O R I G I N A L   A R T I C L E

E n d o c r i n e   C a r e

Impact of Duration of Critical Illness on the Adrenal Glands of Human Intensive Care Patients

Eva Boonen, Lies Langouche, Thomas Janssens, Philippe Meersseman, Hilke Vervenne, Emilie De Samblanx, Zoë Pironet, Lisa Van Dijck, Sarah Vander Perre, Inge Derese, and Greet Van den Berghe

Clinical Division and Laboratory of Intensive Care Medicine (E.B., L.L., T.J., H.V., E.D.S., Z.P., L.V.D., S.V.P.I.D., G.V.d.B.), Department Cellular and Molecular Medicine, and Clinical Department of Internal Medicine (P.M.), University of Leuven (KU Leuven), B-3000 Leuven, Belgium

Downloaded from https://academic.oup.com/jcem/article/99/11/4214/2836670 by guest on 16 August 2022

Context: Adrenal insufficiency is considered to be prevalent during critical illness, although the pathophysiology, diagnostic criteria, and optimal therapeutic strategy remain controversial. During critical illness, reduced cortisol breakdown contributes substantially to elevated plasma cortisol and low plasma ACTH concentrations.

Objective: Because ACTH has a trophic impact on the adrenal cortex, we hypothesized that with a longer duration of critical illness, subnormal ACTH adrenocortical stimulation predisposes to adrenal insufficiency.

Design, Setting and Participants: Adrenal glands were harvested 24 hours or sooner after death from 13 long intensive care unit (ICU)-stay patients, 27 short ICU-stay patients, and 13 controls. Prior glucocorticoid treatment was excluded.

Main Outcome and Measure(s): Microscopic adrenocortical zonational structure was evaluated by hematoxylin and eosin staining. The amount of adrenal cholesterol esters was determined by Oil-Red-O staining, and mRNA expression of ACTH-regulated steroidogenic enzymes was quantified.

Results: The adrenocortical zonational structure was disturbed in patients as compared with controls ($P < .0001$), with indistinguishable adrenocortical zones present only in long ICU-stay patients ($P = .003$ vs. controls). Adrenal glands from long ICU-stay patients, but not those of short ICU-stay patients, contained 21% less protein ($P = .03$) and 9% more fluid ($P = .01$) than those from controls, whereas they tended to weigh less for comparable adrenal surface area. There was 78% less Oil-Red-O staining in long ICU-stay patients than in controls and in short-stay patients ($P = .03$), the latter similar to controls ($P = .31$). The mRNA expression of melanocortin 2 receptor, scavenger-receptor class B, member 1, 3-hydroxy-3-methylglutaryl-CoA reductase, steroidogenic acute regulatory protein, and cytochrome P450 cholesterol side-chain cleavage enzyme was at least 58% lower in long ICU-stay patients than in controls (all $P \leq .03$) and of melanocortin 2 receptor, scavenger-receptor class B, member 1, steroidogenic acute regulatory protein, and cytochrome P450 cholesterol side-chain cleavage enzyme at least 53% lower than in short ICU-stay patients (all $P \leq .04$), whereas gene expression in short ICU-stay patients was similar to controls.

Conclusion and Relevance: Lipid depletion and reduced ACTH-regulated gene expression in prolonged critical illness suggest that sustained lack of ACTH may contribute to the risk of adrenal insufficiency in long-stay ICU patients. ***(J Clin Endocrinol Metab* 99: 4214–4222, 2014)**

ISSN Print 0021-972X   ISSN Online 1945-7197
Printed in U.S.A.
Copyright © 2014 by the Endocrine Society
Received May 21, 2014. Accepted July 11, 2014.
First Published Online July 25, 2014

Abbreviations: BMI, body mass index; CYP11A1, cytochrome P450 cholesterol side-chain cleavage enzyme; HE, hematoxylin and eosin; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; ICU, intensive care unit; IQR, interquartile range; LDL, low-density lipoprotein; LDLR, LDL receptor; MC2R, melanocortin 2 receptor; NEFL, neurofilament light-polypeptide; ORO, Oil-Red-O; POMC, proopiomelanocortin; SCARB1, scavenger-receptor class B, member 1; STAR, steroidogenic acute regulatory protein.

Critical illness, an example of severe physical stress, is hallmarked by increased circulating levels of the stress hormone cortisol. We have shown that, in contrast to previous understanding, these high levels are to a large extent explained by reduced cortisol breakdown, whereas cortisol production is only moderately increased if at all (1). Furthermore, plasma corticotrophin (ACTH) was shown to be low throughout at least the first week of critical illness (1), possibly explained by negative feedback inhibition. Whereas reduced cortisol breakdown could be interpreted as a beneficial adaptation of the body to maintain hypercortisolemia in an economic way, limiting the need for energy-consuming cortisol production in times of low energy availability, sustained low ACTH levels could negatively affect structure and function of the adrenal cortex. Indeed, ACTH exerts important trophic and structural effects on the adrenal cortex (2) and depletion of ACTH in experimental models causes adrenal atrophy (3). Furthermore, ACTH is responsible for both short- and long-term regulation of steroidogenesis (4).

Cortisol cannot be stored in the adrenal gland, so its availability depends on rapid synthesis within the adrenal gland in case of stress. Cortisol is synthesized from cholesterol in the zona fasciculata of the adrenal cortex. The principal source of cholesterol for the adrenal glands is circulating low-density lipoprotein (LDL) cholesterol and to a lesser extent high-density lipoprotein cholesterol, which is taken up via the LDL receptor (LDLR) and the high-density lipoprotein receptor [scavenger-receptor class B, member 1 (SCARB1)], respectively. Twenty percent of the cholesterol required for adrenal cortisol synthesis is newly produced within the adrenal cortex via 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (5). The cholesterol molecules are stored as cholesterol esters in intracellular vesicles, in which they reside until steroidogenesis starts. Whenever more cortisol is needed, a cholesterol esterase rapidly mobilizes cholesterol out of the lipid droplets to the cytoplasm, from which it is transported to the inner membrane of the mitochondria (5, 6). This transport is mediated by the steroidogenic acute regulatory protein (STAR) (7, 8). Within the inner membrane of the mitochondria, cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone, which is the rate-limiting step in steroidogenesis. Afterward, multiple enzymes regulate the further conversion of pregnenolone to cortisol (7).

