Impact of duration of critical illness and level of systemic glucocorticoid availability on tissue-specific glucocorticoid receptor expression and actions: A prospective, observational, cross-sectional human and two translational mouse studies

Arno Téblick, Lisa Van Dyck, Nathalie Van Aerde, Sarah Van der Perre, Lies Pauwels, Inge Derese, Yves Debaveye, Pieter J. Wouters, Ilse Vanhorebeek, Lies Langouche, and Greet Van den Berghe*

Clinical division and Laboratory of Intensive Care Medicine, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium

Summary

Background Reduced glucocorticoid-receptor (GR) expression in blood suggested that critically ill patients become glucocorticoid-resistant necessitating stress-doses of glucocorticoids. We hypothesised that critical illness evokes a tissue-specific, time-dependent expression of regulators of GR-action which adaptively guides glucocorticoid action to sites of need.

Methods We performed a prospective, observational, cross-sectional human study and two translational mouse studies. In freshly-isolated neutrophils and monocytes and in skeletal muscle and subcutaneous adipose tissue of 137 critically ill patients and 20 healthy controls and in skeletal muscle and adipose tissue as well as in vital tissues (heart, lung, diaphragm, liver, kidney) of 88 septic and 26 healthy mice, we quantified gene expression of cortisone-reductase 11β-HSD1, glucocorticoid-receptor-isoforms GRa and GRb, GRa-sensitivity-regulating-co-chaperone FKBP51, and GR-action-marker GILZ. Expression profiles were compared in relation to illness-duration and systemic-glucocorticoid-availability.

Findings In patients' neutrophils, GRa and GILZ were substantially suppressed (p<0.05) throughout intensive care unit (ICU)-stay, while in monocytes low/normal GRa coincided with increased GILZ (p<0.05). FKBP51 was increased transiently (neutrophils) or always (monocytes, p<0.05). In patients' muscle, 11β-HSD1 and GRa were low-normal (p<0.05) and substantially suppressed in adipose tissue (p<0.05); FKBP51 and GILZ were increased in skeletal muscle (p<0.05) but normal in adipose tissue. GRb was undetectable. Increasing systemic glucocorticoid availability in patients independently associated with further suppressed muscle 11β-HSD1 and GRa, further increased FKBP51 and unaltered GILZ (p<0.05). In septic mouse heart and lung, 11β-HSD1, FKBP51 and GILZ were always high (p<0.01). In heart, GRa was suppressed (p<0.05), while normal or high in lung (all p<0.05). In diaphragm, 11β-HSD1 was high/normal, GRa low/normal and FKBP51 and GILZ high (p<0.01). In kidney, 11β-HSD1 transiently increased but decreased thereafter, GRa was normal and FKBP51 and GILZ high (p<0.01). In liver, 11β-HSD1 was suppressed (p<0.01), GRa normal and FKBP51 high (p<0.01) whereas GILZ was transiently decreased but elevated thereafter (p<0.05). Only in lung and diaphragm, treatment with hydrocortisone further increased GILZ.

Interpretation Tissue-specific, time-independent adaptations to critical illness guided GR-action predominantly to vital tissues such as lung, while (partially) protecting against collateral harm in other cells and tissues, such as neutrophils. These findings argue against maladaptive generalised glucocorticoid-resistance necessitating glucocorticoid-treatment.

Funding Research-Foundation-Flanders, Methusalem-Program-Flemish-Government, European-Research-Council, European-Respiratory-Society.

*Corresponding author. E-mail address: greet.vandenberghe@kuleuven.be (G. Van den Berghe).

1 Authors contributed equally.
Glucocorticoid receptor; CIRCI; Glucocorticoid resistance; Critical illness; Sepsis; ICU

Research in context

Evidence before this study

The Medline database was searched through Pubmed up to December 31, 2021, for publications without start date restrictions, using various combinations of the terms “critical illness”, “shock”, “sepsis”, “multiple organ failure”, “glucocorticoid receptor” and “glucocorticoid resistance”. Multiple opinion papers, commentaries, reviews and guidelines postulate that a subset of critically ill patients, typically those presenting with sepsis or septic shock, may suffer from generalised glucocorticoid resistance. This condition was considered an important part of “critical illness-related corticosteroid insufficiency” (CIRCI), and has led to the conduct of multiple randomized controlled trials (RCTs), investigating the impact of further increasing systemic glucocorticoid availability through the administration of stress doses of glucocorticoids. Although results of these trials were inconsistent, practice guidelines recommend treatment with stress doses of hydrocortisone for all septic patients with vasopressor-dependent and -refractory shock. However, high-quality human or translational animal studies investigating the expression and actions of the glucocorticoid receptor at target tissues during critical illness are scarce and were often limited to the study of blood cells. Importantly, impact of illness duration and of the level of systemic glucocorticoid availability has not been investigated.

