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

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31 **Abstract**

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

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

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

39 **Patients and main outcome measures:** 63 patients (remission of CS for ≥ 4 years) and 63 healthy,
40 well-matched controls were compared. In group A (58 patients and 58 controls) serum biomarkers
41 associated with endothelial dysfunction, intima media thickness, pulse wave velocity and pulse wave
42 analysis were studied. In group B (14 patients and 14 controls) endothelium-dependent and
43 -independent vasodilatation was studied in conduit arteries (flow mediated dilation of the brachial
44 artery) and forearm skeletal muscle resistance arteries (vasodilator response to intra-arterial
45 acetylcholine, sodium-nitroprusside and N^G -monomethyl-L-arginine using venous occlusion
46 plethysmography).

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

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

54

55 **Introduction**

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

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

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

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

82 **Subjects and methods**

83 *Subjects*

84 All adult patients of Radboud University Medical Center Nijmegen and Leiden University
85 Medical Center, who had been successfully treated for CS (caused by either an ACTH-producing
86 pituitary adenoma or a benign adrenal adenoma) and were in remission for at least four years, were
87 eligible for inclusion in this multi-center cross-sectional matched case-control study. Remission was
88 defined as absence of clinical signs and symptoms of hypercortisolism and suppression of plasma
89 cortisol to ≤ 50 nmol/l after 1 mg dexamethasone overnight or, if a patient had received radiotherapy of
90 the pituitary gland, a 24-h urinary free cortisol value of < 240 nmol/24 h for men or < 150 nmol/24 h for
91 women. The medical records of all patients were retrospectively reviewed to assess clinical data
92 regarding the etiology of CS, the type of treatments that patients had received, duration of remission,
93 presence of hormonal deficiencies and co-morbidities. Information on the treatment of co-morbidities
94 of the patients can be found in supplemental Table 1.

95 In our study we investigated 63 patients, divided in 2 different patient groups. Group A
96 comprised 58 patients, and group B 14 patients. Nine patients were included in both groups.

97 Group A was the same group of patients that we previously described in our study on body
98 composition, extensive information about the patient selection can be found in that article (16). In
99 short: the following exclusion criteria were applied: untreated (or inadequately treated) hormonal
100 deficiencies, active malignancy or systemic therapy for malignancy in the past, severe inflammatory
101 diseases and psychiatric pathology. Each patient was matched to a control subject with the same
102 gender, age (± 2 years), and body mass index (BMI, ± 2 kg/m²). Control subjects, recruited via
103 advertisements in a local newspaper, had to be healthy and without current use of medication.

104 For the second group of patients (group B, n=14), even stricter exclusion criteria were used:
105 All subjects with hormonal deficiencies, except for adequately treated hypothyroidism (free T4 range
106 8.0-22.0 pmol/l), were excluded. Furthermore, besides the co-morbidities applied for exclusion in
107 Group A, all patients with co-morbidities that are known to affect vascular function or who used
108 medication that may interfere with the cardiovascular system were excluded. In addition to gender, age
109 and BMI, the healthy control subjects were also matched for smoking, ethnicity, and physical activity

110 levels (estimated via metabolic equivalent of task scores and measured for one week with a SenseWear
111 Pro **Armband**[™] (Body Media, Pittsburg, USA)). Female controls were matched for estrogen status and
112 oral contraceptive use.

113 The Medical Ethics Committees of our institutions approved this study and all participants
114 provided written informed consent prior to participation.

115

116

117 ***Methods***

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

121 *Biochemical markers associated with endothelial dysfunction (group A)*

122 Serum concentrations of plasminogen activator inhibitor-1 (PAI-1), intracellular adhesion
123 molecule-1 (ICAM-1) and soluble E-selectin were measured by Multiplex Fluorescent Bead
124 Immunoassays (xMAP technology, Millipore, Billerica, MA, USA) and a Bio-plex microbead
125 analyzer (Luminex, Austin, TX, USA) according to the manufacturer's protocol. Serum concentrations
126 of vascular cell adhesion molecule-1 (VCAM-1) were determined by an enzyme-linked
127 immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