Within minutes after onset of stress, ACTH acutely activates its receptor, the melanocortin 2 receptor (MC2R), on the adrenal cortex, which causes the release of cholesterol from the lipid droplets and increases STAR expression (4). The more long-term impact of ACTH on the adrenal cortex involves increased transcription of genes encoding proteins for cholesterol uptake (*SCARB1*, *LDLR*), cholesterol synthesis (*HMGCR*), and steroidogenesis (*STAR* and *CYP11A1*) as such enhancing the synthetic capacity of the cells by providing a reserve pool for acute steroid demand (4, 5, 7, 9, 10). In addition, ACTH has a direct stimulatory effect on the expression of its own receptor (MC2R), which amplifies the adrenal responsiveness to ACTH (11). Considering the extensive acute and chronic impact of ACTH on the adrenal cortex, persistently low ACTH concentrations profoundly affect the structure and function of the adrenal cortex. This is illustrated by the phenotype of proopiomelanocortin (POMC) deficiency in a knockout mouse model and in human patients, characterized by the loss of adrenocortical zonational structure, adrenocortical lipid depletion, reduced ACTH signaling, and adrenal atrophy/failure (3, 12, 13).

We hypothesized that low plasma ACTH concentrations during prolonged critical illness are associated with reduced trophic ACTH effects on the adrenal cortex, reflected by fewer lipid droplets and reduced gene expression of ACTH-regulated enzymes. Such effects could predispose to symptomatic adrenal failure in the prolonged phase of critical illness (14). To test this hypothesis, we studied adrenal glands, harvested postmortem, from patients who died in the intensive care unit (ICU) after a short or a prolonged critical illness and compared these with adrenal glands from individuals who died acutely out of the hospital. We quantified alterations in size, weight, and microscopic structure of the adrenal glands, the adrenocortical storage of cholesterol esters, and mRNA expression of different key ACTH-regulated proteins of the steroidogenic pathway.

Materials and Methods

Patients and study samples

From patients who died in the ICU and for whom an autopsy was requested by the attending physician, adrenal glands were harvested for this study. Patients who were on chronic glucocorticoid treatment during the preceding 3 months or who had received acute glucocorticoid treatment within 7 days prior to the day of death were excluded. For comparison, adrenal glands were harvested at the occasion of a planned autopsy from individuals who suddenly died out of the hospital, further referred to as control subjects, in collaboration with the emergency and forensic departments of the Leuven University Hospital. Control subjects who were known to suffer from chronic illnesses or were treated with glucocorticoids were excluded. All adrenal glands were harvested within 24 hours of death.

The Leuven University Hospital permits postmortem tissue sampling for academic purposes whenever a patient or his/her legal representative consented upon hospital admission. This is performed via a hospital-wide information and consent proce-

Downloaded from https://academic.oup.com/jcem/article/99/11/4214/2836670 by guest on 16 August 2022

dure, requiring active opting out when not consenting, with opting out remaining possible until the time of death. The study protocol was approved by the Institutional Ethical Review Board of the KU Leuven (ML6625). The study was registered at International Standard Randomized Controlled Trial Number Register (number ISRCTN49306926).

We wanted to investigate the impact of duration of critical illness. To this end, control subjects who died suddenly out of hospital were compared with patients who died after a long ICU stay (>7 d) and with patients who died after a short ICU stay (≤7 d). The sample size was determined by the a priori calculated need of at least 13 patients in each group to detect a difference in lipid staining of about 50% in long ICU-stay patients, with a power of 80% and certainty of 95%. Harvesting continued until this minimal number was reached and all patients who did not have any exclusion criteria and from whom adrenal glands were harvested within 24 hours after death were kept in the study.

Processing of adrenal glands

Immediately after harvesting, adrenal glands were processed for the different analyses. After cleaning from adherent fat, adrenal glands were weighed and photographed to estimate surface area with Image J 1.44p software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland). Adrenal tissue intended for paraffin embedding was immediately placed in 6% paraformaldehyde. Adrenal tissue intended for cryostat sectioning were snap frozen in liquid nitrogen cooled isopentane, whereas tissue intended for gene expression was immediately snap frozen in liquid nitrogen and stored at −80°C until the time of analysis. All the analyses were performed on the left adrenal gland of each patient.

To quantify water content, approximately 50 mg of adrenal gland tissue was weighed before and after 20 hours of lyophilisation (SpeedVac concentrator; Thermo scientific). The difference in weight divided by the start weight represented the water fraction of the tissue. To determine the total protein content, adrenal samples (∼100 mg) were weighed and homogenized in 4× Nonidet P-40-glycerol lysis buffer with phosphatase and protease inhibitors, using ceramic beads and a Precellys 24 homogenizer (6000 rpm, 45 sec; Bertin Technologiesance). Protein content was quantified using Coomassie protein assay reagent (Pierce Biotechnology Inc).

Microscopic analysis of the adrenocortical zonational structure

After embedding in paraffin, 5-μm-thick sections were stained with hematoxylin and eosin (HE). To evaluate adrenocortical zonational structure, HE sections were scored semiquantitatively based on the identification of the three zones in the adrenal cortex at a ×5 magnification using a Leica DM3000 microscope. Two investigators, blinded for group allocation, evaluated all sections separately, and any discrepancy between them was resolved by consensus. Adrenocortical zonational structure was graded as follows: 0′, when the three adrenocortical zones were clearly distinguishable, 1′, for those with a moderately distorted zonational structure, and 2′, when the different zones were no longer distinguishable.

