoassays and ELISA techniques.

Results: Compared to controls mean adipocyte size was larger in PR adipose tissue in patients. The percentage of macrophage infiltration of the PR adipose tissue and PR adipose tissue lysate leptin levels were higher and adiponectin levels were lower in SC and PR adipose tissue lysates in patients. The adiponectin levels were also lower in the SC adipose tissue supernatants of patients. Associations were found between the severity of hypercortisolism and PR adipocyte size.

Conclusions: Cushing's syndrome is associated with hypertrophy of PR adipocytes and a higher percentage of macrophage infiltration in PR adipose tissue. These changes are associated with an adverse local adipokine profile.

Introduction

One of the main physical features in Cushing's syndrome is a centripetal adipose tissue distribution. Previous findings suggest that centripetal adipose tissue expansion in Cushing's syndrome is predominantly caused by hypertrophy of visceral adipocytes and that this adipocyte hypertrophy is a direct consequence of hypersecretion of cortisol [1,2]. In contrast, adipose tissue expansion in generalized obesity is caused by both adipocyte hypertrophy and hyperplasia, in subcutaneous and visceral adipocytes [3].

Adipocyte hypertrophy has been linked to numerous metabolic alterations, including a disturbed blood lipid profile, independent of total adiposity or body mass index (BMI) [4-7], and insulin resistance via reduced activation of insulin receptor substrate (IRS-1) and expression of glucose transporter 4 (GLUT4) protein [8]. There is evidence that the adverse metabolic alterations caused by adipocyte hypertrophy in obesity are mediated by infiltration of macrophages, resulting in a proinflammatory state with an increase in systemic concentrations of proinflammatory cytokines [9-11].

Increased adipocyte size has also been linked to an adverse secretion profile of adipokines [12]. Adiponectin and leptin in particular are of interest, as they are almost exclusively secreted by adipocytes. Multiple reports have shown negative associations between subcutaneous (SC) and visceral adipocyte size and adiponectin release [12,7,13]. Adipocytes, especially in the SC compartment, secrete more leptin as their size increases [12]. These alterations represent an adverse adipokine profile which is related to insulin resistance, endothelial dysfunction and cardiovascular disease [14].

Little is known about adipocyte size, macrophage infiltration and the secretion of adipokines in different fat depots in active Cushing's syndrome. Only one study showed enlarged abdominal fat cells in women with Cushing's syndrome compared with non-obese women and obese women with the android and gynoid types of fat distribution [1]. We hypothesized that, in active Cushing's syndrome, hypercortisolism leads to increased adipocyte size, a secondary increase in macrophage infiltration due to microhypoxia in the rapidly expanding adipose tissue [15] and a disturbed local adipokine profile, especially in the visceral adipose tissue compartments, as in generalized obesity. Therefore, we investigated adipocyte size, macrophage infiltration and the secretion of adipokines in SC and perirenal (PR) adipose tissue compartments in active Cushing's syndrome as compared to gender-, age- and BMI-matched controls.

Methods

Subjects

Adult (>18 years) patients with active Cushing's syndrome (pituitary-dependent Cushing's syndrome, ectopic ACTH production or a cortisol-producing adrenal adenoma) that were scheduled for unilateral or bilateral adrenalectomy were recruited from the Department of Medicine, Division of Endocrinology, Radboud University Medical Center. Active Cushing's syndrome was confirmed in all patients by means of an increased 24-hour urine excretion of cortisol and a lack of suppression of plasma cortisol to ≤50 nmol/l after 1 mg dexamethasone overnight. Control subjects were recruited from all patients that underwent surgery for kidney donation (8 controls) or bariatric surgery (2 controls, the latter needed in order to match for the 2 patients with Cushing's syndrome with the highest BMI; 44.6 kg/m^2 and 40.9 kg/m^2 respectively) . Control subjects were matched for gender, age (± 2 years) and BMI (±2 kg/m^2) and were included in the study before the surgery was done in order to perform matching before analysis of the adipose tissue samples started The Medical Ethics Committee of our institution approved this study (trial registration number: NL3282809110) and all participants provided written informed consent prior to participation.

Procedures

The medical records of all patients with Cushing's syndrome were reviewed to assess clinical data regarding the duration of Cushing's syndrome related physical complaints before the start of medical treatment, the etiology of Cushing's syndrome, comorbidities, the use and duration of medical treatment for Cushing's syndrome before surgery, efficiency of medical treatment defined as the last 24-hour urine excretion of cortisol before adrenalectomy and the severity of hypercortisolism defined as the mean of all 24-hour urine excretions of cortisol measured before adrenalectomy.

Adipose tissue sampling

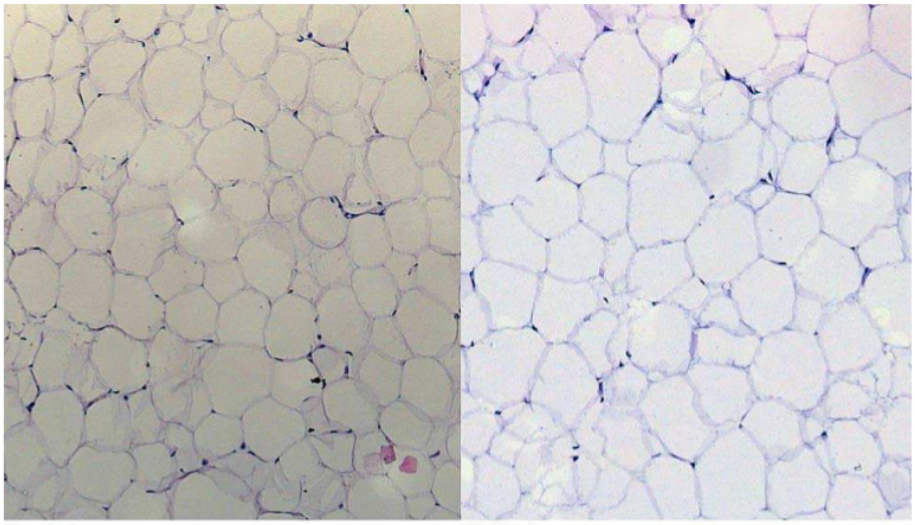
In the patients with active Cushing's syndrome, the PR and SC adipose tissue depots were sampled by the surgeon during adrenalectomy. In the control subjects, the kidney donation procedures (n=8) yielded SC and PR adipose tissue samples and the bariatric surgery procedures (n=2) yielded only SC adipose tissue samples.

Biopsies consisted of approximately 60 mg of adipose tissue. Tissue samples were immediately immersed in 30 ml of transport medium (DMEM Glutamax 5.4g/L, 1% penicillin-streptomycin (p/s) and 1% bovine serum albumin (BSA)) and transported to the laboratory on ice. Adipose tissue was subsequently cleaned by removing visible blood and rinsed with wash buffer (DMEM Glutamax 5.4g/L + 1% p/s + 0,1% BSA). Approximately

20 mg of adipose tissue of each depot was stored in formaldehyde 4% for later histological evaluation. Another 20 mg was snap frozen in liquid nitrogen and stored in -80°C for later determination of adipokine levels. The remaining adipose tissue was immediately cut into very small pieces and incubated for 24h at 37°C in DMEM (Gibco Life technologies, Bleiswijk, the Netherlands) in the presence of 10% fetal calf serum (FCS). After 24 h the supernatant was collected and stored in -20°C upon use. Each tissue sample mass was measured to correct for differences in adipose tissue weight.

Adipose tissue morphometry and macrophage infiltration

Adipocyte size was assessed in a blinded fashion by a single observer with KS-400 digital image analyses. The formaldehyde 4%-fixed samples were embedded in paraffin and 8 µm sections were mounted on glass slides, deparaffinized in xylene and stained with hematoxylin-eosin. Representative examples of this staining are depicted in Figure 1. Adipocyte size of each sample was calculated based on values of at least 500 adipocytes from four to ten microscopic 10x fields, and expressed as mean cross-sectional area per cell (μm^2).



Control Patient

Figure 1. hematoxylin-eosin staining of perirenal adipose tissue sections in a matched couple.

CD68 protein expression was evaluated by immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections. To remove the paraffin, tissues were incubated twice in xylene and successively in 100%, 96% and 70% of alcohol for 5 minutes each step. Antigens were retrieved with citrate buffer for 2 minutes in the microwave (800 W) and 10 minutes at room temperature (Citrate buffer: pH=6.0, 16.4 ml sodium citrate (0.1 M) with

3.6 ml citric acid (0.1M) in 180 ml H_2O). The endogenous peroxidase activity was blocked with 3% of H_2O_2 in methanol for 15 minutes at room temperature. Additionally, any potential endogenous biotin in the samples was blocked in the tissue sections by an avidin/biotin blocking kit according to the manufacturers' protocol (Vector Laboratories, CA, USA). Sections were incubated with 20% goat serum diluted in PBS for 10 minutes and subsequently with the anti-CD68 antibody KP1 (DAKO, 1:8000) diluted in PBS supplemented with 5% goat/rabbit serum, overnight at 4°C. After washing with PBS, sections were incubated with a second HRP-conjugated antibody 1:200 diluted in PBS for 1h at room temperature. The ABC-HRP complex (ABCkit-HRP Vector PK-6101), 1:500 diluted in PBS, was applied to the sections for 30 minutes at room temperature. The substrate solution was added for 7 minutes at room temperature: 0.5 ml of DAB in 9.5 ml of PBS and 10 μl of H_2O_2. Tissues were counterstained with haematoxylin for 30 seconds at room temperature. Slides were dehydrated with consecutive incubation in 70%, 96%, 100% of alcohol and xylene (two times) for 5' each step. Sections were mounted in Permount. Negative controls were used by omitting the primary antibody. Macrophages were counted manually in five microscopic (20x) fields per tissue block, blinded for the analyst. A representative example of this staining is shown in Figure 2. Macrophage count was expressed as a percentage of the total adipocyte number counted per slide.

