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

Title page

Title: Vascular health in patients in remission of Cushing's syndrome is comparable to that in BMI-matched controls.

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Abstract

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 as this topic relates to future development of cardiovascular disease.

Objective: To investigate whether micro- and macrovascular health is impaired after long-term remission of CS, in patients with no or adequately treated co-morbidities.

Design and setting: Cross-sectional case–control study in two tertiary referral centers.

Patients and main outcome measures: 63 patients (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, 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 intra-arterial 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 group A and B.

Conclusion: Vascular health of patients in long-term remission of Cushing's syndrome seems to be comparable to that of healthy gender-, age and BMI matched controls, provided that the patients have no, or adequately controlled co-morbidities. Therefore, the effects of hypercortisolism *per se* on the vasculature may be reversible. This accentuates the need for stringent treatment of metabolic co-morbidities in these patients.

Introduction

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, since 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 if 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 co-morbidity 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 four years, were eligible for inclusion in this multi-center 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 after 1 mg dexamethasone overnight or, if a patient had received radiotherapy of the pituitary gland, a 24-h urinary free cortisol value of <240 nmol/24 h for men or <150 nmol/24 h 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 co-morbidities. Information on the treatment of co-morbidities of the patients can be found in supplemental Table 1.

In our study we investigated 63 patients, divided in 2 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, 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 years), 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 T4 range 8.0-22.0 pmol/l), were excluded. Furthermore, besides the co-morbidities applied for exclusion in Group A, all patients with co-morbidities 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 one week with a SenseWear Pro Armband™ (Body Media, Pittsburg, USA)). 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, Millipore, Billerica, MA, USA) and a Bio-plex microbead analyzer (Luminex, Austin, TX, USA) according to the manufacturer's protocol. Serum concentrations of vascular cell adhesion molecule-1 (VCAM-1) were determined by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

Non-invasive measurements of atherosclerosis and arterial stiffness (group A)

Measurements of carotid intima media thickness (cIMT), pulse wave velocity (PWV) and pulse wave analysis (PWA) were performed according to a highly standardized protocol and performed by the same experienced technician (SH) 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 PWA were measured with applanation tonometry, using SphygmoCor system version 7.1 (Atcor Medical, Sydney. Australia). Central arterial pressure (CAP) and central systolic pressure (CSP) 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 ECG 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 (DT & TS). A 10 MHz multifrequency linear array probe attached to a high-resolution ultrasound machine (T3000; Terason, Burlington, Massachusetts, USA) 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^{o} angle from the torso. A rapid inflation pneumatic cuff (Hokanson, Bellevue, Washington, USA) 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 ($< 60^{o}$). The forearm cuff was inflated to 220mmHg for 5 minutes. Diameter and flow recordings resumed 30 seconds prior to cuff deflation and continued for 5 minutes thereafter. Following a 15 minutes 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 (GTN), which serves as a direct nitric oxide (NO) donor, to detect endothelium-independent vasodilator capacity. This was followed by 5 minutes of continuous recording of brachial artery diameter and blood flow. Post-test 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 following 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 post-deflation 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 <1.0 (29). In such an event, FMD% 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, FMD% 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 measurements using venous occlusion plethysmography (FBF) – measures changes in blood flow (mainly determined by arteriolar resistance arteries in the muscle bed) in response to the infusion of intra-arterial vasoactive medications (25, 31). It therefore mainly assesses microvascular function. FBF was measured at the forearm using ECG-triggered bilateral strain-gauge venous occlusion plethysmography (31). Measurements were performed at 09:00 AM in a quiet, temperature controlled room (22°C). Mercury in silastic strain gauges 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 cuff to 60mmHg. A wrist cuff was inflated to suprasystolic (220mmHg) pressures to exclude the hand circulation from the blood flow during the measurement starting 30 seconds prior to each measurement. A brachial artery catheter (angiocath 20G 1.88in, BD Angiocath) was inserted in the non-dominant arm after local anesthesia (lidocaine 2%), which was elevated slightly above the right atrium. Systolic blood pressure (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. 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, 20mg, Bausch&Lomb; 1-2-4 µg/dL forearm volume/minute) was used to explore endothelium-dependent vasodilatation. SNP (25mg/ml, 2ml, Sigma-Aldrich; 0.2-0.4-0.8 µg/dL forearm volume/minute) was used to explore non-endothelium dependent vasodilatation. Finally, the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA acetate 250 mg, Clinalfa® Basic, Bachem; 0.2-0.4-0.8 µmol/dL forearm volume/minute) 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 100ml 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 6 measurements per drug dose during a measurement period. Besides 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, Chicago, IL). 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 χ^2 test. In group A, stepwise backward multiple linear regression analysis was performed in the patients in order to detect clinical characteristics (etiology of CS, treatment strategies, presence of hormonal deficiencies, use of alcohol, smoking and co-morbidity) 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. $P < 0.05$ 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. Intra-arterial cannulation was not successful in 3 patients and therefore the vasomotor response to intra-arterial 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 only differed from controls with respect to smoking habits (more smokers in the patient group, $P<0.05$).