Quantification of cholesterol ester storage in the adrenal glands

To quantify cholesterol esters stored in the adrenal gland, Oil-Red-O (ORO) staining was performed on 10-μm-thick frozen tissue sections. ORO is a red dye used for staining neutral lipids and therefore stains the cholesterol esters in the lipid droplets of the adrenal cortex. Sections were dried at room temperature, fixed in propylene glycol, and stained overnight in 0.5% ORO (Sigma-Aldrich) in propylene glycol and counterstained with hematoxylin. Digital microscopic images of the ORO stainings were analyzed for the amount of redness per surface unit and the intensity of this redness with an in-house-developed computer algorithm from a plug-in for ImageJ 1.44p. In short, the stained areas were quantified by training a classification algorithm to detect staining in the brightness and color saturation channels of the digitized images. This allows the differently stained areas to be extracted from the overall image, after which their respective surface areas can be calculated by the number of image pixels present in them. As a preprocessing step, image artifacts such as dust spots or staining drops were removed manually. For the intensity of the red staining, the maximal and minimal redness (interpreted as pixel saturation) was determined for each individual image and labeled, respectively, 1 and 0. Every pixel was evaluated against this scale. The mean of all values for all pixels represented the intensity of the staining. The product of the intensity and amount of redness per tissue surface was considered to represent the total amount of available cholesterol esters.

mRNA expression of ACTH-regulated key proteins of the steroidogenic pathway

Total mRNA was extracted from adrenal gland tissue (∼30 mg) using Qiazol lysis reagent (QIAGEN) and subsequently purified with RNAeasy minicolumns (QIAGEN). Samples were treated with deoxyribonuclease to remove all genomic DNA, and 1 μg total mRNA was reverse transcribed using random hexamers. cDNA levels of genes regulated by ACTH signaling [for cholesterol uptake and synthesis (*SCARB1*, *LDLR*, and *HMGCR*) and crucial genes for steroidogenesis (*MC2R*, *STAR*, and *CYP11A1*)] were quantified in real time with the TaqMan gene expression assays using the StepOnePlus system (Applied Biosystems) (Hs00196245_m1, 4310884[E], Hs02758991_g1, Hs03928985_g1, Hs00167984_m1, Hs00986559_g1, Hs00300820_s1, Hs00969821_m1, Hs00181192_m1, and Hs00168352_m1). Individual samples with a copy number coefficient of variation greater than 20% were reanalyzed. Data were expressed relative to expression 18S ribosomal 5 RNA (*RNA18S5*), a housekeeping gene of which the expression was not altered neither by critical illness nor by the duration of illness, and as a fold difference from the mean of the control subjects.

To exclude confounding effects of RNA degradation that may have occurred during the postmortem period, mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was evaluated with two different primer sets designed to amplify fragments at two distinct exons, (exons 6 and 7 and exon 3). To evaluate the relative proportion of the adrenal gland medulla in the tissue samples, cDNA levels for neurofilament light-polypeptide (*NEFL*), a marker for neuronal elements, were also quantified.

Downloaded from https://academic.oup.com/jcem/article/99/11/4214/2836670 by guest on 16 August 2022

Statistical analyses

Statistical analyses were performed with JMP (version10.0; SAS Institute Inc). Data are presented as means ± SD or medians with interquartile range (IQR), as appropriate. All experimental results were analyzed with a nonparametric Wilcoxon singed rank test. Comparison of proportions was done by χ^2 testing. Associations between parameters were analyzed with linear regression. The Pearson determination (R^2) coefficient was calculated and its significance analyzed by ANOVA. No corrections were made for multiple comparisons. The a priori-defined primary comparison was that between long ICU-stay patients and control subjects because this is where the hypothesized differences were expected. Two-sided values of $P < .05$ were considered statistically significant.

Results

The study comprised adrenal glands harvested from 13 long-stay ICU patients, 27 short ICU-stay patients, and 13 control subjects. The characteristics of patients and control subjects are described in Table 1.

Weight, size, and microscopic zonational structure of the adrenal glands

The weight of the adrenal glands, corrected for body weight, was not different among the groups [median 0.10 IQR (0.07–0.12) g/kg in the control subjects, 0.10 (0.08–0.12) g/kg in the short ICU stay patients and 0.08 (0.06–0.10) g/kg in the long ICU stay patients (all $P > .08$)]. Also, the adrenal surface area was similar for all groups [10.2 (8.8–12.2) cm^2 in control subjects, 11.2 (9.1–13.7) cm^2 in the short ICU stay patients, and 11.1 (9.3–16.5) cm^2 in the long ICU stay patients (all $P > .25$)].

The fraction water in adrenal glands from long ICU-stay patients [76% (IQR 72–78)] was larger than in those from control subjects [69% (IQR 62–74); $P = .01$], whereas no difference was observed between short ICU-stay patients [72% (IQR 67–76)] and controls ($P = .22$) or between the 2 ICU patient groups ($P = .08$). There was 21% less protein per milligram tissue in adrenal glands from long ICU-stay patients [74 μg/mg (IQR 67–78)] than

