Decreased Aerobic Exercise Capacity After Long-Term Remission From Cushing Syndrome: Exploration of Mechanisms

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Background: Although major improvements are achieved after cure of Cushing 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 more than 4 years in comparison with matched controls, and to investigate whether the reduction in exercise capacity is related to alterations in muscle tissue.

Methods: Seventeen patients were included. A control individual, matched for sex, estrogen status, age, body mass index, smoking, ethnicity, and physical activity level was recruited for each patient. Maximal aerobic capacity (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 an indicator of mitochondrial content. Furthermore, protein content of endothelial nitric oxide synthase (eNOS) and eNOS phosphorylated on serine^{1177} and of the NAD(P)H-oxidase subunits NOX2, $p47^{phox}$, and $p67^{phox}$ 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, $P < .01$), maximal workload (SD) (176 [49] vs 212 [67] watt, $P = .01$), and oxygen pulse (SD) (12.0 [3.7] vs 14.8 [4.2] ml/beat, $P < .01$) 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 $p67^{phox}$.

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Abbreviations: BMI, body mass index; CS, Cushing syndrome; EE, energy expenditure; TAs, terminal arterioles; METS, metabolic equivalent of task scores; RER, respiratory exchange ratio; VE, minute ventilation; VO_{2peak}, maximal aerobic capacity.

Interpretation: Because 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. **(*J Clin Endocrinol Metab* 105: 1–11, 2020)**

Key Words: Cushing syndrome, long-term remission, VO_{2peak}, muscle capillarization, muscle mitochondrial content

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 patients, independent of the origin of CS, report subjective feelings of fatigue and limitations in their ability to perform exercise (1-3).

The present 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 to 28 years. The second aim was to investigate whether the patients for a given habitual physical activity level (including exercise activities) have a lower maximal aerobic capacity (VO_{2peak}) than healthy matched controls. The third aim was to investigate whether the mechanisms limiting VO_{2peak} in 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, sex, body mass index (BMI), smoking, physical activity level, and ethnicity. These conditions and characteristics were selected because they are known to affect VO_{2peak} independently 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, for example, a lower physical activity level or a higher BMI.

To investigate the potential mechanism(s) that led to a reduction in VO_{2peak} in patients compared to their controls, we measured in percutaneous muscle biopsies 1) mitochondrial content, 2) several measures of capillary density and structure, and 3) the cross-sectional area (CSA) of type 1 and type 2 fibers as measures 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 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 endothelial nitric oxide synthase (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, whereas 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 present study to test the hypothesis that the eNOS/NAD(P)H-oxidase protein ratio is lower in patients than in matched controls and therefore may limit exercise-induced vasodilation of TAs and recruitment of additional capillaries during exercise.

Participants and Methods

Participants

In this cross-sectional, matched, case-control study, patients who were successfully treated for CS 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, duration of postoperative glucocorticoid treatment, and follow-up data on remission, recurrences, and hormonal deficiencies. Adult patients (age > 18 years) in long-term (> 4 years) remission from CS were eligible for this study. Remission was defined as suppression of plasma cortisol to 50 nmol/L or less after 1 mg dexamethasone overnight and absence of clinical signs and symptoms of active hypercortisolism, documented no longer than 1 year before inclusion (7). Individuals 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 patients had been tested after their last pituitary surgery for growth hormone (GH) deficiency by means of an insulin tolerance test, because GH deficiency is known to have a strong influence on $VO_{2\ peak}$ (8). GH deficiency was defined as a maximal GH response less than 15.3 mU/L during an insulin tolerance test (9). Serious comorbidity (ie, 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 control matched for sex, age, BMI, smoking (yes/no), ethnicity, and physical activity level 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). Participants 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) after inclusion to ensure 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) using a progressive, incremental exercise protocol. All participants refrained from alcohol, caffeine, and intensive physical exercise for at least 24 hours before testing. All tests were performed in laboratory conditions with consistent temperature (18°C-20°C) and humidity (35%). All tests started at the same time of day (9:00 AM). Participants were instructed to cycle at 60 to 80 rotations per minute to volitional fatigue or until they reached symptom-limited exhaustion. Spiroergometric equipment (Oxycon Alpha, Jaeger) was used to continuously measure breath-by-breath minute ventilation (V_E), respiratory rate, oxygen consumption (VO2), 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 milliliters 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 (beats per minute) (11). Values were obtained from expired air as 30-second 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) was measured before and 2 minutes after the test. On cessation of exercise, participants reported their rating of perceived exertion using a 0 to 10 Borg scale (12). VO_{2peak} was deemed to have been reached and the test data were included in the analysis when 3 of the following 4 criteria were met: 1) clinical signs of full exhaustion including Borg scale score of 8 or greater, 2) RER of 1.10 or greater at cessation, 3) maximal heart rate within 10 beats of the maximum predicted heart rate (220 − age), and 4) flattening of the VO_2 uptake curve (≤ 150 mL increase during the last minute of exercise) (13).