128

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

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

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

144

145 *Endothelial function (group B)*

146 Brachial artery flow mediated dilation (FMD) is widely accepted to reflect endothelium-
147 dependent and largely nitric oxide-mediated function of conduit arteries (26). Measurements were
148 performed by two experienced vascular sonographers (DT & TS). A 10 MHz multifrequency linear
149 array probe attached to a high-resolution ultrasound machine (T3000; Terason, Burlington,
150 Massachusetts, USA) was used for imaging of the brachial artery in the distal third of the upper arm.
151 Subjects rested in a supine position for at least 15 minutes to enable baseline assessment of arterial
152 diameter and blood flow. The arm was extended and positioned at an 80° angle from the torso. A rapid
153 inflation pneumatic cuff (Hokanson, Bellevue, Washington, USA) was positioned on the forearm
154 immediately distal to the olecranon to provide the forearm ischemic stimulus. After obtaining an
155 optimal image, the probe was manually stabilized and the ultrasound parameters were set to optimize
156 longitudinal B-mode imaging of the lumen-arterial wall interface. Continuous Doppler velocity was
157 measured using the lowest possible insonation angle (< 60°). The forearm cuff was inflated to
158 220mmHg for 5 minutes. Diameter and flow recordings resumed 30 seconds prior to cuff deflation and
159 continued for 5 minutes thereafter. Following a 15 minutes resting period, a 1-minute baseline
160 recording of the brachial artery diameter and flow was taken. Subsequently, brachial artery
161 endothelium-independent vasodilatation was examined after administration of a single spray of
162 sublingual glyceryl trinitrate (GTN), which serves as a direct nitric oxide (NO) donor, to detect
163 endothelium-independent vasodilator capacity. This was followed by 5 minutes of continuous
164 recording of brachial artery diameter and blood flow. Post-test analysis of brachial artery diameter was

165 performed using customized edge-detection and wall tracking software (27). Baseline diameter, flow
166 and shear rate were calculated as the mean of data acquired across the 1-minute preceding the cuff
167 inflation period. Peak diameter following cuff deflation was automatically detected as previously
168 described (28). FMD was calculated as the percentage rise of this peak diameter from the preceding
169 baseline diameter. The time to peak diameter (seconds) was calculated from the point of cuff deflation
170 to the maximum post-deflation diameter. According to a recent study, inadequate scaling for FMD
171 would be present if the upper confidence limit of the regression of the relation between logarithmically
172 transformed base diameter and peak diameter is <1.0 (29). In such an event, FMD% is not an
173 appropriate measure for the estimation of endothelial function. Data were checked for this
174 phenomenon and subsequently allometric modeling was applied (29). Furthermore, FMD% was
175 corrected for shear rate stimulus by adding this factor as a covariate in our analysis (30). The CV for
176 measuring FMD with our protocol is 6.7% (30).

177 Forearm blood flow measurements using venous occlusion plethysmography (FBF) –
178 measures changes in blood flow (mainly determined by arteriolar resistance arteries in the muscle bed)
179 in response to the infusion of intra-arterial vasoactive medications (25, 31). It therefore mainly
180 assesses microvascular function. FBF was measured at the forearm using ECG-triggered bilateral
181 strain-gauge venous occlusion plethysmography (31). Measurements were performed at 09:00 AM in
182 a quiet, temperature controlled room (22°C). Mercury in silastic strain gauges placed around the
183 widest portion of the upper third of both forearms were electrically coupled to a plethysmograph
184 calibrated to measure normalized changes in volume. For each measurement, venous flow was
185 occluded just proximal to the elbow by rapidly inflating a blood pressure cuff to 60mmHg. A wrist
186 cuff was inflated to suprasystolic (220mmHg) pressures to exclude the hand circulation from the blood
187 flow during the measurement starting 30 seconds prior to each measurement. A brachial artery catheter
188 (angiocath 20G 1.88in, BD Angiocath) was inserted in the non-dominant arm after local anesthesia
189 (lidocaine 2%), which was elevated slightly above the right atrium. Systolic blood pressure (BP),
190 diastolic BP, mean arterial BP and heart rate were monitored continuously. The other arm was used as
191 a control for systemic changes in vasomotor tone. To establish resting FBF, we administered 0.9%
192 saline for 30 minutes. Vasoactive agent infusions were then started. Between each series of drug