Table 1. Characteristics of ICU Patients and Control Subjects

	Controls Subjects (n = 13)	Short ICU-Stay Patients (n = 27)	Long ICU-Stay Patients (n = 13)	*P* Value Control Subjects vs Short ICU-Stay Patients	*P* Value Control Subjects vs Long ICU-Stay Patients	*P* Value Short vs Long ICU-Stay Patients
Demography and anthropometry						
Gender, number male, %	9 (69)	14 (52)	9 (69)	.29	1.0	.29
Age, y (mean ± SD)	40.8 ± 17.5	69.7 ± 12.6	67.6 ± 14.1	<.0001	.0017	.62
BMI, kg/m^2) (mean ± SD)	24.9 ± 4.8	26.0 ± 5.5	25.8 ± 3.9	.34	.40	.86
Admission characteristics						
Diagnostic group on admission, n, %	N.A.					.92
Cardiovascular		7 (26)	3 (23)			
Respiratory/esophageal-lung surgery		10 (37)	4 (31)			
Abdominal/gastro intestinal/hepatic		6 (22)	3 (23)			
Other		4 (15)	3 (23)			
APACHE II (mean ± SD)	N.A.	34.6 ± 9.9	34.8 ± 6.0			.70
SIRS, n, %	N.A.	27 (100)	12 (92)			.14
Sepsis, n, %	N.A.	18 (67)	11 (85)			.23
Patient characteristics at study time						
Cause of death						
Refractory shock	N.A.	12 (44)	0 (0)			.004
Therapy withdrawal forfutility	N.A.	15 (56)	13 (100)			
Sudden out-of-hospital death[a]	13 (100)	N.A.	N.A.			
SIRS, n, %	N.A.	26 (96)	12 (92)			.58
Sepsis, n, %	N.A.	16 (59)	11 (85)			.10
Duration of stay in ICU prior to death, d [median (IQR)]	N.A.	2 (1–5)	16 (13–21)			<.0001
Time interval between death and autopsy, h [mean ± SD]	17.5 ± 4.5	15.0 ± 6.8	15.4 ± 4.8	.28	.31	.79

Abbreviations: APACHE II, Acute Physiology and Chronic Health Evaluation II; N.A., not available; SIRS, systemic inflammatory response syndrome. The BMI is the weight in kilograms divided by the square of the height in meters. APACHE II ranges from 0 to 71, with higher scores indicating a greater severity of illness. SIRS is determined by the BONE criteria.

[a] Sudden out-of-hospital deaths comprised the following: trauma (n = 2), cardiac arrest (n = 4), drowning (n = 2), electrocution (n = 1), gunshot (n = 1), acute hemorrhage (n = 1), pulmonary embolism (n = 1), and sudden unexpected death of epilepsy (n = 1).

Downloaded from https://academic.oup.com/jcem/article/99/11/4214/2836670 by guest on 16 August 2022

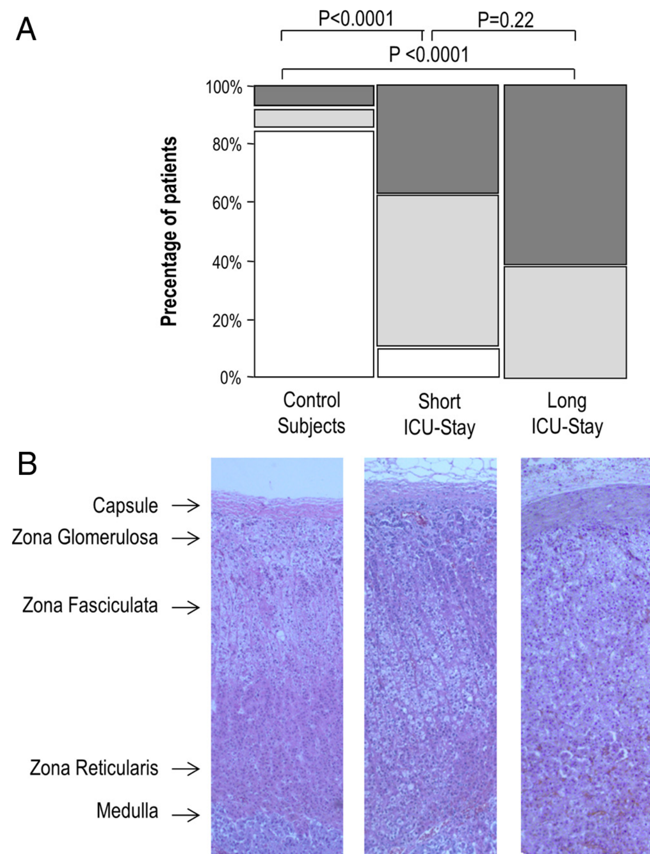

Figure 1. Semiquantitative scoring of the loss of adrenocortical zonational structure. A, The fraction of patients with score 0, indicating a normal zonational structure, is depicted in white. The fraction of patients with score 1, indicating a moderately distorted zonational structure, is depicted in light gray. The fraction of patients with score 2, indicating that the different zones were no longer distinguishable, is depicted in dark gray. B, The illustrations are representative examples of HE staining images for each group. Photographs were taken at a ×5 magnification.

in those from control subjects [94 μg/mg (IQR 71–101), $P = .03$] and also 23% less protein in short ICU-stay patients [72 μg/mg (IQR 66–83)] than in control subjects ($P = .02$). Adrenal protein content in both ICU patient groups was similar ($P = .89$).

Microscopic semiquantitative scoring revealed disturbed adrenocortical zonational structure in both ICU patient groups as compared with control subjects ($P < .0001$) (Figure 1). As compared with the control subjects, an indistinguishable zonational structure was significantly more often present only in the long ICU-stay patients ($P = .003$).

Quantification of adrenal cholesterol ester storage

Computerized quantification of the ORO staining revealed that in adrenal glands from long ICU-stay patients, there were 78% less cholesterol esters stored than in those from controls ($P = .03$) and also 78% less than in those from short ICU-stay patients ($P = .03$), whereas short ICU-stay patients were not different from controls (Figure 2).

mRNA expression of ACTH-regulated proteins of the steroidogenic pathway

To assess any confounding effect of postmortem loss of mRNA quality, the degree of mRNA degradation was investigated. For this purpose, glyceraldehyde-3-phosphate dehydrogenase mRNA expression was measured with two primers directed toward a different exon. There was a tight correlation between the expression using both primers ($R^2 = 0.94$; $P < .0001$), suggesting that RNA degradation was limited.

mRNA expression of *NEFL* was similar in long ICU-stay patients [0.13 (IQR 0.06–0.77)], in short ICU-stay patients [0.13 (IQR 0.04–1.15)], and in control subjects [0.24 (IQR 0.04–1.70)] (all $P > .80$), indicating that tissue samples of all study groups contained comparable amounts of adrenal medulla. The amounts of mRNA encoding for *MC2R, SCARB1, HMGCR, STAR, and CYP11A1* were at least 58% lower in long ICU-stay patients than in control subjects (all $P < .03$) (Figure 3). In addition, compared with short ICU-stay patients, mRNA expression of *MC2R, SCARB1, CYP11A1, and STAR* was at least 53% lower in long ICU-stay patients (all $P < .04$). No differences were observed between short ICU-stay patients and control subjects.