Physical activity levels

Average daily 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) were assessed using an activity monitor (Sensewear Pro3) around the upper right arm. The activity monitor measured physical activity 24 hours 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 the vastus lateralis muscle using the percutaneous needle biopsy technique under local anesthesia (1% lidocaine) as previously described (15). The vastus lateralis muscle was chosen because it is easy to access by percutaneous biopsy and the fact that this muscle makes a significant contribution to the workload 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) and frozen in liquid nitrogen–cooled isopentane (Sigma-Aldrich). 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 COXIV (Invitrogen) and myosin heavy chain type I (A4.840-c, DSHB, developed by Dr Blau), followed by incubation with appropriate secondary antibodies (Alexa Fluor goat antimouse immunoglobulin G_{2a} 488 and Alexa Fluor goat antimouse immunoglobulin M 546, respectively) and a wheat germ agglutinin (WGA) Alexa Fluor 350 conjugate (to visualize the cell border) (Invitrogen).

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 antimouse immunoglobulin M 546 secondary antibody in combination with Ulex Europaeus-fluorescein isothiocyanate (FITC) conjugate

(UEA-I-FITC; Sigma-Aldrich) and the WGA-350 conjugate. A Leica DMI600B microscope with a 40×/0.6 NA objective, coupled to a Leica DFC365 FX CCD microscope camera (Leica Microsystems), was used to obtain digital images of cross-sectionally orientated muscle. DAPI (4′,6-diamidino-2-phenylindole) UV (340-380 nm) and FITC (465-495 nm) excitation filters both 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). A total of 50 ± 11 fibers per muscle cross-section were analyzed. Fluorescence staining intensity was used to indicate differences between patients and their matched controls 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 was used to estimate skeletal muscle eNOS, eNOS-P- ser^{1177}, NOX2, $p47^{phox}$, and $p67^{phox}$ 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 so the eNOS-P-ser^{1177}/eNOS ratio was calculated for individual vessels. Methods to assess endothelial-specific and membrane-specific NOX2 content have also been described previously (18, 19). Assessment of endothelial-specific $p47^{phox}$ and $p67^{phox}$ apart from using different primary antibodies uses 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) and p-eNOS ser^{1177} (Cell Signaling Technology). For assessment of NOX2 $p47^{phox}$ and $p67^{phox}$ content, sections were double-stained with antibodies against NOX2, $p47^{phox}$, or $p67^{phox}$ (all kind gifts from Prof Mark Quinn, Montana State University). All sections were then incubated with appropriate secondary antibodies (Invitrogen) in combination with the endothelial marker UEA-I-FITC (Sigma-Aldrich). A plasma membrane marker, WGA-633 (Invitrogen), was also included when staining samples for NOX2.

Images were acquired using an inverted confocal microscope (Zeiss LSM-710, Carl Zeiss) with a 40× NA oil immersion objective. Alexa Fluor 405 was excited using the 405-nm line of the diode laser and detected with 371 to 422 nm emission. FITC fluorescence was excited with a 488-nm line of the argon laser and detected with 493 to 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 to 623 nm and 638 to 747 nm emission, respectively. Identical settings were used for all image captures within each participant.

All image analysis was performed using ImagePro Plus 5.1 (Media Cybernetics Inc). 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. Because eNOS and eNOS ser^{1177} phosphorylation had been stained on the same sections, it was possible to establish each eNOS ser^{1177}/eNOS ratio on an individual-vessel basis because 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). Data are expressed as mean and SDs unless stated otherwise. Before analysis, data were 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 the Spearman correlation coefficient. The level for significance was set at α equal to .05 or less.