Added value of this study

In this study, we documented and characterised glucocorticoid receptor (GR) expression and action hereof during critical illness in multiple target tissues (isolated neutrophils, isolated monocytes, skeletal muscle, subcutaneous adipose tissue, heart, lung, diaphragm, kidney, liver), in relation to increasing duration of critical illness and to increasing levels of systemic glucocorticoid availability. The results revealed that the expression profiles of key regulators of local glucocorticoid action, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), GRα isoform, and FK506 binding protein 51 (FKBP51), are altered in a tissue-specific manner during critical illness, illustrating an adaptive guidance of glucocorticoid actions towards vital tissues, most strikingly the lung, that most need it, while protecting other cells such as neutrophils that would otherwise suffer from harmful immune suppressive effects. Only in lung, the adjacent diaphragm and adipose tissues, a further increase in circulating glucocorticoids resulted in more GR-action, which again seems appropriate.

Implications of all evidence available

These data argue against a maladaptive generalised glucocorticoid resistance that would necessitate treating patients with sepsis or septic shock with stress doses of glucocorticoids. Instead, the data provided evidence for useful adaptations taking place in response to sepsis or other critical illnesses that result in selective and controlled target tissue effects of stress-induced glucocorticoid availability.

Introduction

Critical illness, evoked by sepsis or other causes of hyperinflammation, is hallmarked by a swift and substantial increase in systemic glucocorticoid availability, a vital part of the stress response. Some patients, typically those presenting with sepsis or septic shock, are thought to suffer from insufficiently elevated systemic glucocorticoid availability superimposed on peripheral glucocorticoid resistance, together interpreted as lack of glucocorticoid action which necessitates treatment with high (stress) doses of hydrocortisone. The presence of this condition, referred to as “critical illness-related corticosteroid insufficiency” (CIRCI), as well as its diagnostic criteria remain debated. Also, the results from large scale randomized controlled trials (RCTs) assessing the impact of stress doses of hydrocortisone on outcome remain inconsistent. Nevertheless, practice guidelines recommend treatment with stress doses of hydrocortisone for all septic patients with vasopressor-dependent and -refractory shock.

Endogenously produced glucocorticoids, cortisol in humans and corticosterone in rodents, as well as exogenously administered glucocorticoids, primarily target the glucocorticoid receptor (GR) to exert wide-ranging genomic and non-genomic effects. The magnitude of tissue-specific glucocorticoid and GR-actions depends on the local cellular availability of the GR ligand and on the expression and function of the GR. Local glucocorticoid availability is determined by the amount of circulating ligand (“systemic cortisol/glucocorticoid availability”) and by tissue-specific expression of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), the enzyme that converts inert cortisone into metabolically active cortisol. Although the GR is coded by a single gene, alternative splicing yields multiple GR messenger ribonucleic acid (mRNA) transcripts. The GR alpha isoform (GRα) is able to bind ligand and is the primary
is form that, after translocation to the nucleus, signals most glucocorticoid effects. Prior to ligand-binding and translocation to the nucleus, the GRα resides in the cytoplasm, in a multi-protein complex of which the co-chaperone protein FK506 binding protein 5 (FKBP51) is a glucocorticoid-induced negative regulator of affinity and nuclear translocation of the GRα.15 The lesser expressed GR beta isoform (GRβ) has a C-terminal domain that is unable to bind glucocorticoids and is considered a dominant inhibitor of the GRα. During health, tight regulation of both cellular glucocorticoid availability and of GR isoform and co-chaperone expression is crucial to maintain tissue homeostasis.16