193 infusions, FBF was allowed to return to basal value during a 20 minute resting period, during which
194 solvent (0.9% saline for acetylcholine (Ach) and 5% glucose for sodium-nitroprusside (SNP)) was
195 infused to maintain a constant infusion rate. Ach (Miochol-E intraocular solution, 20mg,
196 Bausch&Lomb; 1-2-4 µg/dL forearm volume/minute) was used to explore endothelium-dependent
197 vasodilatation. SNP (25mg/ml, 2ml, Sigma-Aldrich; 0.2-0.4-0.8 µg/dL forearm volume/minute) was
198 used to explore non-endothelium dependent vasodilatation. Finally, the nitric oxide synthase inhibitor
199 N^G-monomethyl-L-arginine (L-NMMA acetate 250 mg, Clinalfa® Basic, Bachem; 0.2-0.4-0.8
200 µmol/dL forearm volume/minute) was infused to investigate the contribution of nitric oxide to basal
201 vascular tone. Each substance-dose was infused for 5 minutes. FBF values are reported in milliliters
202 per minute per 100ml of forearm volume. The baseline value is a mean of all measurements during the
203 baseline measurement period. The values during drug infusion are a mean value of the last 6
204 measurements per drug dose during a measurement period. Besides changes in blood flow, the blood
205 flow ratio between the infusion and control arm was also calculated to correct for possible systemic
206 effects(32). The CV of FBF has been reported to be 8-10% during stimulation (31, 33).

207

208 **Statistical methods**

209 Data were analyzed using SPSS 20.0 statistical package for Windows (SPSS Inc, Chicago,
210 IL). Data were expressed as mean ± SD, unless mentioned otherwise. Data distributions were analyzed
211 and logarithmic transformation was performed before statistical testing when appropriate. Differences
212 between patients and controls were tested with paired t-tests. Differences in categorical variables were
213 analyzed using the χ^2 test. In group A, stepwise backward multiple linear regression analysis was
214 performed in the patients in order to detect clinical characteristics (etiology of CS, treatment strategies,
215 presence of hormonal deficiencies, use of alcohol, smoking and co-morbidity) that are predictors of
216 vascular function. A stepwise backward multiple linear regression analysis could not reliably be
217 performed in group B because of the small sample size. P < 0.05 was considered statistically
218 significant.

219 **Results**

220 *Subject characteristics*

221 Table 1 shows the clinical characteristics of the patients and control subjects for group A, and
222 Table 2 for group B. Intra-arterial cannulation was not successful in 3 patients and therefore the
223 vasomotor response to intra-arterial drug infusions was investigated in 11 patients and controls (Table
224 3). Adequate matching was reflected by the fact that no differences between patients and controls were
225 present in both groups in gender, age and BMI. In group A patients only differed from controls with
226 respect to smoking habits (more smokers in the patient group, $P < 0.05$).

227

228 *Biochemical markers associated with endothelial dysfunction (Group A)*

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

231

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

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

237

238 *Endothelial function (Group B)*

239 No statistically significant differences were found between patients and controls in all FMD
240 measurements (Table 4). Furthermore, no statistically significant differences were found between
241 patients and controls regarding FBF or blood flow ratio responses at baseline or in response to the
242 incremental doses of Ach, SNP and L-NMMA (all $p > 0.09$) (Figure 1).

243

244 *Stepwise backward multiple linear regression analysis (Group A)*

245 Having DM predicted both a higher PWV ($p = 0.01$) and higher sVCAM-1 levels ($p < 0.01$).
246 Subgroup analysis was performed for these two outcomes after exclusion of all matched patient-

247 control couples containing a patient with DM. This did not lead to significant differences between
248 patients and controls (PWV $p=0.796$; sVCAM-1 $p=0.865$). Being a smoker was a predictor for a
249 higher AIx ($p<0.01$). Subgroup analysis, after exclusion of all patient-control couples with a smoker,
250 did not lead to a significant difference between patients and controls (AIx; $p=0.078$).

251 Mineralocorticoid replacement was a predictor for higher E-selectin levels ($p<0.01$). Subgroup
252 analysis, after exclusion of all couples with mineralocorticoid users, did not lead to a significant
253 difference between patients and controls (E-selectin; $p=0.913$). Thyroid hormone replacement was a
254 predictor for higher sVCAM-1 levels ($p<0.01$). Subgroup analysis after exclusion of couples with
255 thyroid hormone users did not lead to a significant difference between patients and controls (sVCAM-
256 1; $p=0.504$).