Results

Baseline characteristics

Seventeen patients in long-term remission from CS, and 17 healthy controls matched for sex, 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

The mean VO_{2peak} of patients in long-term remission from CS was significantly lower compared to matched controls ($P < .01$) (Fig. 1). A significantly lower maximal workload ($P = .01$) and shorter test duration ($P = .02$) was observed in patients in long-term remission from CS compared to the control group. V_E was significantly lower in patients in long-term remission from CS ($P = .02$). This lower V_E consisted of a lower respiratory rate ($P = .047$) with comparable tidal volumes in the patients and their controls. Furthermore, oxygen pulse was lower in the patients compared to their controls ($P = .01$). The peak heart rate, RER, and posttest blood lactate concentrations were not statistically different between the groups (Table 2) but occurred at a lower absolute workload ($P = .01$) in the former CS patients.

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).

Table 1. Characteristics of patients and controls

	Patients (n = 17)	Controls (n = 17)	*P*
Sex: female/male, no.	15/2	15/2	1.00
Age at time of test, y	45.7 ± 11.1	45.2 ± 10.1	.89
Age at diagnosis, y	34.0 ± 10.2	–	–
Duration of remission: median (range), y	11.3 (4–28)	–	–
Height, cm	171.6 ± 6.3	174.1 ± 6.3	.25
Weight, kg	73.9 ± 8.1	76.1 ± 9.8	.50
BMI, kg/m^2	25.1 ± 2.4	25.1 ± 2.6	.95
Systolic BP, mm Hg	126 ± 13	130 ± 12	.32
Diastolic BP, mm Hg	81 ± 7	82 ± 2	.70
Cushing syndrome type, No. (%)		–	–
Pituitary	10 (58.8)		
Adrenal	7 (41.2)		
Duration of postoperative glucocorticoid treatment, d		–	–
Pituitary	371 (203)		
Adrenal	397 (298)		
Treated hypothyroidism, No. (%)	4 (23.5)	–	–
Estrogen status in women, No. (%)			
Sufficient (premenopausal)	10 (66.7)	10 (66.7)	1.00
Insufficient (postmenopausal)	5 (33.3)	5 (33.3)	
Smoking, No. (%)			
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.
Abbreviations: BMI, body mass index; BP, blood pressure.

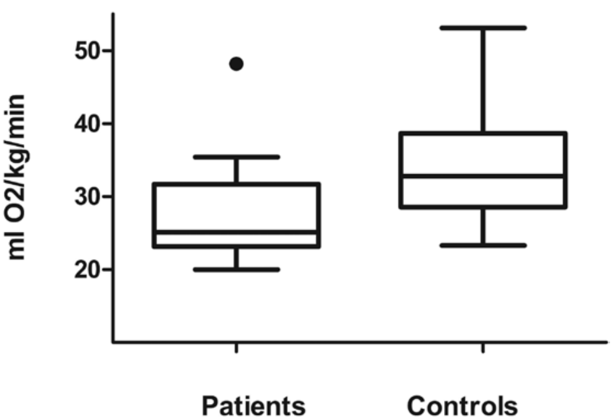

Figure 1. VO_{2peak} of patients in long-term remission of Cushing syndrome compared to matched controls. Tukey boxplot with whiskers indicating 1.5 interquartile range of the lower and upper quartile and black dot indicating an outlier.

Correlations between aerobic exercise capacity and clinical characteristics

In the patient group, older age (r = –0.62, P < .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 < .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 = .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 < .01)) (Table 3).

Skeletal muscle capillarization and mitochondrial content

Thirteen matched pairs provided informed consent to undergo skeletal muscle biopsy. Owing to technical problems (frost damage) and/or a small biopsy sample size in 1 of the 2 members of a matched pair, mitochondrial content and skeletal muscle capillarization could not be determined in 5 matched pairs. No differences were found between the patients and their matched controls with regard to muscle total fiber CSA, 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 (Tables 3 and 4).