One of the proposed mechanisms behind CIRCI is the presence of a critical illness-induced, generalised dysfunction of GRα-mediated glucocorticoid actions at target tissues, commonly referred to as ‘glucocorticoid resistance’3. This assumption was largely based on small studies documenting downregulation of GRα expression or upregulation of the GRβ in blood cells, mostly during the acute phase of critical illness.17-20 These findings were considered as a rationale for administering high (stress) doses of glucocorticoids, up to 200 mg/day of hydrocortisone which is the equivalent of >10 times the usual substitution dose, to overcome such resistance.4,5,21 However, it is currently unknown whether the altered expression profile of the GR isoforms also affects local GR-action, as most studies did not include downstream markers hereof, such as ‘glucocorticoid-induced leucine zipper’ (GILZ) nor GR-co-regulators 11β-HSD1 and FKBP51.22 In addition, it is unclear whether very high systemic glucocorticoid availability, brought about either endogenously or by exogenous administration, results in a proportionate rise in cellular GR-action. Furthermore, the available data on GR isoform and co-chaperone expression in blood cannot be extrapolated to other cells and tissues, as it is well known, at least during health, that expression and function of the GR isoforms is highly cell- and tissue type-specific.14

We hypothesised that during critical illness, in the face of the illness-induced elevated systemic glucocorticoid availability, expression of 11β-HSD1, the GR isoforms -α and -β, and FKBP51 is altered in an adaptive, tissue-specific and possibly time-dependent manner, to titrate local GR-action depending on the tissue-specific needs. In addition, we hypothesised that further increasing systemic glucocorticoid availability not necessarily results in increased local GR-action as it may also drive further adaptive alterations in the expression profile of 11β-HSD1, the GR isoforms, and FKBP51 that prevent such an increased action.

**Methods**

**Human study**

**Study participants and sample size calculation.** To study the impact of critical illness, and the duration hereof, on the expression and actions of the GR in immune cells (separately and freshly isolated neutrophils and monocytes), skeletal muscle, and (subcutaneous) adipose tissue, in relation to the level of systemic glucocorticoid availability, we made use of samples collected in the CROSS trial. This is a prospective, observational, cross-sectional study performed in five medical/surgical intensive care units (ICUs), part of a single division of the University Hospitals of Leuven, Belgium, and in demographically-matched healthy control subjects designed to study the dynamics of epigenetic, metabolic and endocrine alterations during critical illness (ISRCTN17621057). Between 11/1/2017 and 03/09/2020, all adult (>18 years of age) patients admitted to one of the participating ICUs were screened for eligibility (exclusion criteria of the main study: referral from another non-participating ICU, readmission to the ICU within the same hospitalization period, pre-admission treatment with systemic glucocorticoids, no vital organ support or no arterial or venous catheter, ‘do not resuscitate’ code interfering with current needed life support, patients with human immunodeficiency virus or hepatitis C virus). For the current study, all patients included in the trial and in whom a skeletal muscle biopsy was performed, were included (exclusion criteria/contraindications to perform a skeletal muscle biopsy: history of neuromuscular disorders or admission because of a neuromuscular disorder, unable to walk without assistance prior to ICU admission, increased bleeding risk during procedure including a known coagulation disorder, platelet count below 50000/mm³, prothrombin time activity below 40% or therapeutic use of anticoagulating or thrombolytic agents). To study the impact of duration of illness, patients were clustered in one of four predefined time cohorts, based on the day of sampling (cohort 1: ICU admission to ICU-day 3, cohort 2: ICU-day 4 to ICU-day 7, cohort 3: ICU-day 8 to ICU-day 14, cohort 4: ICU-day 15 to ICU-day 28; individual sampling days per patient are displayed in supplementary Figure 1). Based on previous studies on GRα expression in blood cells, we estimated that 30 patients per cohort would allow to detect a 50% decrease in GRα expression with a Cohen d effect size of 0.74, an α-error <0.05 and >80% power.17 Due to the outbreak of the Coronavirus Disease 2019 (COVID-19) pandemic, further patient recruitment became difficult, necessitating an early stop. At that time, sample size in each cohort had reached between 20 and 47 participants, which allowed to detect a Cohen d effect size of 0.51 with an alpha error <0.05 and >80% power.