Biochemical markers associated with endothelial dysfunction (Group A)

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

Non-invasive measurements of atherosclerosis and arterial stiffness (Group A)

cIMT, PWV and CAP were not different between patients and controls (Table 4). A trend towards a statistically significant difference between the two groups was found for the AIx ($P=0.056$). Atherosclerotic plaques were detected in 10 patients and 10 controls. Plaque thickness was not significantly different between patients and controls.

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 L-NMMA (all $p>0.09$) (Figure 1).

Stepwise backward multiple linear regression analysis (Group A)

Having DM predicted both a higher PWV ($p=0.01$) and higher sVCAM-1 levels ($p<0.01$). 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=0.796; sVCAM-1 p=0.865). Being a smoker was a predictor for a higher AIx ($p<0.01$). 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=0.078). Mineralocorticoid replacement was a predictor for higher E-selectin levels ($p<0.01$). Subgroup analysis, after exclusion of all couples with mineralocorticoid users, did not lead to a significant difference between patients and controls (E-selectin; p=0.913). Thyroid hormone replacement was a predictor for higher sVCAM-1 levels ($p<0.01$). Subgroup analysis after exclusion of couples with thyroid hormone users did not lead to a significant difference between patients and controls (sVCAM-1; p=0.504).

Discussion

In this study we investigated micro- and macrovascular health in patients in long-term remission of CS who had no, or adequately treated co-morbidities 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. who investigated arterial endothelial function, with FMD, in a group of 12 patients shortly after remission (>3 months) of CS (22). 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 NO synthase (34, 35) and reduced acetylcholine induced vasodilation of mouse resistance arteries (34) and rat aorta's (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 to 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 presence of coronary artery disease detected by computed tomography, respectively) compared to gender-, age- and BMI matched controls (18, 23). However, the patients in these studies had significantly more uncontrolled metabolic co-morbidities than their matched controls. In our study population the co-morbidities in Group A were adequately treated (16), and the patients in Group B had no known co-morbidities (except for treated hypothyroidism in 4 patients).

A more recent publication of Colao et al. (19) also supports our findings. This study measured differences in cIMT and artery stiffness between active disease and one 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 blood pressure, LDL- and HDL-cholesterol levels were not different between the patients and the BMI-matched control group, but were significantly more adverse in the patients compared to 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 co-morbidities as age-, gender- and BMI matched healthy subjects, have comparable vascular health. This accentuates the need for stringent treatment of metabolic co-morbidities 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 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-levels and smoking predicted a higher AIx but this did not affect the results of the total group. Moreover the trend towards 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 (e.g. etiology of CS, treatment strategies, hormonal deficiencies) negatively affected

311 vascular health parameters. This is in contrast to previous studies, where for example the use of
312 glucocorticoid replacement therapy was associated with an increased cardiovascular risk (10).

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

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

329 A multitude of epidemiological studies reported an increased cardiovascular risk and
330 standardized mortality (SMR) in patients in long-term remission of CS compared to an age and gender
331 but not BMI matched reference population (1). As patients in remission of CS tend to have an overall
332 higher BMI and waist circumference than the general population this may negatively affect
333 cardiovascular risk and SMR. Furthermore these studies did not analyze potential differences between
334 patients with- and without co-morbidities. However it may be possible that cardiovascular risk is still
335 elevated in the healthiest patients in remission of CS because of a persistent effect of the prior
336 hypercortisolism on other organs than the vasculature e.g. 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, vascular health of patients in long-term remission of Cushing's syndrome seems to be comparable to that of healthy gender-, age and BMI-matched controls, provided that the patients have no, or adequately controlled co-morbidities. 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 co-morbidities in these patients.