Discussion

The results of this human postmortem study suggest that important alterations occur in the adrenal gland during the course of critical illness. In long ICU-stay patients, but not in the short ICU-stay patients, severe cholesterol ester depletion and substantially reduced mRNA expression of ACTH-regulated key enzymes for steroidogenesis were observed. These observations suggest that with an extended duration of critical illness, a significant loss of ACTH signaling in the adrenal cortex may have important functional consequences.

The loss of adrenocortical zonational structure already in the acute phase of critical illness confirmed the results of previous studies performed in small groups of septic patients (15, 16). Although postmortem changes could have played a role, these should then apply to both acute out-of-hospital deaths and to patients who died in the ICU, which was not the case in our study. Furthermore, severe abnormalities were more prevalent among the long ICU-stay patients only. Another interesting observation was that the adrenal glands of long ICU-stay patients tended to weigh less, not more, than those of short ICU-stay patients, whereas the adrenal glands contained somewhat more water and less protein. This is not what one would expect when the adrenal gland would receive pro-

Downloaded from https://academic.oup.com/jcem/article/99/11/4214/2836670 by guest on 16 August 2022

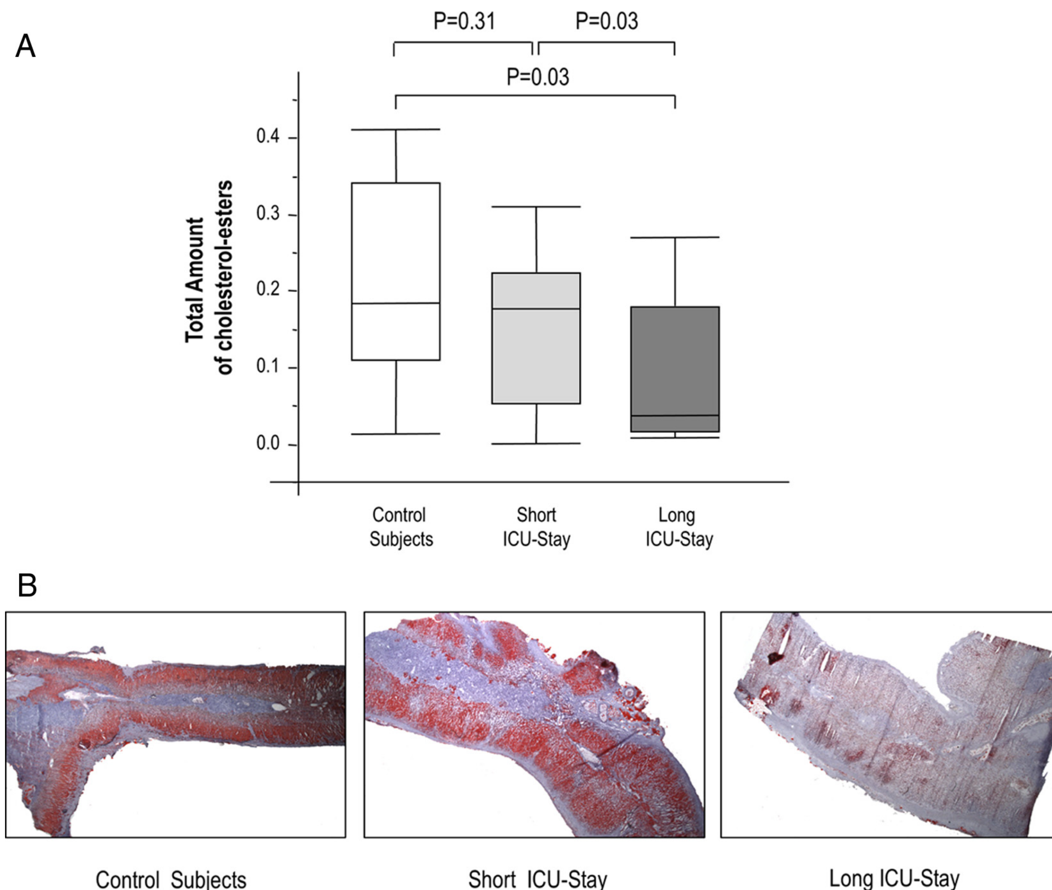

Figure 2. Quantification of the total amount of cholesterol esters in the adrenal glands. A, The total amount of cholesterol esters was calculated by the product of ORO staining intensity and area. Boxes represent medians and interquartile ranges and whiskers represent first quartile-1.5IQR and third quartile+1.5IQR. *P* values for group comparisons were determined by the Wilcoxon rank-sum test. B, The illustrations are representative examples of ORO staining images for each group. Photographs were taken at a ×1.25 magnification.

longed ACTH stimulation in sustained critical illness, which would cause adrenal hypertrophy and hyperplasia (17). Hence, because smaller adrenal glands and loss of zonational structure are also observed in POMC-deficient mice (3), ACTH deprivation with time in the ICU could have played a role. Low plasma ACTH concentrations have been repeatedly reported during critical illness (1, 18, 19), and we recently showed that the nocturnal ACTH secretion rate was suppressed in ICU patients (20). Such ACTH suppression could be in part due to feedback inhibition exerted by elevated cortisol levels brought about by reduced cortisol breakdown (1). This situation is comparable with sustained exogenous administration of hydrocortisone, which is known to suppress ACTH and with time can cause adrenal atrophy (21).