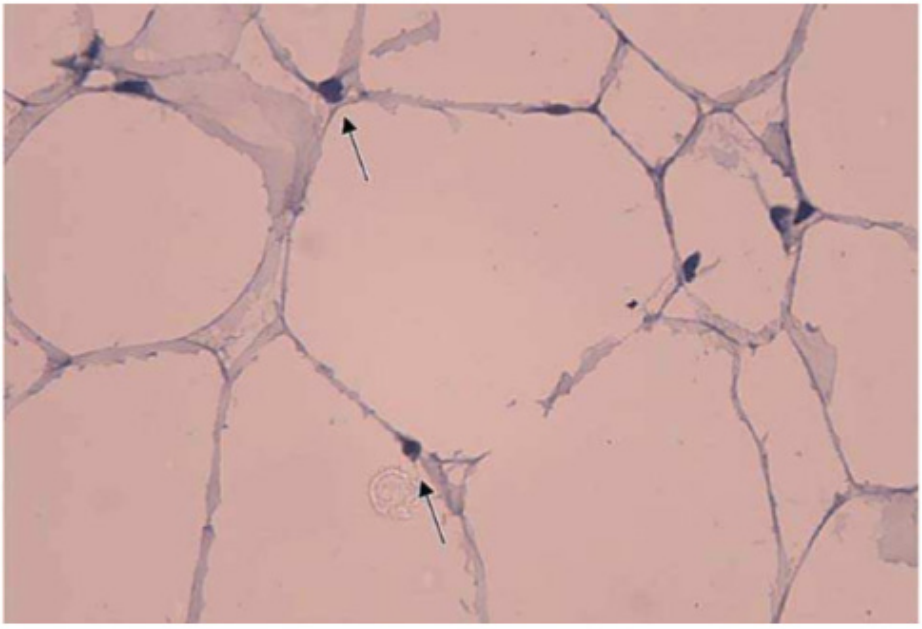


Figure 2. CD68 protein expression on immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections.

Adipokine levels in supernatants and adipose tissue lysates

The snap frozen adipose tissue samples were homogenized. Tissues were weighed and for every 3 mg of tissue, 10µl lysis buffer (Millipore, Billerica, USA), supplemented with cOmplete and PhosSTOP (both Roche diagnostics, Mannheim, DE), was added in order to correct for weight differences between samples. Leptin concentrations in lysates and supernatants were measured by using multiplex fluorescent bead immunoassays (Luminex xMAP technology, Millipore, Billerica, USA), according to the manufacturer's instructions. Total adiponectin concentrations in lysates and supernatants were determined by ELISA, according to the manufacturer's instructions (R&D systems, Minneapolis, USA).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Only the paired adipose tissue samples were analyzed because of the meticulous matching on gender, age and BMI. Comparison between matched patients and controls was performed using a paired t-test. Comparisons of categorical variables were performed using the χ^2-test. Pearson's correlations were performed in order to explore the associations between different parameters. Outcomes were considered statistically significant at a P-value < 0.05. All statistical analyses were performed with SPSS 22.0 statistical analysis package.

Results

Subject characteristics

Table 1 shows the subject characteristics for the patients with Cushing's syndrome and their controls. Adequate matching was confirmed by the observation that no statistically significant differences between patients and controls were observed for gender, age and BMI.

Adipose tissue morphometry and macrophage infiltration

Due to different surgical techniques that were used not all adipose tissue compartments could be sampled in all subjects. The SC compartment was sampled in 10 patients and 10 controls and the PR compartment was sampled in 10 patients and 8 controls, yielding a total of 38 adipose tissue samples. Analyses were only performed when the samples of both the patient and the control were present for that specific adipose tissue compartment.

In the patients with active Cushing's syndrome, mean±SD adipocyte size in the PR tissue depot was significantly higher compared to matched controls (3794±1994 μm^2 versus 1950±881μm^2, $p<0.05$) (Table 2). No difference was observed with respect to adipocyte size in the SC tissue depot between the patients with active Cushing's syndrome and their controls.

In the patients with active Cushing's syndrome, a higher percentage of macrophage infiltration compared to the control group was seen in the PR adipose tissue depot (2.8±2.7% versus 0.5±0.5%, p<0.05). However, no difference was found with respect to macrophage infiltration in the SC tissue compartment in the patients versus controls.

Table 1. Clinical characteristics of patients with active Cushing's syndrome and controls

	Patients (n=10)	Controls (n=10)	*P*-value
Gender (n): male/female	1/9	1/9	1.00
Age: mean (SD) (years)	45.3 (20.5)	44.5 (18.9)	0.93
Age at diagnosis: mean (SD) (years)	43.2 (21.5)	-	-
BMI : mean (SD) (kg/m^2)	29.1 (8.5)	30.5 (8.0)	0.71
Cushing type: n (%)			
Pituitary	5 (50)	-	-
Adrenal	2 (20)	-	-
Ectopic	3 (30)	-	-
Control type: n (%)			
Kidney donation	-	8 (80)	-
Bariatric surgery	-	2 (20)	-
Fat depots sampled: n (%)			
Subcutaneous	10 (100)	10 (100)	-
Perirenal	10 (100)	8 (80)	-
Comorbidities[1]: n (%)			
Hypertension	6 (60)	1 (10)	0.06
Diabetes mellitus	2 (20)	0 (0)	0.47
Hypercholesterolemia	1 (10)	1 (10)	1.00
Medical treatment: n (%)			
Ketoconazole	7 (70)	-	-
Ketoconazole and metopirone	1 (10)	-	-
None	2 (20)	-	-
Duration of medical treatment: mean (SD) (months)	14.9 (8.3)	-	-
Disease duration before treatment: mean (SD) (months)	15.0 (14.9)	-	-
Efficiency of medical treatment[2]: mean (SD) (nmol/24h)	197.7 (219.5)[4]	-	-
Severity of hypercortisolism[3]: mean (SD) (nmol/24h)	402.1 (313.6)[4]	-	-

BMI: body mass index.

[1] Defined as the use of medication to treat these comorbidities.

[2] Defined as the last 24-hour urine excretion of cortisol before adrenalectomy

[3] Defined as the mean of all 24-hour urine excretions of cortisol measured before adrenalectomy

[4] Normal values for 24-hour urine excretion: 30-150 nmol/24h (female), 35-240 nmol/24h (male)

Adipokine levels in adipose tissue supernatants and lysates

In the patients with active Cushing's syndrome, a higher leptin level in PR adipose tissue lysates was detected compared to their controls (14389±8433 pg/ml versus 8413±11114 pg/ml, p<0.05) (Table 2). This difference was not detected in the SC adipose tissue depot lysates or in the supernatants.

Table 2. Paired comparisons of adipose tissue measurements in patients with active Cushing's syndrome and controls

	N	Patients		Controls		*P*-value
		mean	SD	mean	SD	
Adipocyte size SC (μm^2)	10	4819	915	3665	1572	0.102
Adipocyte size PR (μm^2)	8	3794	1994	1950	881	**0.025**
Macrophages SC (%)	10	2.0	2.0	1.4	0.9	0.301
Macrophages PR (%)	8	2.8	2.7	0.5	0.5	**0.031**
Leptin SCS (pg/ml)	10	3307	4672	3913	3184	0.734
Leptin PRS (pg/ml)	8	874	1200	693	846	0.684
Leptin SCL (pg/ml)	10	14210	10979	21556	24787	0.359
Leptin PRL (pg/ml)	8	14389	8433	8413	11114	**0.024**
Adiponectin SCS (pg/ml)	10	14835	10097	27703	10571	**0.020**
Adiponectin PRS (pg/ml)	8	13794	10958	13904	9394	0.965
Adiponectin SCL (pg/ml)	10	26655	10987	54512	31291	**0.005**
Adiponectin PRL (pg/ml)	8	28015	12862	39021	11160	**0.042**

SCS, subcutaneous fat supernatant; PRS, perirenal fat supernatant; SCL, subcutaneous fat lysate; PRL, perirenal fat lysate.
Values in bold signify P <0.05
Note: the male couple was present in all comparisons.

A lower adiponectin level in supernatants was detected in the patients with active Cushing's syndrome in the SC tissue depot compared to their controls (14835±10097 pg/ml versus 27703±10571 pg/ml, p<0.05). No difference was found in the PR depot. A lower adiponectin level was detected in the adipose tissue lysates of the SC and PR tissue depots compared to their controls (26655±10987 pg/ml versus 54512 ±31291 pg/ml and 28015±12862 pg/ml versus 39021±11160 pg/ml respectively, p<0.05 in both depots).