257 **Discussion**

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

265 Our findings that endothelial function recovers after remission of CS are in line with the study
266 of Akaza et al. who investigated arterial endothelial function, with FMD, in a group of 12 patients
267 shortly after remission (>3 months) of CS (22). They found that the impaired FMD in active CS was
268 reversible after remission. Previous studies have shown that in vitro (cell culture) and in vivo (mouse)
269 exposure of endothelial cells to glucocorticoids reduced the mRNA and/or protein content of
270 endothelial NO synthase (34, 35) and reduced acetylcholine induced vasodilation of mouse resistance
271 arteries (34) and rat aorta's (36). Therefore Akaza et al. [22] proposed that endothelial dysfunction in
272 active CS is largely accounted for by the direct effect of hypercortisolism on vascular endothelium and
273 that this is reversible after treatment.

274 On the other hand, five other studies observed persistent impaired vascular health after
275 remission of CS (17, 18, 20, 21, 23). However, in three of these studies there was either a short period
276 of remission (17) or a pediatric study population (20, 21), so these studies are not comparable to our
277 study. The studies reported by Colao et al. (18) and Barahona et al. (23) are more comparable. They
278 both found a higher prevalence of atherosclerosis (measured by cIMT and presence of coronary artery
279 disease detected by computed tomography, respectively) compared to gender-, age- and BMI matched
280 controls (18, 23). However, the patients in these studies had significantly more uncontrolled metabolic
281 co-morbidities than their matched controls. In our study population the co-morbidities in Group A
282 were adequately treated (16), and the patients in Group B had no known co-morbidities (except for
283 treated hypothyroidism in 4 patients).

284 A more recent publication of Colao et al. (19) also supports our findings. This study measured
285 differences in cIMT and artery stiffness between active disease and one year after remission of CS in
286 25 patients. There was a significant decrease in both variables between active disease and remission.
287 After 1 year of remission both variables did not differ from a gender-, age- and BMI-matched control
288 group as used in our study, but they were still higher than in controls with a lower BMI, matched only
289 for gender and age. Moreover, diastolic blood pressure, LDL- and HDL-cholesterol levels were not
290 different between the patients and the BMI-matched control group, but were significantly more
291 adverse in the patients compared to the controls with a lower BMI. This emphasizes the importance of
292 strict matching of each patient to a healthy individual of at least the same gender, age and BMI if one
293 wants to investigate the effect of the previous period of hypercortisolism *per se*.
294 Taken both our results and the previous findings into account, we conclude that patients in remission
295 of CS, who are equally well-controlled for co-morbidities as age-, gender- and BMI matched healthy
296 subjects, have **comparable** vascular health. This accentuates the need for stringent treatment of
297 metabolic co-morbidities in these patients. Interestingly, the normalized vascular health seems to be
298 irrespective of the fact that these patients have, as we have previously shown, a more centripetal
299 adipose tissue distribution and adverse adipokine profile than their age-, gender- and BMI matched
300 controls (16). **However, the patients in our study are relatively young, and vascular problems are more**
301 **frequent as age increases. So even though we did not find indications for impaired vascular health at**
302 **approximately 50 years of age, the fact that persistent central adiposity and an adverse adipokine**
303 **profile are still present after long term remission of CS may mean patients still are at higher vascular**
304 **risk later in life.**

305 As could be expected in group A, DM was associated with a higher PWV and higher sVCAM-
306 levels and smoking predicted a higher AIX but this did not affect the results of the total group.
307 Moreover the trend towards a higher AIX in the patient group disappeared after correcting for
308 smoking. Interestingly, except for an association between mineralocorticoid replacement and E-
309 selectin levels and the use of thyroid hormone replacement and VCAM-1 levels, no other patient
310 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

337 was not supported by a small study (39). Therefore further research is necessary to investigate these
338 issues.