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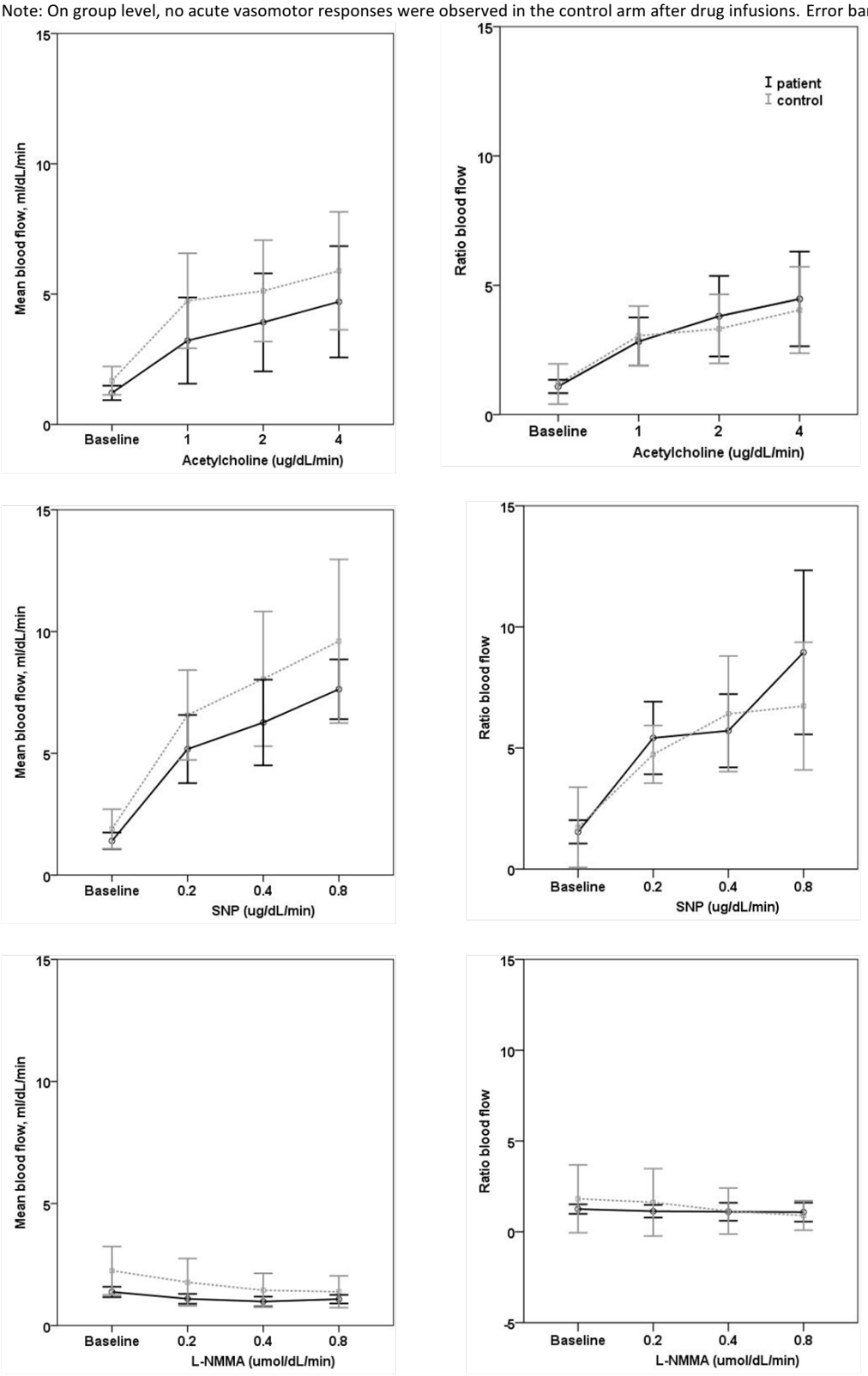
449

Figure legend:

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.



457 Table 1: Group A: Clinical characteristics of patients in long-term remission of Cushing's syndrome
458 and healthy controls

	Patients (n=58)	**Controls (n=58)**	***P*-value**
Gender (n): male/female	12/46	12/46	
Age: mean (± SD) (years)	50.8(12.3)	51.2(12.4)	0.863
BMI: mean (± SD) (kg/m$^{2)}$	26.5(4.2)	26.3(4.1)	0.793
Duration of remission: median (± range) (years)	13.6 ± 8.0		
Smoking (yes/no)	14/44	5/53	0.024*
Pack-years (± SD)	11.5(15.6)	6.9(13.9)	
Alcohol consumption: yes/no	10/48	13/45	0.485
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)	-	-
Growth hormone deficiency	15(25.9)	-	-
Thyroid hormone deficiency	25(43.1)	-	-
Mineralocorticoid deficiency	11(19.0)	-	-
Testosterone deficiency	6/12(50.0)	-	-
Estrogen deficiency [1]	25/46(54.3)	29/46 (63.0)	-
Co-morbidities: 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)	-	-