Correlation analysis in patients with active Cushing's syndrome

In the patients with active Cushing's syndrome, associations of PR adipocyte size with PR fat lysate leptin levels (*r* 0.692, *p* 0.026) and with PR fat lysate adiponectin levels (*r* -0.768, *p* 0.009) were found. No such associations were found for the SC compartment. No associations were found between adipocyte size and macrophage infiltration in the patients. An association was found between the severity of hypercortisolism and PR adipocyte size

(*r* 0.730, *p* 0.017). The duration of Cushing's syndrome related physical complaints was not associated with any of the outcome measures.

Discussion

In this study we investigated adipocyte size, macrophage infiltration, and local adipose tissue levels of adipokines in different fat depots in active Cushing's syndrome compared to age-, gender- and BMI-matched control subjects. We found that adipocyte size is larger in the PR adipose tissue compartment during active Cushing's syndrome. There was also a higher percentage of macrophage infiltration in the PR adipose tissue compartment. With regard to the adipokine levels in active Cushing's syndrome we found higher leptin levels in PR adipose tissue lysates, lower adiponectin concentrations in SC supernatants and lower adiponectin levels in SC and PR adipose tissue lysates. These findings are highly relevant because changes in the function of adipose tissue have been associated with the development of cardiovascular and metabolic adverse effects.

This is the first study to show that PR adipocyte size is larger in patients with active Cushing's syndrome. One previous study already described a larger omental adipocyte size during active Cushing's syndrome [1]. We did not find a larger adipocyte size in SC adipose tissue of active Cushing's syndrome. This can possibly be explained by the fact that most effects of glucocorticoids on adipose tissue activity and lipid accumulation in humans are seen on visceral rather than SC fat [16].

Macrophage infiltration into different adipose tissue compartments has not been studied before in active Cushing's syndrome. This study showed a higher percentage of macrophage infiltration in PR adipose tissue in active Cushing's syndrome compared to matched controls. This result is in concordance with the findings in generalized obesity where adipocyte hypertrophy is accompanied by death of adipocytes and macrophage recruitment [17]. It is suggested that, at least in part, this is caused by hypoperfusion leading to an inadequate oxygen supply in the face of expanding adipose tissue [15]. Adipose tissue expansion may occur even more rapidly in Cushing's syndrome compared to common obesity. Infiltrated macrophages interact with adipocytes in a paracrine manner increasing the production of proinflammatory adipokines and reducing the production of anti-inflammatory adiponectin [18] as found in this study. Similar to the other parameters, these differences were not found in SC adipose tissue. The finding of a statistically significant association between the severity of hypercortisolism and PR adipocyte size supports the theory that this finding is a direct consequence of hypercortisolism.

Local adipokine concentrations in different adipose tissue compartments have not been studied before in active Cushing's syndrome. Increased serum leptin levels in obesity and Cushing's syndrome are associated with insulin resistance, atherogenesis and cardiovascular disease [14,19]. In this study, the higher PR leptin levels in active Cushing's syndrome adipose tissue lysates could be explained by the higher adipocyte size in this compartment, which is supported by the statistically significant correlation between adipocyte size and leptin levels in this compartment. Our result is in accordance with previous findings of increased serum leptin levels during active Cushing's syndrome [20,21,19]. In our study no differences in local adipokine concentrations were seen in the SC compartments between patients and controls, which is in accordance with the preferential effect of glucocorticoids on visceral adipose tissue. Furthermore, this finding suggests that the increase in serum leptin levels previously found in Cushing's syndrome in remission is probably caused by increased leptin production by the PR adipose tissue compartment [22].

Serum adiponectin levels are markedly decreased in obesity and are associated with insulin resistance [14]. In this study, we found lower adiponectin levels in SC supernatants and lower adiponectin levels of SC and PR fat lysates in patients with active Cushing's syndrome. As with leptin levels, an association was found with adipocyte size. Adipocyte size was inversely correlated with PR fat lysate adiponectin levels. Multiple studies have investigated serum adiponectin levels in active Cushing's syndrome. However, these studies have reported conflicting data [23-25]. The findings in the current study suggest that the lower serum adiponectin level in ex-patients with Cushing's syndrome as recently described by our group [22] could be the result of decreased adiponectin production in SC and PR adipose tissue.

A strength of the current study is that a broad spectrum of adipose tissue characteristics was investigated in different adipose tissue compartments of patients with active Cushing's syndrome. Furthermore, the meticulous matching between patients and controls makes that our findings are independent of gender, age and BMI and are more likely to be an effect of the hypercortisolism per se.

The main limitation of this study is the small sample size which is caused by the low incidence of Cushing's syndrome on the one hand and the rarity of patients with Cushing's syndrome that require adrenalectomy on the other hand. Therefore it is possible that we have missed relevant differences. Furthermore, the cross-sectional character of this study limits our ability to establish causal relations. A final limitation is that ex-vivo adipokine production in this study does not necessarily correspond to the excretion in-vivo as the tissues are removed from their natural environment.

In conclusion, the central adipose tissue distribution in active Cushing's syndrome appears primarily to be caused by adipocyte hypertrophy. This is accompanied by and associated with an adverse local adipokine profile in these adipose tissue compartments. Furthermore, the number of macrophages in PR adipose tissue in active Cushing's syndrome is increased. These combined findings could contribute to the disturbed blood lipid profile, insulin resistance, endothelial dysfunction and cardiovascular disease that are frequently seen during active Cushing's syndrome. Future research should be performed to further elucidate the role of adipose tissue parameters as assessed in this study, both during active Cushing's syndrome and after remission, as these mechanisms might also play a role in patients on chronic glucocorticoid treatment.

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Chapter 9

Summary and general discussion

This thesis focuses on quality of life (QoL) and long-term physical consequences of endocrine diseases, specifically thyroid carcinoma, Cushing's syndrome (CS) and acromegaly.

Part 1 Long-term health related quality of life after treatment of thyroid carcinoma and acromegaly

Thyroid carcinoma

Cancer patients are at increased risk for distress [1-3]. The Distress Thermometer (DT) and Problem List (PL) are short tools validated and recommended for distress screening in cancer patients [1, 4]. The objective of **chapter 2** was to investigate the level of distress and problems experienced by survivors of differentiated, non-medullary, thyroid carcinoma (DTC), using the DT and PL and whether this correlates with clinical and demographical variables. All 205 DTC patients under follow-up at our institution were asked to fill in the DT and PL, hospital anxiety and depression scale (HADS) and the illness cognition questionnaire (ICQ). Receiver Operator Characteristic analysis (ROC) was used to establish the optimal DT cut-off score according to the HADS. Correlations of questionnaire scores with data on diagnosis, treatment and follow-up collected from medical records were analyzed. Of the 159 respondents, 145 agreed to participate (118 in remission, median follow-up 7.2 years (range 3 months to 41 years)). Of these, 34.3% rated their distress score ≥ 5, indicating clinically relevant distress according to ROC analysis. Patients reported physical (86%) more than emotional problems (76%) as sources of distress. DT scores correlated with HADS scores and ICQ subscales. No significant correlations were found between DT scores and clinical or demographical characteristics except for employment status.

Subjective estimates of distress levels by medical professionals are often inaccurate and misleading [5, 6]. The DT is an easy-to-use instrument that helps identify treatable problems and specific issues that may cause distress, allowing physicians to address these issues more effectively. The use of the DT and PL potentially improves communication between patients and healthcare providers and thereby patient care and satisfaction [7] . In addition, it may also help to use limited consultation time more effectively [8]. **Chapter 2** was the first study evaluating the level of distress using the DT and PL in DTC patients. Surprisingly, the 34.3% of subjects with distress score ≥ 5 was comparable to other studies with similar response rates, including patients with other types of cancer such as lung cancer, breast cancer and leukemia, having a more aggressive course and being associated with more physical burden than patients with DTC [7, 9-12]. In consistence with findings in other types of cancer [7], no significant correlations were found between level of distress and either clinical or demographic characteristics, with the exception of employment status. The role of employment status in impaired quality of life in patients

with chronic conditions including patients with cancer is not clear as literature data are lacking. It has been suggested that unemployment influences distress levels in patients with cardiovascular disease [13]. Although not completely comparable with DTC survivors, this might support the need for reintegration strategies for patients in the work process after they have been cured. In conclusion, the prevalence of distress is high in patients with DTC even after long-term remission. Physical and emotional problems were the main sources of distress. The DT and PL are useful and time efficient screening instruments for psychosocial distress in DTC patients and could easily be incorporated into daily practice. The findings in **chapter 2** highlight the importance of routine psychological distress screening even in a later phase after treatment.

Screening for distress has already been implemented in our clinic as an integral part of the follow-up of patients with thyroid carcinoma and it is currently recommended in the Dutch thyroid carcinoma treatment guidelines (with reference to **chapter 2** of this thesis, www.oncoline.nl/schildkliercarcinoom). Future research in this field should focus on the clinical benefits of prospective and systematic screening for distress in patients with DTC, as well as best intervention strategies to treat this distress.