Severe depletion of cholesterol esters was observed only in the adrenal glands harvested from long ICU-stay patients and not in those from the short ICU-stay patients. Because normal adrenocortical synthesis and release of cortisol relies predominantly on the availability of cholesterol stored in the adrenal cortex, the observed lipid depletion may theoretically contribute to the inability of long ICU-stay patients to acutely release enough cortisol in response to a second hit (14). Also, the gene expression of one of the cholesterol uptake receptors, SCARB1, as well as of HMGCR, the enzyme responsible for de novo cholesterol production, was not increased at all and was significantly down-regulated in the adrenal glands of the long ICU-stay patients only. Furthermore, the expression of genes encoding for the ACTH receptor (*MC2R*) and the steroidogenic proteins (*STAR*, *CYP11A1*) showed a comparable expression profile, with reduced expression levels in long ICU-stay patients only. Given that ACTH stimulates the expression of these genes, sustained ACTH deprivation could explain these findings (4, 5, 7, 9, 10). Indeed, the presentation is again highly reminiscent of the alterations observed in POMC-deficient mice (12).

Our observation that mRNA expression of the different key genes in steroidogenesis was not up-regulated in critically ill patients does not support a role for ACTH or ACTH-independent stimulators of cortisol synthesis such as neuropeptides, endothelin, and cytokines, (2) because all would require an activation of the steroidogenic genes (22). It actually further supports our previous finding that

Downloaded from https://academic.oup.com/jcem/article/99/11/4214/2836670 by guest on 16 August 2022

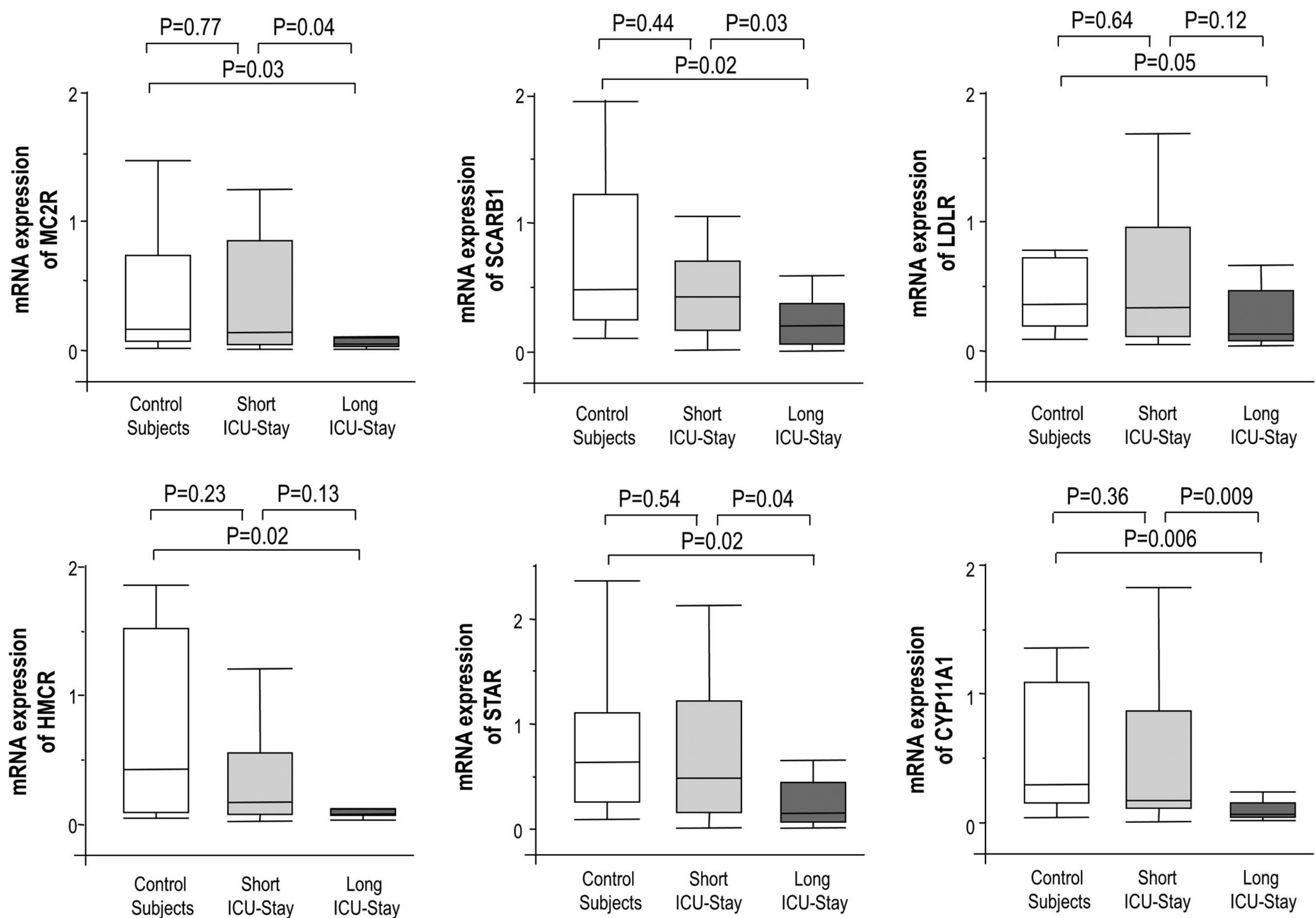

Figure 3. mRNA expression of ACTH-regulated proteins of the steroidogenic pathway. The mRNA data are expressed, normalized to RNA18S, as a fold difference from the mean of the controls. Boxes represent medians and interquartile ranges and whiskers represent first quartile-1.5IQR and third quartile+1.5IQR. *P* values for group comparisons were determined by the Wilcoxon rank-sum test.

high plasma cortisol in critically ill patients is to a large extent due to reduced cortisol breakdown (1, 20). Unfortunately, given the nature of the study, blood samples were not available to correlate the adrenocortical pathology and gene expression findings with plasma ACTH and cortisol concentrations. It appears that especially ACTH pulsatility is critical for adequate transcriptional activation of steroidogenic genes (23). We recently showed, by serial blood sampling and deconvolution analysis, that it is specifically the pulsatile nocturnal ACTH release that is suppressed during critical illness, with reduced ACTH pulse mass in the presence of unaltered ACTH pulse frequency (20). Possibly, in the acute phase of critical illness, the pulsatile ACTH signal is still sufficient to prevent the down-regulation of ACTH responsive genes in the adrenal cortex. However, when increased feedback inhibition exerted by high cortisol levels is maintained into the prolonged phase of critical illness, and as a consequence pulsatile ACTH is suppressed for a longer time, this might reach a critical threshold below which steroidogenic transcription becomes impaired.