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

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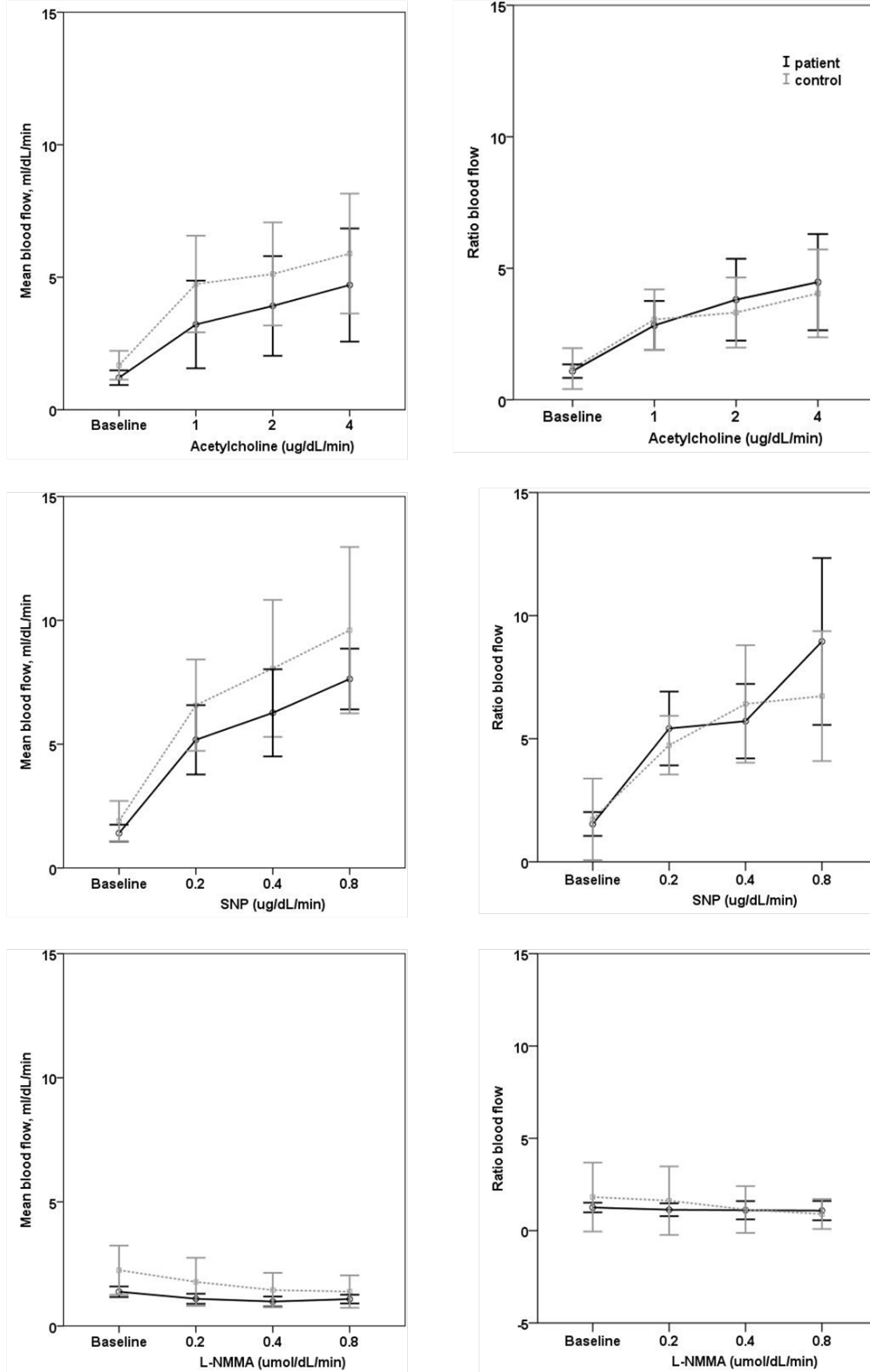
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449

450 **Figure legend:**

451 Figure 1: Change in forearm blood flow from baseline in response to infusion of different vasoactive
452 agents in increasing dosages.