Shoulder complaints could be one of the physical sources of distress and are frequently reported after surgical treatment for thyroid carcinoma (TC). However, no specific literature on this topic was available for these patients and hence, its impact on QoL was unknown. Therefore, the objective of **chapter 3** was to assess the prevalence of shoulder-related complaints and its relation to QoL and clinical characteristics after TC surgery. The prevalence of shoulder complaints and its relation to clinical characteristics and QoL after TC surgery in 109 patients was compared to 81 healthy controls. Main outcome measures were prevalence of self-reported shoulder complaints, results of the Disabilities of the Arm, Shoulder and Hand questionnaire (DASH) and the European Organization for Research and Treatment of Cancer, Quality of Life Questionnaire-C30 (EORTC QLQ-C30). TC patients, on average 10.2 years after thyroid carcinoma surgery, reported a 58.7% prevalence of shoulder-related complaints, which was significantly more than the 13.6% reported by healthy controls ($P<0.01$). TC patients scored worse than healthy controls on most of the different subscales of the DASH and EORTC QLQ-C30. Bivariate association analysis identified level V neck dissection as being associated with the prevalence of shoulder complaints and DASH score. Furthermore, spinal accessory nerve (SAN) damage and employment status were associated with DASH score. Prevalence of shoulder complaints and DASH scores were significantly correlated to several EORTC-QLQ30 scores. Only 11.9% of TC patients retrospectively reported having received preoperative information on possible shoulder complaints and only 34.9% of TC patients retrospectively reported having

received additional care for their shoulder complaints. As expected, SAN damage during surgery was significantly associated with worse DASH scores.

The results reported in **chapter 3** in TC patients are comparable to the results of studies in patients undergoing surgical treatment for other head and neck cancers. These studies also used questionnaires on self-reported QoL and complaints. One of these studies showed that especially extensive neck dissections are associated with shoulder complaints, which is an important source of long-term morbidity and QoL impairment [14-17]. Dissections extending into level V are known to cause a higher incidence of shoulder complaints [18]. This is consistent with the finding of an association between level V neck dissection and shoulder complaints with worse DASH scores in the bivariate analysis in **chapter 3**. However, we were surprised to find that a large proportion of patients in our series who did not undergo extensive ND reported shoulder-related complaints still many years after the primary treatment. This has led us to consider that, apart from the obvious possible causes of shoulder complaints after head and neck surgery, other factors may play a role as well. One can envisage that, even in the absence of a reported SAN damage, traction, devascularisation, transsection of the fine branches or adhesive capsulitis may contribute to a temporary or a persistent shoulder discomfort. The second unexpected finding was that patients with a lower TNM stage reported more shoulder discomfort. We hypothesize that this may be partially related to the fact that in higher stage, locally extensive disease and not completely removable tumor, one might prefer performing a more limited surgery aimed to preserve functionality and QoL. On the other hand, we cannot exclude that psychological factors might induce reporting bias as well. Patients with extensive disease may experience more burden because of the worse prognosis of the disease and may pay less attention to shoulder discomfort. In contrast, patients with less extensive disease, having a better long-term prognosis, may focus more on optimal QoL and functionality required for instance for work rehabilitation. Regression analysis identified employment status to be significantly associated with shoulder complaints and DASH scores in the TC patients. Because **chapter 3** is a cross-sectional study it is difficult to make causal inferences about the relation between employment status and DASH scores. It is however possible that patients with worse DASH scores are more often unemployed due to their complaints especially as a previous review article found that 46% of patients recovering from head and neck cancer surgery gave up work solely due to shoulder disability [19]. In conclusion, the results of **chapter 3** suggest that the prevalence of self-reported shoulder complaints after TC surgery is high. It represents an important issue in the management of these patients and is associated with a worse QoL.

Patients undergoing TC surgery should be informed about the findings in **chapter 3** preoperatively. Postoperative physical examination of the shoulder is warranted and physiotherapy should be started as soon as possible when complaints are present or can be expected. Future research in this field should be aimed at the prospective assessment of shoulder complaints and analysis of the specific etiology of the shoulder complaints, in order to tailor the treatment and prevention strategies.

Acromegaly

Acromegaly is associated with impaired QoL and causes anatomical disproportions [20], which may contribute to the decreased QoL after successful treatment [21-25]. The Derriford appearance scale 59 (DAS59) is a questionnaire measuring psychological distress and disruptions to everyday life associated with self-consciousness of appearance. The aim of **chapter 4** was to investigate the psychological distress and dysfunction related to self-consciousness about appearance and its effect on QoL in patients in long-term remission of acromegaly. All patients (>18 years old) treated in our hospital for acromegaly in the past were invited to participate. A gender-, age- and body mass index-matched control group was provided by the patients themselves. Participants were asked to complete the modified DAS59- and research and development 36- (RAND-36) questionnaires, the acromegaly quality of life questionnaire (AcroQoL) and a sociodemographic questionnaire. Differences between patient- and control-groups and correlations between questionnaire scores and clinical characteristics were analyzed. Of the 120 respondents, 73 agreed to participate [all cured or under biochemical control, median remission time 10.5 years (range 2.3–43.6 years)]. Of these, 46.6 % reported self-consciousness about their appearance compared to 22.8% of the 57 healthy controls. Twenty-nine of the patients (85.3 %) pointed out their face to be a prominent source of self-consciousness. Significant correlations were found between the scores of the DAS59 and the AcroQoL and RAND-36 questionnaires in patients.

The significant correlations between the DAS59 scores and the AcroQoL and RAND-36 scores found in **chapter 4** indicate that the concerns about appearance are clearly related to an impaired quality of life and general well-being. An impressing 85.3 % of the patients who reported self-consciousness of appearance in our cohort indicated that their face is the main source of discontent. **Chapter 4** therefore clearly demonstrates that patients in long-term remission of acromegaly still have significant problems with their facial appearance. The findings in **chapter 4** are in line with the findings of previous studies that found that the most affected dimension of the AcroQoL was appearance [22, 23]. A positive perception of appearance is related to successful psychological functioning and well-being [26]. Therefore, the self-consciousness about appearance and impaired QoL in patients in long-term remission of acromegaly are serious as these patients are already in

disease remission and these disturbances seem to persist. The concerns about appearance in patients in remission of acromegaly are most likely caused by persisting anatomical alterations as craniofacial disproportions remain present, even after long-term remission [27]. Plastic/craniofacial surgery is not widely performed in (ex) acromegaly patients. However individualized procedures, for example reduction of the vertical dimension of the body of the mandible, rhinoplasty, posterior displacement of the prominent frontal bone, recession and remodeling of cheeks (zygoma) or correction of mandibular prognathism can greatly improve appearance and potentially reduce self-consciousness and associated distress about appearance [28-30]. In conclusion, even after long-term remission of acromegaly, a large number of patients are self-conscious about their appearance, leading to psychological distress and disruptions to everyday life and a decreased QoL.

The findings in **chapter 4** highlight the importance of the fact that physicians should not ignore self-consciousness about appearance in patients treated for acromegaly but have to address these concerns during follow-up. Patients should be encouraged to discuss their concerns about their appearance and should be offered individualized advise about corrective interventions to improve not only their appearance but also functionality. Future research in this field should be directed at prospective assessment of appearance in the follow-up of these patients and efficacy of further treatment options like psychological support or plastic/craniofacial surgery. Furthermore, it would be interesting to know when the reversible changes in facial features of acromegalic patients come to a halt, as this would be the moment most suitable for surgical treatment of remaining deformities.

Part 2 Long-term physical sequelae of Cushing's syndrome

Glucocorticoid receptor (GR) polymorphisms modulate glucocorticoid (GC) sensitivity and are associated with altered metabolic profiles. We [31] and others [32, 33] have shown that some adverse metabolic and cardiovascular characteristics, and changes in body composition persist after treatment of CS. Although these adverse metabolic and cardiovascular characteristics are common in these patients, their incidence and severity vary among patients. This variation seems not to be explained by differences in level of cortisol excess or disease duration alone. Therefore, a variable sensitivity to GC's possibly plays a role in modulating the effect of cortisol excess [34]. Therefore, the aim of **chapter 5** was to evaluate the presence of GR polymorphisms (BclI (rs41423247), N363S (rs56149945), ER22/23EK (rs6189/rs6190), and 9β (rs6198)) and investigate their associations with metabolic alterations in patients in long-term remission of CS. Sixty patients in long-term remission of CS were genotyped. Associations between GR polymorphisms and multiple vascular-, body composition- and metabolic-parameters were

investigated. **Chapter 5** showed that carriers of the 9β polymorphism have a higher systolic blood pressure and lower resistin levels. The GC sensitizing BcII polymorphism is associated with an adverse cardiometabolic risk factor profile: higher fat percentages of extremities and legs, higher serum leptin and E-selectin levels, and higher intima media thickness in carriers versus non-carriers.

In the general population, it has been demonstrated that cardiovascular and metabolic risk and body composition are affected by lifelong overactivation or relative inactivation of GC signaling due to GR polymorphisms. Our observation in **chapter 5** that altered glucocorticoid sensitivity due to GR polymorphisms modulates cardiometabolic risk factors in cured CS patients is in line with these well-known findings in the general population [35-38]. CS patients in remission carrying the minor allele of the 9β polymorphism had a higher systolic blood pressure, which is concordant with previous findings of an increase in carotid atherosclerosis, and a higher incidence of coronary heart disease in carriers of this polymorphism [39]. The minor allele of the BcII polymorphism was associated with an increased fat percentage of the extremities and legs in the remitted CS patients. These body areas only contain subcutaneous adipose tissue. As leptin is preferentially produced in subcutaneous adipose tissue (SAT), carriers also had higher leptin levels [40]. High leptin levels have been widely recognized as an independent cardiovascular risk factor associated with insulin resistance. It also has a pathogenic role in atherothrombosis and endothelial dysfunction. Furthermore, a higher level of E-selectin was found in carriers of the minor allele of the BcII polymorphism. This may reflect increased endothelial activation and progressing atherosclerosis in the BcII carriers. This is supported by the observations in **chapter 5** that the intima media thickness (IMT) is higher in BcII carriers. In conclusion, the study in **chapter 5** is one of the first to suggest that GR polymorphisms modulate cardiometabolic risk factors in patients in long-term remission of CS [41].