Skeletal muscle eNOS, eNOS-P-ser^{1177}, NOX2, p47^{phox}, and p67^{phox} protein content

In 8 matched pairs skeletal muscle eNOS and eNOS-P-ser^{1177} protein content and eNOS ser^{1177} phosphorylation (Fig. 2), and in 7 matched pairs NOX2, p47^{phox}, and p67^{phox} protein content were determined specifically in the endothelial layer of muscle capillaries and terminal arterioles (Fig. 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

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

	Patients	Controls	P/C ratio	*P*
VO_{2peak}: (mL O_2/kg/min)	28.0 ± 7.0	34.8 ± 7.9	0.80	<0.01[b]
HR, bpm	174 ± 16	180 ± 13	0.97	0.22
Workload, watt	176 ± 49	212 ± 67	0.83	0.01[a]
V_E, L/min	89.4 ± 27.3	101.0 ± 19.8	0.89	0.02[a]
Respiratory rate, b/min	38 ± 8	42 ± 6	0.90	<0.05[a]
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, min	12.0 ± 2.4	14.3 ± 3.8	0.84	0.02[a]
VO_2/HR, mL/beat	12.0 ± 3.7	14.8 ± 4.2	0.83	0.01[a]
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 h, < 1.5 METS)	10.7 ± 1.7	10.4 ± 1.6	1.03	0.54
Daily active h, > 3 METS)	4.1 ± 1.6	4.9 ± 1.8	0.84	0.12

Data are presented as mean ± SD.
Abbreviations: EE, energy expenditure; cal, calories; HR, heart rate; METS, metabolic equivalent of task scores; P/C ratio, mean of the ratio of the indicated variable measured in the patient and its matched control; RER, respiratory exchange ratio; V_E, minute ventilation; VO_{2peak}, maximal aerobic capacity.
[a]*P* less than .05.
[b]*P* less than .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	< .01[b]
HR, bpm	173 ± 18	178 ± 15	0.98	.43
Workload, watt	158 ± 33	192 ± 35	0.82	.01[a]
V_E, L/min	80.8 ± 19.4	95.1 ± 15.9	0.85	.02[a]
Respiratory rate, b/min	37 ± 8	42 ± 6	0.88	.06
RER	1.23 ± 0.08	1.17 ± 0.08	1.05	.10
Lactate, mmol/L	9.1 ± 2.6	10.3 ± 2.6	0.88	.24
Test duration, min	11.5 ± 2.2	14.0 ± 3.9	0.82	.03[a]
VO_2/HR, mL/beat	10.6 ± 1.8	13.9 ± 3.4	0.76	< .01[b]
Daily EE, cal	2288 ± 308	2374 ± 257	0.96	.24
Daily active EE, cal	408 ± 247	459 ± 250	0.89	.52
Daily sedentary h (< 1.5 METS)	11.1 ± 1.3	10.7 ± 1.4	1.04	.53
Daily active h (> 3 METS)	3.8 ± 1.4	4.6 ± 1.9	0.83	.17

Data are presented as mean ± SD.
Abbreviations: EE, energy expenditure; cal, calories; HR, heart rate; METS, metabolic equivalent of task scores; P/C ratio, mean of the ratio of the indicated variable measured in the patient and its matched control; RER, respiratory exchange ratio; V_E, minute ventilation; VO_{2peak}, maximal aerobic capacity.
[a]*P* less than .05.
[b]*P* less than .01.

the NAD(P)H-oxidase subunits between 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, the aerobic exercise capacity of 17 patients in long-term remission from CS was compared to the aerobic exercise capacity of 17 healthy participants individually matched for sex, 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 controls. 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 or 8 patient/matched

Table 4. Skeletal muscle capillarization and mitochondrial content

	No.	Patients	Controls	P/C ratio	*P*
Mitochondrial content type 1 fibers	7	18.9 ± 4.3	19.4 ± 5.3	0.97	.89
Mitochondrial content type 2 fibers	7	13.8 ± 4.1	14.4 ± 4.2	0.96	.84
Capillary contacts type 1 fibers	8	4.3 ± 1.1	4.9 ± 1.5	0.88	.43
Capillary contacts type 2 fibers	8	3.3 ± 0.9	3.5 ± 1.0	0.94	.58
Average total fiber cross-sectional area, mm^2	8	4527 ± 897	4846 ± 2364	0.93	.71
Average type 1 fiber cross-sectional area, mm^2	8	5220 ± 1205	5251 ± 1836	0.99	.97
Average type 2 fiber cross-sectional area, mm^2	8	3834 ± 1221	4439 ± 3033	0.86	.53
Average total fiber perimeter, mm^2	8	320 ± 57	310 ± 90	1.03	.76
Average type 1 fiber perimeter, mm^2	8	350 ± 84	322 ± 77	1.09	.48
Average type 2 fiber perimeter, mm^2	8	290 ± 53	297 ± 108	0.97	.81
Total capillary-fiber perimeter exchange	8	4.9 ± 0.9	5.6 ± 1.1	0.88	.20
Type 1 capillary-fiber perimeter exchange	8	5.1 ± 1.3	6.4 ± 1.4	0.80	.14
Type 2 capillary-fiber perimeter exchange	8	4.6 ± 1.0	4.7 ± 1.0	0.98	.74
Capillary density, capillaries/mm^2	8	569 ± 91	596 ± 93	0.95	.58