The presented study has several limitations and some strengths. First, inevitably for a human study on this topic, the adrenal glands were harvested at the occasion of autopsy, and although this was performed within 24 hours after death, postmortem artifacts cannot be fully excluded. However, because the corpses were kept cooled until harvesting of the adrenal glands, this problem may have been limited, supported by the mRNA quality analysis that revealed minimal mRNA degradation. Furthermore, not all studied genes were down-regulated in the prolonged phase of illness because *LDLR, NEFL, and 18S ribosomal 5 RNA* were unaltered, which speaks against postmortem artifacts explaining the between group differences. Cholesterol depletion could also theoretically be exacerbated by acute consumption of available cholesterol droplets in the agonal phase of illness (24, 25). However, this would again apply to all ICU patients and to the sudden out-of-hospital deaths alike. Also, adrenocortical lipid depletion was previously observed in an animal model of sepsis-induced critical illness, a model that does not suffer from the above-mentioned methodological issues that are inevitable in human studies (16).

Second, acute out-of-hospital deaths used as control subjects were matched for body mass index (BMI) and

Downloaded from https://academic.oup.com/jcem/article/99/11/4214/2836670 by guest on 16 August 2022

gender but turned out to be younger than the ICU patients. However, research by Hornsby (26) revealed no impact of age on the zona fasciculata. Third, the lack of blood samples did not allow assessing the association between the observed changes in the adrenal glands and the circulating levels of ACTH and cortisol. Hence, in vivo animal studies are required to further study the influence of duration of critical illness, and hereby the duration of ACTH deprivation, on the adrenocortical morphology and function during critical illness. However, despite these limitations, the strength of this study was that no signs of increased steroidogenesis could be found in the adrenal glands of a large and heterogeneous population of patients, being treated for a variety of illnesses in ICU, with or without sepsis. This observation further corroborated our previous findings in vivo, indicating that other mechanisms than ACTH drive elevated plasma cortisol concentrations during sustained critical illness (1, 20). Also, this is the first study to investigate the impact of critical illness on the adrenal gland at the tissue level in such a large population. Most previous studies investigated only the adrenal gland indirectly, via the cortisol response to ACTH injection (27), a test that remains highly debated as to what it reveals about adrenal functional reserve (28, 29).

In conclusion, the changes observed in adrenal glands harvested within 24 hours after death from long-stay and short-stay ICU patients and from sudden out-of-hospital control deaths suggest that extended duration of critical illness profoundly affects adrenocortical structure and function, which could be related to ACTH deprivation. Depletion of cholesterol esters and reduced expression of ACTH-regulated genes involved in steroidogenesis may contribute to the risk of adrenal insufficiency in the prolonged phase of critical illness.

Acknowledgments

We thank the emergency and the forensic departments as well as the staff of the mortuary of the Leuven University Hospital for all the help with the harvesting of the adrenal glands. The Department of Pathology is acknowledged for their expert advice on the adrenal ORO staining. We also thank the clinical research assistants Alexandra Hendrickx and Sylvia Van Hulle for their help with data collection.

Author contributions included the following: G.V.d.B. and E.B. designed and conducted the study and are fully responsible for data analysis. They had full access to all data and take responsibility for data integrity and for the accuracy of the analysis. E.B., H.V., P.M., and G.V.d.B. included the patients and collected the data. E.B., E.D.S., Z.P., L.V.D., S.V.P., and I.D. performed the microscopic and molecular analyses. E.B., L.L., T.J., and G.V.d.B. analyzed the data. E.B., L.L., and G.V.d.B. interpreted and vouch for the data. E.B., L.L., and G.V.d.B. wrote the first draft, which was reviewed and approved by all authors, who jointly decided to publish. No confidentiality agreements existed between the sponsors and authors/institutions.

The abstract was accepted as a late breaker for The Endocrine Society/International Congress of Endocrinology Annual Meeting in Chicago, Illinois, June 21–24, 2014.

Address all correspondence and requests for reprints to: Greet Van den Berghe, MD, PhD, Clinical Division and Laboratory of Intensive Care Medicine, KU Leuven, Herestraat 49, B-3000 Leuven, Belgium. E-mail: greet.vandenberghe@med.kuleuven.be.

This work was supported by the Fund for Scientific Research Flanders (Belgium) Grant FWO G.0417.12 (to G.V.d.B.). G.V.d.B., via the University of Leuven (KU Leuven), receives long-term structural research support from the Methusalem Program funded by the Flemish Government and holds an European Research Council Advanced Grant AdvG-2012-321670 from the Ideas Program of the European Union seventh framework program.

Disclosure Summary: The authors have no conflict of interest to disclose.