In the context of personalized healthcare, the findings in **chapter 5** may implicate that, after treatment of CS, carriers of GR polymorphisms are candidates for a more stringent follow-up regarding cardiovascular and metabolic health. Furthermore, patients who were treated with glucocorticoids for other diseases and even healthy subjects, carrying these polymorphisms, might also be candidates for a more stringent follow-up regarding cardiovascular and metabolic health and the development of the metabolic syndrome, as was recently debated [42]. The results of **chapter 5** need, however, to be interpreted with caution, and further research in larger CS populations is needed to validate these findings. In addition, future studies should delineate to what extent the observed associations also apply to prolonged episodes of exposure to physiologically elevated cortisol levels (e.g., severe stress) or exogenous glucocorticoids as medical treatment.

Patients with CS suffer from endothelial dysfunction and premature atherosclerosis [43-48]. However, it is uncertain to what extent vascular health recovers after long-term remission. This is highly relevant as this topic relates to future development of cardiovascular disease. Therefore, the aim of **chapter 6** was to investigate whether micro- and macrovascular health is impaired after long-term remission of CS, in patients without or adequately treated co-morbidities. Sixty-three patients (with remission of CS for ≥ 4 years) and 63 healthy, well-matched controls were compared. In group A (58 patients and 58 controls) serum biomarkers associated with endothelial dysfunction, IMT, pulse wave velocity and pulse wave analysis were studied. In group B (14 patients and 14 controls) endothelium-dependent and-independent vasodilatation was studied in conduit arteries (flow mediated dilation (FMD) of the brachial artery) and forearm skeletal muscle resistance arteries (vasodilator response to intra-arterial acetylcholine, sodium-nitroprusside and NG-monomethyl-L-arginine using venous occlusion plethysmography). There were no significant differences between the outcome measures of vascular health of patients and controls in group A and B.

Our finding in **chapter 6** that after remission of CS endothelial function is comparable to that in healthy controls, is in line with previous research that investigated arterial endothelial function with FMD [49]. Previous studies have shown that in vitro and in vivo exposure of endothelial cells to glucocorticoids reduced the mRNA and/or protein content of endothelial NO-synthase [50, 51] and reduced acetylcholine-induced vasodilation of resistance arteries [50] and the aorta [52]. Therefore, it can be proposed that endothelial dysfunction in active CS is largely accounted for by the direct effect of hypercortisolism on vascular endothelium and that this is reversible after cure of CS. In contrast, five other studies observed persistent impaired vascular health after remission of CS [32, 53-56]. However, three of these five studies included patients that were in remission for only a short period of time or a pediatric population and are therefore not comparable to the study in **chapter 6.** Two of the five studies can be compared with our study. They both found a higher prevalence of atherosclerosis (measured by carotid IMT and presence of coronary artery disease detected by computed tomography respectively) compared to gender-, age- and BMI-matched controls [32, 56]. However, the patients in these studies had significantly more uncontrolled metabolic comorbidities than their matched controls. In **chapter 6** the comorbidities in group A were adequately treated [31], and the patients in group B had no known comorbidities (except for adequately treated hypothyroidism in 4 patients). Another publication supports our findings [57]. This study measured differences in cIMT and artery stiffness between active disease and one year after remission of CS. After 1 year of remission both variables did not differ from a gender-, age- and BMI-matched control group, but they were still higher than in controls with a lower BMI, matched only for gender and age. We conclude that patients in remission of CS, who are equally well-controlled

for comorbidities as age-, gender- and BMI-matched healthy subjects, have comparable vascular health. Interestingly, the normalized vascular health seems to be irrespective of the fact that these patients have, as we have previously shown, a more centripetal adipose tissue distribution and adverse adipokine profile than their age-, gender- and BMI matched controls [31]. However, the patients in **chapter 6** are relatively young, and vascular problems are more frequent as age increases. So even though we did not find indications for impaired vascular health at approximately 50 years of age, the fact that persistent central adiposity and an adverse adipokine profile are still present after long-term remission of CS may suggest that patients still are at higher vascular risk later in life. In conclusion, vascular health of patients in long-term remission of CS seems to be comparable to that of healthy gender-, age and BMI-matched controls, provided that the patients have no, or adequately controlled comorbidities. Therefore, the effects of the previous hypercortisolism per se on the vasculature may be reversible.

The findings in **chapter 6** underscores the need for stringent individualized treatment of metabolic comorbidities in CS patients, even after remission. We need to be aware of the fact that cardiovascular risk is still elevated even in the healthiest patients in remission of CS because of a persistent effect of the prior hypercortisolism on other organs than the vasculature e.g., the myocardium [58, 59]. This might be a focus of future research.

Although biochemical cure and concomitant improvement of phenotype can be achieved in most patients with CS, a decreased quality of life, mild cognitive impairment and a centripetal fat distribution often persist after biochemical cure [31, 60]. Whether the level of physical fitness is decreased in patients biochemically cured of CS has not been investigated. Therefore, the aim of **chapter 7** was to investigate physical fitness level, as measured by peak oxygen uptake (VO2 peak) during a maximal exercise stress test, and to explore its relation with current daily activity level and skeletal muscle alterations, in patients in long-term (>4 years) remission of CS compared to matched controls. Of all patients treated for CS in our hospital and the Leiden University Medical Center between 1984 and 2009, 17 patients were eligible for the study based on the preset inclusion criteria (long-term (>4 years) remission of CS without hormonal deficiencies, except for hypothyroidism adequately substituted with levothyroxine (4 patients)). For each patient a gender-, estrogen status-, age-, BMI-, smoking-, ethnicity-, and physical activity level- (confirmed by actometer data) matched control subject was recruited from the general population. Physical fitness was assessed in all patients and controls during a maximal exercise stress test on a bicycle ergometer with an incremental exercise protocol. In 8 unselected patients who agreed to the procedure and their matched controls, skeletal muscle tissue of the vastus lateralis muscle was biopsied and measures were made of cross-sectional areas, capillarization, and oxphos complex IV (COXIV) protein content as

indicator of mitochondrial content. Furthermore, protein content of eNOS and eNOS phosphorylated on serine[1177] and of the NAD(P)H-oxidase subunits NOX2, p47phox and p67phox were measured in the vascular endothelial layer. Patients showed a lower mean VO_{2peak} (SD) (28.0 (7.0) vs. 34.8 (7.9) ml O_2/kg bw/min, *P*<0.01), -maximal workload (SD) (176 (49) vs. 212 (67) watt, *P*=0.01) and -oxygen pulse (SD) (12.0 (3.7) vs. 14.8 (4.2) ml/beat, *P*<0.01) at VO_{2peak}. No differences were seen in muscle fiber type specific cross-sectional area, -capillarization measures, -mitochondrial content and -protein content of eNOS, eNOS-P-ser[1177]**,** NOX2, $p47^{phox}$ and $p67^{phox}$.

Our finding in **chapter 7,** that patients in long-term remission of CS demonstrate a lower level of physical fitness (VO2peak) compared to the matched group of healthy controls independent of daily physical activity levels, could have different explanations. The patient group was not receiving any medical treatment at the time of testing, except for thyroid hormone substitution in 4 patients, and none of the patients had further hormonal deficiencies. A subgroup analysis after exclusion of the patients on thyroid hormone substitution did not change our main findings. **Chapter 7** does not provide evidence for the hypothesis that a lower physical fitness level could be caused by a difference in muscle fiber type specific cross-sectional area, -capillarization measures, -mitochondrial content and -protein content of eNOS, eNOS-P-ser[1177], NOX2, $p47^{phox}$ and $p67^{phox}$. We cannot exclude that the lower VO2peak, as found in our study, is caused by a lower functional capacity of mitochondria. The finding of comparable lactate levels immediately after exercise at VO_{2peak} in the patients and their matched controls does not support this option, but patients had a lower work load and possibly an earlier rise in lactate. A lower VO2peak could also be explained by a decrease in recruited muscle mass during exercise [61] or deconditioning of the muscle. A previous publication of our group has provided evidence for the hypothesis that lower extremity muscle mass is decreased in patients in long-term remission of CS as these patients had an almost 3 cm smaller thigh circumference compared to gender-, age- and BMI-matched controls [31]. However, the observation that the cross-sectional area of the type I and type II fibers did not differ between patients and their matched controls in the current study excludes this explanation. With regard to blood supply, **chapter 6** of this thesis showed that functioning of larger conductance- and resistance vessels in patients in remission of CS is comparable to that of healthy controls, making this explanation less likely. There are a number of other factors that can negatively influence physical fitness, such as decreases in pulmonary diffusing capacity and cardiac output. These parameters were not directly investigated in **chapter 7**. However, our patients showed no decreases in oxygen saturation during ergometry testing, which makes a decreased pulmonary diffusing capacity unlikely. With respect to the cardiovascular system, a lower oxygen pulse was detected in the patients in long-term remission. This finding might be caused by a limitation in exercise cardiac output [62, 63], e.g. due to coronary artery disease. There is evidence that, despite

long-term biochemical cure of CS, these patients have more coronary artery disease [56]. However, caution is required in interpreting these results as the oxygen pulse might also be lower due to respiratory constraints and deconditioning. In conclusion, **chapter 7** is the first study that demonstrates that patients in long-term remission of CS have a lower physical fitness level when compared to a well-matched healthy control group. In addition, **chapter 7** shows that this finding is independent of current daily activity levels. **Chapter 7** is the first study to generate evidence that there are no differences between patients and matched controls in the cross-sectional area of muscle fiber types, any of the capillary measures, and mitochondrial content. There were also no significant differences in the protein content of eNOS, eNOS-P-ser^{1177}, NOX2, p47^{phox} and p67^{phox}.