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

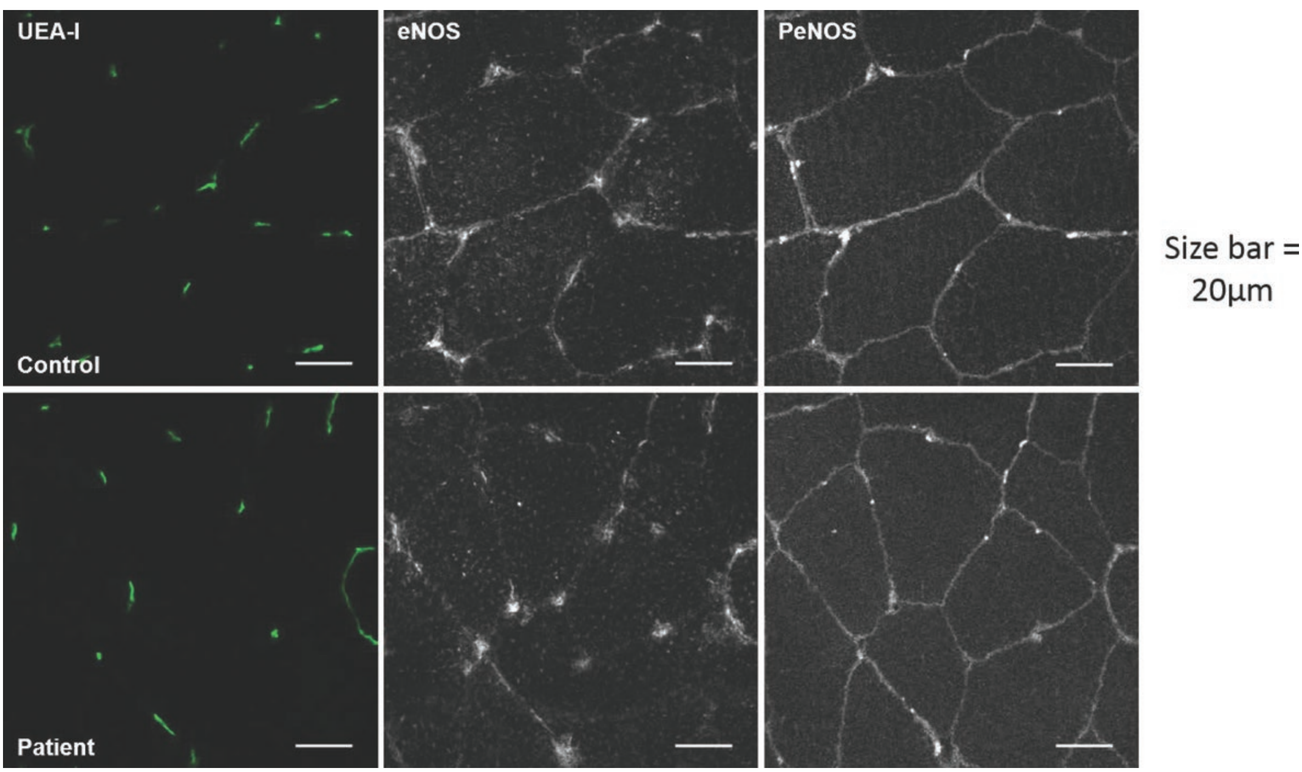

Figure 2. Comparison of the eNOS and eNOS-P-ser[1177] (eNOS phosphorylated on serine[1177]) content in skeletal muscle capillaries and arterioles in a former patient and the matched control. Cross-sectional images of muscle fibers were generated with a confocal immunofluorescence microscope. Capillaries 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 visualized with specific primary and secondary antibodies on the same section so the eNOS/PeNOS fluorescence intensity ratio could be measured in each individual microvessel.