459
460 BMI: body mass index; CS: Cushing's syndrome.
461 * P<0.05
462 **P<0.01
463 Note[1]: Secondary hypogonadotropic hypogonadism or a postmenopausal state without the use of
464 chronic estrogen replacement.
465

Table 2: Group B (Flow Mediated dilation): Clinical characteristics of patients in long-term remission of Cushing's syndrome and healthy controls

	Patients (n=14)	**Controls (n=14)**	***P*-value**
Gender (n): male/ female	2/12	2/12	1.00
Age at time of test: mean (SD) (years)	46.8 (11.8)	45.7 (10.9)	0.79
Duration of remission: median (range) (years)	12.9 (4.8-29.4)	-	-
BMI: mean (SD) (kg/m^2)	25.6 (2.3)	25.6 (2.5)	0.98
Cushing's syndrome type: n Pituitary Adrenal	 7 7	-	-
Treated hypothyroidism: n	4	-	-
Estrogen status in females: n Sufficient Insufficient	 7 5	 7 5	1.00

BMI: body mass index

Table 3: Group B (venous occlusion plethysmography): Clinical characteristics in long-term remission of Cushing's syndrome and healthy controls

	Patients (n=11)	**Controls (n=11)**	***P*-value**
Gender (n): male/ female	2/9	2/9	1.00
Age at time of test: mean (SD) (years)	45.6 (13.2)	45.8 (12.1)	0.98
Duration of remission: median (range) (years)	12.8 (4.8-28.8)	-	-
BMI: mean (SD) (kg/m^2)	25.7 (1.7)	25.3 (2.7)	0.62
Cushing's syndrome type: n Pituitary Adrenal	 5 6	-	-
Treated hypothyroidism: n	3	-	-
Estrogen status in females: n Sufficient Insufficient	 5 4	 5 4	1.00

BMI: body mass index

Table 4:Micro- and macrovascular health parameters in patients in long-term remission of Cushing's syndrome and matched controls.

Variable	Patients		N	Controls		N	*P*-value
GROUP A	Mean	95%-CI		Mean	95%-CI		
Serum biomarkers							
*ICAM-1 (pg/ml)	280.4	226.7-346.7	57	314.9	234.7-422.4	57	0.545
*PAI (pg/ml)	1810.8	1505.8-2163.1	57	1940.5	1653.9-2276.7	57	0.497
*VCAM-1 (pg/ml)	670.0	615.1-729.9	57	682.4	637.3-730.6	57	0.721
*E-Selectin (pg/ml)	40.0	35.7-44.6	57	38.5	34.6-43.0	57	0.661
Non-invasive measurements of arterial stiffness and atherosclerosis							
CAP (mmHg) (HR75)	10.1	8.8-11.5	52	9.4	7.6-11.3	52	0.457
Aortic AIx (HR75)	26.0	23.2-28.8	53	23.1	19.6-26.6	53	0.056
PWV (m/s)	8.4	8.0-8.9	58	8.3	7.8-8.8	58	0.648
Mean cIMT (mm)	0.75	0.72-0.78	58	0.75	0.72-0.77	58	0.617
Plaque thickness (mm)	2.66	1.94-3.38	10	1.95	1.71-2.18	10	0.092
GROUP B							
Measurements of flow mediated dilation							
Baseline diameter (mm)	3.60	3.33-3.86	14	3.56	3.30-3.82	14	0.839
FMD (%)	5.13	4.10-6.15	14	6.22	4.72-7.72	14	0.125
GTN (%)	18.6	15.5-22.0	14	19.4	15.0-22.9	14	0.691
Time to peak diameter (s)	40.3	33.4-47.3	14	54.3	42.1-66.6	14	0.059
SR_{AUC} (s, 10^3)	30323	25530-35115	14	32164	26471-37857	14	0.597

Note[1] *: For ln-transformed data the geometric means and back-transformed 95%-CI were calculated to enable clinical interpretation of the outcomes.

Note[2]: For plaque thickness the comparison between the groups was performed using an unpaired t-test

ICAM-1, intracellular adhesion molecule 1; PAI-1, plasminogen activator inhibitor 1; VCAM-1, vascular cell adhesion molecule 1; CAP, central augmented pressure; AIx, augmentation index; cIMT, carotid intima media thickness; PWV, pulse wave velocity; HR75, corrected for a heart rate of 75 beats per minute. FMD, flow mediated dilation; GTN, glyceryltrinitrate; SR_{AUC}, shear rate area under the curve; CI, confidence interval

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).