The findings in **chapter 7** could be an explanation for the persisting complaints of fatigue and lack of energy in these patients and confirm that biochemical cure does not lead to complete functional recovery in CS patients. These findings may also have implications for patients on chronic GC therapy. Further research is needed to elucidate the exact cause of the impaired physical fitness and whether a standardized comprehensive physical recovery program could restore physical fitness after long-term remission of CS. The finding of a decreased oxygen pulse in patients during exercise testing warrants further investigation into cardiac function in future research.

As previously mentioned, CS is associated with centripetal obesity. Little is known about the effects of chronic hypercortisolism on adipose tissue morphology and physiology. This is highly relevant because changes in the function of adipose tissue have been associated with the development of cardiovascular and metabolic adverse effects [64-68]. The aim of **chapter 8** was to analyze changes in fat cell size, macrophage infiltration and local adipose tissue adipokine profiles in perirenal and subcutaneous fat depots in patients with active CS. Subcutaneous (SC), and perirenal (PR) adipose tissue of 10 patients with active CS were compared to adipose tissue of 10 gender-, age- and BMI-matched controls with regard to adipocyte size, macrophage infiltration and adipose tissue leptin and adiponectin levels in adipose tissue lysates and conditioned supernatants. In the patients with CS, adipose tissue was collected during adrenal surgery and in the controls during a kidney donation procedure or during bariatric surgery. Compared to controls, adipocyte size was significantly larger in PR adipose tissue in patients with active CS. There was no difference in adipocyte size in SC adipose tissue between patients and controls. The percentage of macrophage infiltration of the PR adipose tissue and PR adipose tissue lysate leptin levels were higher and adiponectin levels were lower in SC and PR adipose tissue lysates in patients with CS compared to controls. The adiponectin levels were also lower in the SC adipose tissue supernatants of patients with CS compared to controls.

Chapter 8 is the first study to show that PR adipocyte size is larger in patients with active CS. One previous study described a larger visceral adipocyte size during active CS [69]. We did not find a larger adipocyte size in SC adipose tissue of active CS. This can possibly be explained by the fact that most effects of GC on adipose tissue activity and lipid accumulation in humans are seen in visceral rather than in SC fat [70]. Macrophage infiltration into different adipose tissue compartments has not been studied before in active CS. **Chapter 8** shows a higher percentage of macrophage infiltration in PR adipose tissue in active CS compared to matched controls. These results are in concordance with the findings in generalized obesity where adipocyte hypertrophy is accompanied by death of adipocytes and macrophage recruitment [71]. It is suggested that, at least in part, this is caused by hypoperfusion leading to an inadequate oxygen supply in the face of expanding adipose tissue [72]. Infiltrated macrophages interact with adipocytes in a paracrine manner increasing the production of proinflammatory adipokines and reducing the production of anti-inflammatory adiponectin [73] as found in **chapter 8**. Similar to the other parameters, these differences were not found in SC adipose tissue. Local adipokine concentrations in different adipose tissue compartments have not been studied before in active CS. Increased serum leptin levels in obesity and CS are associated with insulin resistance, atherogenesis and cardiovascular disease [74, 75]. In **chapter 8**, the higher PR leptin levels in active CS adipose tissue lysates could be explained by the higher adipocyte size in this compartment, which is supported by the statistically significant correlation between adipocyte size and leptin levels. Our results are in accordance with previous findings of increased serum leptin levels during active CS [75-77]. In **chapter 8** no differences in local adipokine concentrations were found in the SC compartments between patients and controls, which is in accordance with the preferential effect of glucocorticoids on visceral adipose tissue. Furthermore, this finding suggests that the increase in serum leptin levels previously found in CS in remission [31] is probably caused by increased leptin production by the PR adipose tissue compartment. Serum adiponectin levels are markedly decreased in obesity and are associated with insulin resistance [74]. In **chapter 8**, we found lower adiponectin levels in SC supernatants and lower adiponectin levels of SC and PR fat lysates in patients with active CS. As with leptin levels, an association was found with adipocyte size. Adipocyte size was inversely correlated with PR fat lysate adiponectin levels. Multiple studies have investigated *serum* adiponectin levels in active CS. However, these studies have reported conflicting data [56, 78, 79]. The findings in **chapter 8** suggest that the lower *serum* adiponectin level in patients with cured CS as recently described by our group [31] could be the result of decreased adiponectin production in SC and PR adipose tissue. In conclusion, the central adipose tissue distribution in active CS appears primarily to be caused by adipocyte hypertrophy. This is accompanied by and associated with an adverse local adipokine profile in these adipose tissue compartments. Furthermore, the number of macrophages in PR adipose tissue in active CS is increased.

Future research should be performed to further elucidate the role of adipose tissue parameters as assessed in **chapter 8**, both during active CS and after remission, as these mechanisms might also play a role in patients on chronic glucocorticoid treatment.

General conclusion and future perspectives

The general conclusion of this thesis is that the consequences of endocrine tumors, specifically thyroid carcinoma, CS and acromegaly sustain after biochemical/oncological cure. This thesis has shown that there is an aftermath of treatment with long-term adverse effects on QoL and long-term physical consequences. These long-term effects deserve the attention of physicians and other healthcare providers and should be incorporated into the information provided to patients, insurance companies, employers etc. The goal of future research in this field should be to improve patient care after "cure". Important for future research in the field of rare endocrine diseases, such as thyroid carcinoma, acromegaly and CS, is to join efforts and to perform more multicenter research in an international context, as this would significantly strengthen and increase the impact of the research. Prospective interventional studies towards improving the recovery of patients with endocrine diseases are also badly needed. The European Reference Network Rare Endocrine Diseases provides a promising opportunity to extend our collaboration internationally and to facilitate dissemination and implementation of the results.

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Chapter 10

Nederlandstalige samenvatting (voor niet-medici)

Het menselijke hormonale systeem bestaat uit meerdere hormoonklieren. Hormonen reguleren een groot aantal functies van ons lichaam. Normaal gesproken hebben deze hormoonklieren een vast formaat en wordt de hoeveelheid hormonen die zij uitscheiden strikt gereguleerd. Soms echter, ontstaat er overmatige of onvoldoende productie van een bepaald hormoon of ontstaat er groei van een hormoonklier door goed- of zelfs kwaadaardige tumorgroei. Dit kan leiden tot ziekte. In dit proefschrift worden een drietal van deze ziekten onderzocht; *schildklierkanker*, *acromegalie* en het *syndroom van Cushing*.

Schildklierkanker is een ziekte van de schildklier welke is gelegen aan de voorzijde van de hals (zie figuur 4 van hoofdstuk 1). Schildklierhormoon is verantwoordelijk voor het reguleren van meerder processen in het lichaam. *Schildklierkanker* kan ontstaan vanuit verschillende celtypen in de schildklier. Het eerste symptoom van *schildklierkanker* is vaak het voelen van een zwelling in de hals/schildklierregio. Soms kan ook een opgezette lymfeklier een eerste teken van de ziekte zijn. De meeste zwellingen in de hals zijn echter goedaardig en slechts 5% van deze zwellingen zijn kanker. Latere symptomen kunnen pijnklachten zijn en soms heesheid door ingroei van de kanker in de zenuwen van de stembanden. Schildklierkanker leidde in 2010 tot 36,000 doden wereldwijd. Behandeling van schildklierkanker bestaat over het algemeen uit het operatief verwijderen van de tumor, gevolgd door een nabehandeling met radioactief jodium.

Acromegalie is een ziekte die meestal wordt veroorzaakt door een verhoogde aanmaak van groeihormoon in de hypofyse. Soms kan de verhoogde aanmaak ook voortkomen uit tumoren elders in het lichaam. De hypofyse is een hormoonklier, ter grootte van een erwt, die onder aan het brein hangt. Door een overschot aan groeihormoon kan reusgroei ontstaan indien iemand nog niet voorbij de puberteit is. Bij volwassenen ontstaat ook groei, echter niet meer in de lengte. Kenmerkende symptomen van *acromegalie* zijn groei van het voorhoofd, neus, kaak, handen en voeten (zie figuur 6 van hoofdstuk 1). Tevens kunnen er klachten ontstaan zoals gewrichtspijnen, dikker worden van de huid, verlaging van de stem, hoofdpijn, snurken en problemen met het zien. Complicaties van de ziekte kunnen verder suikerziekte en een hoge bloeddruk zijn. De ziekte komt het meest voor op middelbare leeftijd. Mannen en vrouwen krijgen de ziekte even vaak. De behandeling van de ziekte bestaat over het algemeen uit medicatie en operatief verwijderen van de tumor.