control pairs. Because the patient/matched control ratio for each of the measured variables was close to 1.0 (Tables 4 and 5), this leads to the conclusion that the reduction in VO_{2peak} in the patients must be the result of impairment in the blood supply to the exercising muscle or of a reduction in the efficiency of mitochondrial respiration (lower adenosine triphosphate/oxygen ratio) in the exercising muscle. This conclusion is important because 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 sex, age, BMI, and current physical activity level because careful matching for these variables between individual patients and their control was performed. The lower aerobic exercise capacity also did

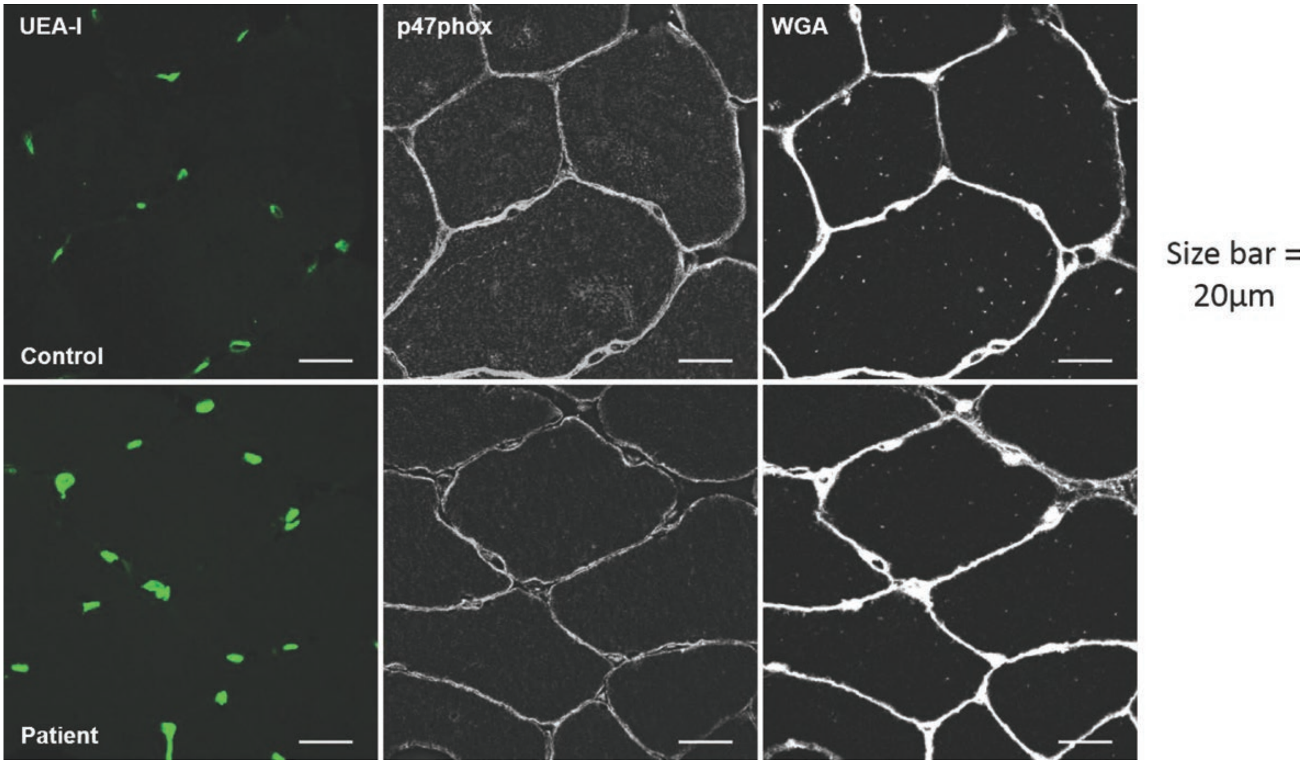

Figure 3. Comparison of the p47^{phox} content (images in the middle) in skeletal muscle capillaries and arterioles (stained with UEA-I in images on the left) and in the plasma membrane of the skeletal muscle fibers (stained with WGA in images on the right) in a former patient and the matched control. The 3 stains were applied to a single cross-section for each individual. P47^{phox} is present both in the endothelial mask of the capillaries and arterioles and mask of the plasma membrane of the skeletal muscle fibers (both in the former patient and the matched control).