453 Note: On group level, no acute vasomotor responses were observed in the control arm after drug infusions. Error bars: 95% CI.
454



455
456

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 (\pm SD) (years)	50.8(12.3)	51.2(12.4)	0.863
BMI: mean (\pm SD) (kg/m ²)	26.5(4.2)	26.3(4.1)	0.793
Duration of remission: median (\pm range) (years)	13.6 \pm 8.0		
Smoking (yes/no)	14/44	5/53	0.024*
Pack-years (\pm 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 Note¹: Secondary hypogonadotropic hypogonadism or a postmenopausal state without the use of

464 chronic estrogen replacement.

465

466 Table 2: Group B (Flow Mediated dilation): Clinical characteristics of patients in long-term remission
 467 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	7		
Adrenal	7		
Treated hypothyroidism: n	4	-	-
Estrogen status in females: n			
Sufficient	7	7	1.00
Insufficient	5	5	

468 BMI: body mass index

469

470 Table 3: Group B (venous occlusion plethysmography): Clinical characteristics in long-term remission
 471 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	5		
Adrenal	6		
Treated hypothyroidism: n	3	-	-
Estrogen status in females: n			1.00
Sufficient	5	5	
Insufficient	4	4	

472 BMI: body mass index

473

474 Table 4: Micro- and macrovascular health parameters in patients in long-term remission of Cushing's
 475 syndrome and matched controls.
 476
 477

Variable	Patients		N	Controls		N	P-value
	Mean	95%-CI		Mean	95%-CI		
GROUP A							
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

478 Note¹ *: For ln-transformed data the geometric means and back-transformed 95%-CI were calculated
 479 to enable clinical interpretation of the outcomes.

480 Note²: For plaque thickness the comparison between the groups was performed using an unpaired t-
 481 test

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

487

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 (μ mol/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

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

493 Note 1: In our hospitals patients continue to visit our outpatient clinic at least once a year after remission of Cushing's
494 syndrome (CS). During that visit patients are screened for the presence of hypertension, diabetes mellitus and
495 hypercholesterolemia. If needed treatment is initiated. (If they already had hypertension, diabetes mellitus or
496 hypercholesterolemia during the active phase of CS, we try to taper medication and if possible to stop medication to see if
497 it is still needed).The choice of which medication is used was dependent on the preferences of the individual physicians and
498 patients, but usually metformin was the first choice for diabetes mellitus type 2, simvastatin was the first choice for
499 hypercholesterolemia and a thiazide diuretic or an ace-inhibitor were the first choice for hypertension. The effect of
500 treatment was monitored regularly (each 3-6 months) and treatment was adjusted till treatment goals (a blood pressure of
501 < 140/90 mmHg, a HbA1c < 53 mmol/mol and a LDL-cholesterol of < 3.5 mmol/l) were reached.

502 Note 2: In case a patient had CS of pituitary origin biochemical evaluation is carried out on the fourth day postoperatively to
503 evaluate the function of the pituitary gland (after glucocorticoid substitution had been stopped for at least 24 hours), by
504 measurement of fasting (08:00 h) plasma cortisol, ACTH, thyrotropin, free thyroxine, gonadotropins, testosterone or
505 estradiol and insulin-like growth factor type-1. If basal plasma cortisol is lower than 200 nmol/l substitution therapy with
506 hydrocortisone, 30 mg a day, was prescribed. Patients were re-evaluated every 2–4 weeks during the first 3 months after TS
507 and thereafter at 2–3 months intervals during the first year. The fasting plasma cortisol concentration was measured at
508 each visit. If a patient received glucocorticoid substitution therapy postoperatively, the dose was reduced and stopped, if
509 possible, between 3 and 12 months after TS. Thereafter the integrity of the hypothalamic–pituitary–adrenal axis was
510 assessed by an insulin tolerance test. Growth hormone deficiency is tested with a growth hormone stimulation test. If a
511 hormonal deficiency is present substitution is initiated to reach reference values.

512 Note 3: Hypertension is defined as a blood pressure \geq 140/90 mmHg. Diabetes mellitus is defined as a HbA1c \geq 6.5% (\geq 48
513 mmol/mol), a fasting glucose of \geq 7.0 mmol/L (126 mg/dL) or a non fasting glucose \geq 11.1 mmol/l (199 mg/dl).
514 Hypercholesterolemia is defined as a LDL cholesterol of > 3.5 mmol/l or non-HDL cholesterol of > 4.0 mmol/L (in case no
515 other co-morbidities are present; otherwise we use stricter criteria).