Het *syndroom van Cushing* wordt veroorzaakt door een verhoogde aanmaak van het hormoon cortisol (stresshormoon) aangestuurd vanuit de eerder genoemde hypofyse of de bijnieren. Vaak wordt het echter ook veroorzaakt door medicamenteus toedienen van cortisol in de vorm van medicijnen zoals prednison. De bijnieren zijn gelegen boven de nieren. Cortisol is van groot belang bij het reguleren van meerdere metabole processen in het lichaam, maar ook bij het functioneren van het immuunsysteem. Symptomen van het *syndroom van*

Cushing bestaan uit een hoge bloeddruk, dikker worden van de buik, striae (huidstriemen), vetophoping in de nek, een rood bol gelaat, spierzwakte en een kwetsbare, slecht genezende huid met veel blauwe plekken (zie figuur 5 van hoofdstuk 1). Bij vrouwen kan er ook een overmatige haargroei en onregelmatige menstruatie optreden. Twee tot drie per miljoen mensen per jaar krijgen deze ziekte. Meest aangedaan zijn mensen tussen de 20 en 50 jaar oud. Vrouwen krijgen de ziekte 3 keer vaker dan mannen. De behandeling van de ziekte bestaat over het algemeen uit medicatie en operatieve verwijdering van de tumor.

Voor alle bovengenoemde ziekten geldt dat we inmiddels goed weten hoe we ze moeten behandelen. Veel van deze patiënten kunnen we genezen, waardoor de overleving bij deze ziekten over het algemeen goed is. Uit wetenschappelijk onderzoek wordt steeds meer duidelijk dat deze ziekten, op de lange termijn na genezing, forse lichamelijke gevolgen kunnen hebben en ook de kwaliteit van leven sterk kunnen beïnvloeden. Daarom was het doel van de onderzoeken in dit proefschrift om te bekijken wat de lange termijn effecten zijn op fysiek- en psychosociaal vlak van de eerder genoemde ziekten, waardoor deze effecten beïnvloed worden en hoe we hier iets aan kunnen doen.

Hoofdstuk 1, de introductie, geeft een overzicht van de eerder beschreven hormoonklieren en de daarbij behorende ziektebeelden. Tevens wordt er een overzicht gegeven van de wetenschappelijke literatuur die reeds bestaat omtrent de lange termijn gevolgen van bovengenoemde ziekten. Dit als opmaat voor de introductie van de studies die in dit proefschrift beschreven staan. In deze introductie worden de redenen gegeven waarom we de verschillende studies hebben uitgevoerd en worden de doelen van de verschillende studies beschreven.

Deel 1 Gezondheid gerelateerde kwaliteit van leven na behandeling voor schildklierkanker en acromegalie

Patiënten die *schildklierkanker* hebben gehad blijken zelfs na jarenlange genezing nog een verminderde kwaliteit van leven te ervaren. Dit wordt onder andere veroorzaakt door “distress” die zij ervaren op emotioneel, psychisch of sociaal vlak zich uitend als gevoelens van kwetsbaarheid, verdriet, angst, depressie, paniek en een sociaal isolement. De mate van “distress” bij patiënten van schildklierkanker was nooit eerder objectief gemeten en er was geen eerder onderzoek beschikbaar naar de oorzaken van deze “distress”. In **hoofdstuk 2** onderzochten wij daarom de mate van “distress” bij deze patiënten door middel van de “distress” thermometer en de “probleemlijst” (een tweetal vragenlijsten). Hieruit bleek dat er bij 34.3% van de 145 deelnemers sprake was van significante “distress” meest veroorzaakt door lichamelijke (86%) en emotionele (76%) problemen. Tevens bleek bij deze patiënten

de kwaliteit van leven sterk verminderd te zijn. Op basis van de verzamelde data is niet duidelijk te voorspellen welke mensen last krijgen van meer "distress". Wel bleek er een duidelijke relatie te bestaan tussen "distress" en werkloosheid. Uit **hoofdstuk 2** blijkt dat de gebruikte vragenlijsten nuttige screeningsinstrumenten zijn om "distress" te detecteren. Het gebruik van de vragenlijsten is een goede eerste stap om het gesprek met de patiënt aan te gaan over de problemen die nog spelen. Dit gesprek is weer een eerste stap naar een eventuele oplossing van het probleem. Gebruik van de vragenlijsten is inmiddels standaard onderdeel van de landelijke *schildklierkanker* zorg. Toekomstig onderzoek moet uitwijzen of dit ook leidt tot voordelen voor de patiënt.

Schildklierkanker wordt vaak operatief behandeld. We weten van hoofd-, halschirurgie bij andere ziekten en van verhalen van *schildklierkanker* patiënten uit de spreekkamer dat er na de ingreep vaak sprake blijft van schouderklachten. Bij deze groep patiënten was echter nooit eerder onderzocht hoe vaak dit precies voorkomt en wat de mogelijke oorzaken hiervan zijn. In **hoofdstuk 3** hebben we dit onderzocht met een set vragenlijsten (DASH, EORTC QLQ-C30). Maar liefst 58.7% van de patiënten die gemiddeld meer dan 10 jaar geleden geopereerd waren aan schildklierkanker rapporteerden schouderklachten in vergelijking met maar 13.6% van de gezonde controlepersonen met vergelijkbare leeftijd en geslacht. **Hoofdstuk 3** liet tevens zien dat er een relatie lijkt te bestaan tussen het verwijderen van de lymfeklieren in een bepaald deel van de hals (regio 5), het beschadigen van een zenuw in dit gebied (nervus accessorius) en schouderklachten. Tevens bleek er een relatie te bestaan tussen schouderklachten en werkloosheid. Ook bleken de schouderklachten een duidelijk verband te hebben met een verminderde kwaliteit van leven. Wij concluderen uit deze bevindingen dat patiënten die deze operaties ondergaan vooraf geïnformeerd dienen te worden over deze mogelijke gevolgen. Na de operatie is het noodzakelijk om naar deze klachten te vragen en om zo nodig de schouder verder te onderzoeken. Indien noodzakelijk dient in een vroeg stadium gestart te worden met fysiotherapie om verdere problemen in de toekomst te voorkomen. Toekomstig onderzoek dient uit te wijzen of deze aanpak inderdaad de klachten verminderd, of er meer duidelijke oorzaken van de klachten geïdentificeerd kunnen worden en wat de hierbij behorende behandelingen dienen te zijn.

Bij patiënten genezen van *acromegalie* is gebleken dat meerdere factoren een negatieve invloed kunnen hebben op de kwaliteit van leven, waaronder de manier van behandelen met medicijnen, blijvende gewrichtsklachten en een groeihormoon gebrek na de behandeling. Tevens weten we uit eerder onderzoek van onze groep dat het uiterlijk van deze patiënten nooit meer wordt zoals het vroeger was. Uit wetenschappelijk onderzoek blijkt dat het uiterlijk een factor is met een sterke invloed op de kwaliteit van leven bij deze patiënten tijdens de ziekte. Er is echter nooit onderzocht of dit nog steeds het geval is geruime tijd na genezing. In **hoofdstuk 4** onderzochten we daarom door middel van vragenlijsten (DAS59,

RAND36, AcroQoL) in welke mate er sprake is van zelfbewustzijn van het uiterlijk en in welke mate dit negatieve invloed heeft op de kwaliteit van leven. Hieruit bleek dat bij 73 patiënten die gemiddeld meer dan 10 jaar genezen waren er bij 46.6% van de patiënten sprake was van een verhoogd zelfbewustzijn van het uiterlijk in vergelijking met 22.8% in de algemene populatie. Van deze mensen gaf 85.3% aan dat het gezicht de bron van het verhoogde zelfbewustzijn was. Tevens werd er een duidelijk verband gevonden tussen het verhoogde zelfbewustzijn van het uiterlijk en een verminderde kwaliteit van leven. Deze bevindingen maken duidelijk dat artsen zich bewust dienen te zijn van dit probleem. Dit probleem dient bespreekbaar gemaakt te worden en er dient met individuele patiënten nagedacht te worden over eventuele behandelingen in de vorm van psychologische begeleiding danwel plastische of reconstructieve chirurgie. Toekomstig onderzoek moet uitwijzen of aandacht voor dit probleem ook daadwerkelijk leidt tot vermindering van de klachten hieromtrent bij patiënten. Tevens dient bekeken te worden wanneer het optimale moment voor chirurgisch corrigeren van het afwijkende gelaat zou zijn, omdat we nog niet precies weten wanneer een patiënt na genezing stopt met vergroeien.