Table 5. Skeletal muscle eNOS, eNOS ser^{1177}, NOX2, p47phox, and p67phox protein content

	No.	Patients	Controls	P/C ratio	*P*
eNOS protein content	8	86.0 ± 29.5	75.5 ± 21.7	1.14	.13
eNOS ser^{1177} phosphorylation	8	81.8 ± 14.5	75.9 ± 17.6	1.08	.16
eNOS ser^{1177}/eNOS ratio	8	1.14 ± 0.4	1.20 ± 0.4	0.95	.59
Endothelial-specific NOX2 protein content	7	86.3 ± 28.5	88.0 ± 36.7	0.98	.72
Membrane-specific NOX2 protein content ratio	7	66.9 ± 22.1	70.6 ± 23.9	0.95	.22
p47phox	7	118.9 ± 38.3	120.7 ± 45.6	0.99	.95
p67phox	7	100.9 ± 24.3	112.3 ± 19.4	0.90	.23

The content of these proteins was measured as their fluorescence intensity as described in detail in "Methods."
Abbreviations: eNOS, endothelial nitric oxide synthase; P/C ratio, mean of the ratio of the indicated variable measured in the patient and its matched control.

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 the existing literature (13). The patient group studied was not receiving any medical treatment at the time of testing, except for thyroid hormone substitution in 4 patients. None of the patients had other hormonal deficiencies, nor other comorbidities, like hypertension or impaired glucose tolerance/diabetes. Therefore, the treatment outcome of these specific patients can be considered as a "best-case scenario," and any observed difference in outcome is likely to be, at least in part, a persisting effect of preexposure 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 from CS are physically deconditioned during their previous episode of active CS and/or have a more sedentary lifestyle. However, 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 short-term physical rehabilitation program 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 the still-improving health status in the patients because 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 from CS for more than 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 from CS for > 2 years) and controls. In addition, the patients in the present study have been in remission for more than 4 years and did not have comorbidities so it is unlikely that their health status is still improving. Furthermore, in active CS there is muscle atrophy with a diminished cross-sectional diameter of the muscle fibers (16). In the present study, the muscle fiber CSA of type I and type II fibers is exactly the same in patients and their individually matched controls 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 from CS. First, it could be the result of a reduced supply of arterial blood and therefore of oxygen-borne and blood-borne fuel 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 leg blood flow in patients with long-standing remission from 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 CSA 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-hour 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% to 20% led to significant 5% to 10% increases in eNOS protein content in both studies (6, 19), whereas 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 present 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-hour EE was the same in the patients in remission from CS and their individually matched controls. We also matched patients and their controls for BMI because 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 adenosine diphosphate/oxygen 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 lower leg muscle mass was reduced in patients in long-term remission from CS in comparison to the general population (23). This theoretically could also explain the reduction in VO_{2peak} that we observe in patients during incremental exercise. We can exclude that this is the case, however, in the present study because 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 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 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.

As a limitation of this study, it should be mentioned that measurements of dehydroepiandrosterone sulfate (DHEA-S) were not included in this study because this measurement was not part of our routine clinical practice.

The data in this study might also have relevance for patients treated with exogenous glucocorticoids. Previous research has shown that acute glucocorticoid administration had a minimal effect on exercise capacity and performance measures in healthy men, whereas short-term glucocorticoid administration of 5 to 7 days improved performance (cycling time to exhaustion, maximal force while hopping, and knee extensor endurance time) (32). To our knowledge, there are no studies investigating the effects of long-term glucocorticoid use (of similar duration as the exposure to excess glucocorticoids experienced by CS patients before surgery) on exercise capacity in healthy individuals.

In conclusion, this is the first study that demonstrates that patients in long-term remission from 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 ratio of the protein content of eNOS and producing NO and of the subunits of NAD(P)H-oxidase–producing superoxide anions. These findings need validation in a prospective study with a larger cohort of patients making multiple measurements over a 6- to 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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