References

1. **Boonen E, Vervenne H, Meersseman P, et al.** Reduced cortisol metabolism during critical illness. *N Engl J Med*. 2013;368:1477–1488.
2. **Bornstein SR, Chrousos GP.** Clinical review 104: adrenocorticotropin (ACTH)- and non-ACTH-mediated regulation of the adrenal cortex: neural and immune inputs. *J Clin Endocrinol Metab* 1999; 84:1729–1736.
3. **Coll AP, Challis BG, Yeo GS, et al.** The effects of proopiomelanocortin deficiency on murine adrenal development and responsiveness to adrenocorticotropin. *Endocrinology*. 2004;145:4721–4727.
4. **Simpson ER, Waterman MR.** Regulation by ACTH of steroid hormone biosynthesis in the adrenal cortex. *Can J Biochem Cell Biol* 1983;61:692–707.
5. **Liu J, Heikkila P, Meng QH, Kahri AI, Tikkanen MJ, Voutilainen R.** Expression of low and high density lipoprotein receptor genes in human adrenals. *Eur J Endocrinol*. 2000;142:677–682.
6. **Kraemer FB, Shen WJ.** Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis. J Lipid Res 2002;43:1585–1594.
7. **Stocco DM, Clark BJ.** Regulation of the acute production of steroids in steroidogenic cells. *Endocr Rev*. 1996;17:221–244.
8. **Stocco DM, Clark BJ.** The role of the steroidogenic acute regulatory protein in steroidogenesis. *Steroids*. 1997;62:29–36.
9. **Lehoux JG, Lefebvre A, Belisle S, Bellabarba D.** Hormonal regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA in the rat adrenal gland. *J Steroid Biochem*. 1989;34:379–384.
10. **Lehoux JG, Fleury A, Ducharme L.** The acute and chronic effects of adrenocorticotropin on the levels of messenger ribonucleic acid and protein of steroidogenic enzymes in rat adrenal in vivo. *Endocrinology*. 1998;139:3913–3922.
11. **Lebrethon MC, Naville D, Begeot M, Saez JM.** Regulation of corticotropin receptor number and messenger RNA in cultured human adrenocortical cells by corticotropin and angiotensin II. *J Clin Invest*. 1994;93:1828–1833.
12. **Karpac J, Czyzewska K, Kern A, Brush RS, Anderson RE, Hochgeschwender U.** Failure of adrenal corticosterone production in POMC-deficient mice results from lack of integrated effects of

Downloaded from https://academic.oup.com/jcem/article/99/11/4214/2836670 by guest on 16 August 2022

POMC peptides on multiple factors. *Am J Physiol Endocrinol Metab*. 2008;295:E446–E455.

13. **Krude H, Gruters A.** Implications of proopiomelanocortin (POMC) mutations in humans: the POMC deficiency syndrome. *Trends Endocrinol Metab*. 2000;11:15–22.
14. **Barquist E, Kirton O.** Adrenal insufficiency in the surgical intensive care unit patient. *J Trauma*. 1997;42:27–31.
15. **Bollen TL, van Santvoort HC, Besselink MG, van Ramshorst B, van Es HW, Gooszen HG.** Dutch Acute Pancreatitis Study Group. Intense adrenal enhancement in patients with acute pancreatitis and early organ failure. *Emerg Radiol*. 2007;14:317–322.
16. **Polito A, Mansart A, Louiset E, et al.** Human and experimental septic shock are characterized by depletion of lipid droplets in the adrenals. *Intensive Care Med*. 2010;36:1852–1858.
17. **Ferreira JG, Cruz CD, Neves D, Pignatelli D.** Increased extracellular signal regulated kinases phosphorylation in the adrenal gland in response to chronic ACTH treatment. *J Endocrinol*. 2007;192:647–658.
18. **Michalaki M, Margeli T, Tsekouras A, Gogos CH, Vagenakis AG, Kyriazopoulou V.** Hypothalamic-pituitary-adrenal axis response to the severity of illness in non-critically ill patients: does relative corticosteroid insufficiency exist? *Eur J Endocrinol*. 2010;162:341–347.
19. **Vermes I, Beishuizen A, Hampsink RM, Haanen C.** Dissociation of plasma adrenocorticotropin and cortisol levels in critically ill patients: possible role of endothelin and atrial natriuretic hormone. *J Clin Endocrinol Metab*. 1995;80:1238–1242.
20. **Boonen E, Meersseman P, Vervenne H, et al.** Reduced nocturnal ACTH-driven cortisol secretion during critical illness. *Am J Physiol Endocrinol Metab* 2014;306(8):E883–E892.
21. **Hopkins RL, Leinung MC.** Exogenous Cushing's syndrome and glucocorticoid withdrawal. Endocrinol Metab Clin North Am 2005;34:371–384, ix.
22. **Lacroix A, Ndiaye N, Tremblay J, Hamet P.** Ectopic and abnormal hormone receptors in adrenal Cushing's syndrome. *Endocr Rev*. 2001;22:75–110.
23. **Spiga F, Waite EJ, Liu Y, Kershaw YM, Aguilera G, Lightman SL.** ACTH-dependent ultradian rhythm of corticosterone secretion. *Endocrinology*. 2011;152:1448–1457.
24. **O'Donnell WM, Fajans SS, Weinbaum JG.** Human adrenal cortex after administration of ACTH and cortisone; morphologic changes. *AMA Arch Intern Med*. 1951;88:28–35.
25. **Symington T, Duguid WP, Davidson JN.** Effect of exogenous corticotropin on the histochemical pattern of the human adrenal cortex and a comparison with the changes during stress. *J Clin Endocrinol Metab*. 1956;16:580–598.
26. **Hornsby PJ.** Aging of the human adrenal cortex. *Sci Aging Knowledge Environ* 2004;2004:re6.
27. **Annane D, Sebille V, Troche G, Raphael JC, Gajdos P, Bellissant E A.** Three-level prognostic classification in septic shock based on cortisol levels and cortisol response to corticotropin. *JAMA* 2000; 283:1038–1045.
28. **Dellinger RP, Levy MM, Rhodes A, et al.** Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. *Crit Care Med*. 2013;41:580–637.
29. **Marik PE, Pastores SM, Annane D, et al.** Recommendations for the diagnosis and management of corticosteroid insufficiency in critically ill adult patients: consensus statements from an international task force by the American College of Critical Care Medicine. *Crit Care Med*. 2008;36:1937–1949.

Downloaded from https://academic.oup.com/jcem/article/99/11/4214/2836670 by guest on 16 August 2022