Deel 2 Lichamelijke effecten op lange termijn na genezing van het syndroom van Cushing

De lange termijn effecten op het lichaam van het *syndroom van Cushing* zijn de laatste jaren in toenemende mate een onderwerp van wetenschappelijk onderzoek. Onze groep en anderen hebben aangetoond dat er vele metabole veranderingen en veranderingen van het hartvaatstelsel kunnen blijven bestaan na “genezing” van de ziekte. Tevens blijken deze patiënten, op lange termijn, een dikkere buik te houden en blijkt de uitscheiding van vethormonen (leptine, resistine en adiponectine) door het vetweefsel gestoord te blijven. De mate waarin patiënten klachten houden verschilt echter sterk tussen de verschillende patiënten. We weten dat mensen verschillend kunnen reageren op een verhoogd cortisol gehalte in het bloed, doordat zij verminderd of juist meer gevoelig kunnen zijn voor dit hormoon, door genetische veranderingen van de receptor voor dit hormoon. Nooit eerder was onderzocht of deze genetische veranderingen de lange termijn effecten van het *syndroom van Cushing* beïnvloeden. Daarom hebben we in **hoofdstuk 5** gekeken naar de aanwezigheid van deze genetische veranderingen in cortisol gevoeligheid bij patiënten genezen van het *syndroom van Cushing*. Hieruit bleek dat dragers van de zogenaamde 9β mutatie van de cortisol receptor na genezing een hogere bloeddruk hebben dan de niet-dragers van deze mutatie. Dragerschap van de zogenaamde BclI mutatie bleek geassocieerd te zijn met een verhoogd vetpercentage in de armen en benen. Tevens bleek er bij dragers van deze mutatie sprake te zijn van een verhoogde leptine waarde in het bloed. Dit is een stof die vooral wordt geproduceerd in het onderhuidse vetweefsel en die geassocieerd is

met een verhoogde kans op ziekten van het hart en de bloedvaten en met verminderde gevoeligheid voor insuline en daardoor suikerziekte. Als laatste bleek dragerschap van deze mutatie geassocieerd te zijn met een verhoogde waarde in het bloed van de stof E-selectine. Dit is een stof die verband houdt met de vorming van aderverkalking. Dit blijkt ook uit het feit dat dragerschap van de BclI mutatie in **hoofdstuk 5** samenhing met een verhoogde" intima media dikte" van de bloedvaten. Dit betekend dat er bij deze patiënten meer sprake was van beginnende aderverkalking dan bij niet-dragers van de mutatie. **Hoofdstuk 5** toont dus aan dat de lange termijn effecten van het *syndroom van Cushing* beïnvloed worden door mutaties van de cortisol receptor. Dit betekend dat dragers van deze mutaties mogelijk intensiever gevolgd moeten worden na genezing van het *syndroom van Cushing* met meer aandacht voor hartvaatziekten en suikerziekte. Behalve voor patiënten met het *syndroom van Cushing* kunnen de bevindingen in **hoofdstuk 5** ook van belang zijn voor mensen die langere tijd cortisol in medicamenteuze vorm gebruikt hebben zoals prednison. Toekomstig onderzoek dient gericht te zijn op het reproduceren van onze bevindingen in een grotere groep patiënten. Tevens dient onderzocht te worden in welke mate deze mutaties bijdragen aan de lange termijn effecten van cortisol in medicamenteuze vorm.

In de afgelopen jaren hebben meerdere wetenschappelijke onderzoeken bekeken wat de lange termijn effecten van het *syndroom van Cushing* zijn voor de gezondheid van de bloedvaten. Resultaten van deze onderzoeken waren tegenstrijdig en uitgevoerd met beperkte middelen in kleine groepen patiënten. Daarom hebben wij in **hoofdstuk 6** getracht de gezondheid van de kleine en grote bloedvaten van patiënten genezen van het *syndroom van Cushing* in kaart te brengen. We deden metingen van stoffen in het bloed geassocieerd met vaatschade, deden metingen die een inschatting geven van de gezondheid van de binnenbekleding van de bloedvaten, ofwel het endotheel, en maten of er sprake was van aderverkalking bij deze (ex)patiënten. Uit de resultaten van **hoofdstuk 6** bleek dat er geen verschillen zijn in gezondheid van de bloedvaten tussen patiënten die langer dan 4 jaar genezen zijn van het *syndroom van Cushing* en gezonde controlepersonen die vergelijkbaar waren met de patiënten op het gebied van leeftijd, geslacht en body mass index (BMI). In vergelijking met eerdere studies die wel verschillen lieten zien bleek dat de groep patiënten in **hoofdstuk 6** veel beter behandeld waren voor bijkomende ziekten zoals hoge bloeddruk en suikerziekte. Dit toont aan dat het goed controleren op deze bijkomende ziekten en op tijd behandelen hiervan waarschijnlijk maken dat de gezondheid van de vaten vergelijkbaar blijft met die van gezonde personen. We weten immers dat hoge bloeddruk en suikerziekte de bloedvaten kunnen beschadigen. Deze beschadigingen brengen uiteindelijk een grote kans op bijvoorbeeld hartinfarcten en herseninfarcten met zich mee.

Na genezing van het *syndroom van Cushing* blijft er vaak sprake van een verminderde kwaliteit van leven, welke onder andere wordt veroorzaakt door vermoeidheid en een verminderd vermogen zich in te spannen. Het was echter onbekend of er na genezing van de ziekte sprake is van verminderde fysieke fitheid die deze vermoeidheid kan verklaren. Daarom hebben we dit onderzocht in **hoofdstuk 7** van dit proefschrift. Dit hebben we gedaan door middel van een maximale inspanningstest op een speciaal geprepareerde fiets en metingen van de dagelijkse activiteiten van de patiënten door middel van een bewegingsmonitor. Tevens hebben we gekeken naar de bloedvoorziening en energiehuishouding in de spieren door middel van een biopt uit het bovenbeen. **Hoofdstuk 7** laat zien dat er bij patiënten >4 jaar genezen van het *syndroom van Cushing* nog steeds sprake blijkt te zijn van een verminderde fysieke fitheid en dat dit onafhankelijk is van hun dagelijkse activiteiten niveau. Op het gebied van de spiervezels werden geen verschillen tussen ex-patienten en gezonde personen gevonden die dit verschil in fysieke fitheid kunnen verklaren. Wel werden tijdens de inspanningstest aanwijzingen gevonden voor een verminderde pompfunctie van de hartspier. Het is belangrijk in de toekomst verder onderzoek te verrichten naar dit onderwerp aangezien de oorzaak van de verminderde fitheid uit ons onderzoek nog niet geheel duidelijk is geworden. De bevindingen in **hoofdstuk 7** kunnen wel een verklaring zijn voor de blijvende vermoeidheidsklachten die regelmatig ervaren worden door patiënten na genezing van het *syndroom van Cushing*. Onze bevindingen reiken wellicht verder dan alleen deze patiënten, omdat ditzelfde fenomeen mogelijk ook op zou kunnen treden na langdurig gebruik van geneesmiddelen die cortisol bevatten zoals het eerder genoemde prednison. Tevens zou het interessant zijn om in de toekomst te bekijken of deze klachten te verhelpen zijn middels een revalidatieprogramma.

Zoals eerder genoemd is het *syndroom van Cushing* sterk geassocieerd met een centrale vetverdeling, ofwel een ophoping van vet in de buikregio. De precieze effecten die een overmaat aan bijnierhormoon op lokaal buikvet heeft waren nog niet goed bekend. Daarom hebben we in **hoofdstuk 8** gekeken of de toename in buikvet tijdens het *syndroom van Cushing* vooral wordt veroorzaakt door opzwellen van de reeds aanwezige vetcellen of door een toename van het aantal vetcellen. Tevens hebben we gekeken of deze veranderingen gepaard gingen met een toename van het aantal ontstekingscellen in het vetweefsel en veranderingen in de uitscheiding van een aantal vethormonen; leptine en adiponectine. Dit hebben we gedaan door tijdens verschillende operaties die al gepland stonden voor het verwijderen van een bijnier (dit is een behandeling voor het *syndroom van Cushing)* ook een beetje onderhuids vet en vet uit de buikholte te verwijderen. Dit onderzoek is van belang, omdat onwenselijke veranderingen in deze parameters verband houden met verhoging van het risico op hart- en vaatziekten en suikerziekte. Uit **hoofdstuk 8** bleek dat er bij patiënten met het *syndroom van Cushing* vooral sprake is van opzwellen van de reeds aanwezige vetcellen, dat het aantal ontstekingscellen in het vet inderdaad toeneemt

en dat dit gepaard gaat met een onwenselijke verstoring van de uitscheiding van de eerder genoemde vethormonen. Opvallend hierbij was dat deze veranderingen vrijwel alleen optreden in het vetweefsel uit de buikholte en dat het onderhuidse vet vrijwel niet wordt beïnvloed.

Hoofdstuk 9 van dit proefschrift is een samenvatting van alle bevindingen en een algemene discussie met betrekking tot de gevonden resultaten. Tevens worden er in dit hoofdstuk aanbevelingen gedaan over de richting van het toekomstige onderzoek. De algemene conclusie van dit proefschrift is dat hormonale ziekten vaak niet eindigen na de normalisering van de waarden in het bloed. We hebben aangetoond dat er een lange nasleep kan zijn zowel op het gebied van kwaliteit van leven als op het vlak van lichamelijk klachten/ effecten. Deze lange termijn effecten verdienen de aandacht van de behandeld artsen en andere hulpverleners. Tevens zouden onze bevindingen opgenomen moeten worden in de voorlichting die de patiënt krijgt over zijn of haar ziekte en in de informatie aan bijvoorbeeld werkgevers en verzekeraars. Het algemene doel van toekomstig onderzoek dient het optimaliseren van de zorg na genezing van deze patiënten te zijn.

10

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