**Data and sample collection.** Demographics and illness characteristics at ICU admission were documented (Table 1). At the day of testing, whole blood was drawn from the arterial line of patients, and via a single venous puncture from healthy control subjects. Blood samples
were collected in the morning in pre-chilled EDTA tubes and centrifuged at 4°C. Obtained plasma samples were stored at −80°C until analysis. Neutrophils (Cluster of differentiation (CD)66B+ CD16+) and classical monocytes (CD14+) were immediately and separately isolated using immunomagnetic negative selection kits from EasySep™, as per manufacturer’s instruction (Stemcell Technologies). Obtained cell concentrations were measured with Scepter™ 2.0 cell counter (Merck) with 60° technologies. Obtained cell concentrations were measured with competitive radio-immunoassay (Immunotech cat. no. IM1841 and DIAsource cat. no. R-AJ-100, respectively). Plasma concentrations of albumin were measured with bromocresol green colorimetric method (Sigma-Aldrich, cat. no. MAK124-1KT). Plasma free cortisol was estimated using the previously validated adapted Coolens’ formula23, 24:

\[ \sqrt{\frac{0.0167 + (G - T) \frac{1}{2(1 + N^2)}}{2(1 + N^2)}} + T \frac{1}{(1 + N^2)K} - 0.0167 + (G - T) \frac{1}{2(1 + N^2)} \]

where G = plasma CBG concentration (in µmol/L), T = plasma total cortisol concentration (in µmol/L), K = affinity of CBG for cortisol = 3.107 M⁻¹, and N²=1.74/43 x individual albumin concentration(g/L).

A dichotomized categorical variable for two levels of systemic (free) glucocorticoid availability, with very high versus without very high systemic glucocorticoid availability, was constructed as follows: All patients not receiving synthetic glucocorticoid treatment were allocated either to the category ‘with very high systemic glucocorticoid availability’ when their plasma free cortisol was ≥3.096 µg/dl, which was the 75th percentile of untreated patients, or to the category ‘without very high systemic glucocorticoid availability’ when their plasma cortisol concentration was ≤3.096 µg/dl.

### Table 1: Baseline demographics and illness characteristics.

| Demographics | Cohort 1 N= 47 | Cohort 2 N= 37 | Cohort 3 N= 33 | Cohort 4 N= 20 | Controls N= 20 | p-value |
|--------------|----------------|---------------|---------------|---------------|---------------|---------|
| Age — years  | 64 ± 9 (14-9)  | 61 ± 9 (10-4) | 62 ± 9 (14-4) | 65 ± 3 (14-7) | 59 ± 0 (8-6) | 0.54    |
| Male sex     | 31 (66%)       | 30 (81%)      | 16 (48%)      | 11 (55%)      | 14 (70%)      | 0.05    |
| BMI          | 26 ± 0 (4-5)   | 26 ± 0 (6-2)  | 26 ± 0 (6-9)  | 28 ± 0 (7-8)  | 25 ± 7 (2-8)  | 0.71    |
| History of   |               |               |               |               |               |         |
| Diabetes     | 6 (13%)        | 9 (24%)       | 5 (15%)       | 1 (5%)        | 1 (5%)        | 0.25    |
| Malignancy   | 13 (28%)       | 10 (27%)      | 10 (30%)      | 4 (20%)       | 2 (10%)       | 0.49    |
| APACHE II    | 26 ± 2 (8-1)   | 28 ± 9 (8-5)  | 31 ± 5 (8-0)  | 30 ± 9 (8-3)  | NA            | 0.03    |
| Sepsis on admission | 27 ± 5%       | 21 ± 5%       | 23 ± 7%       | 16 ± 8%       | NA            | 0.22    |
| Cardio-thoracal | 18 (38%)     | 14 (38%)      | 13 (39%)      | 4 (20%)       | NA            | 0.88    |
| Abdominal    | 11 (23%)       | 7 (19%)       | 7 (21%)       | 8 (40%)       |               |         |
| Transplant   | 5 (11%)        | 4 (11%)       | 4 (12%)       | 2 (10%)       |               |         |
| Trauma/Burns | 6 (13%)        | 6 (16%)       | 2 (6%)        | 2 (10%)       |               |         |
| Neurological | 3 (6%)         | 2 (5%)        | 5 (15%)       | 2 (10%)       |               |         |
| Other        | 4 (9%)         | 4 (11%)       | 2 (6%)        | 2 (10%)       |               |         |
| Mortality    |               |               |               |               |               |         |
| In ICU       | 3 (6%)         | 6 (16%)       | 5 (15%)       | 7 (35%)       | NA            | 0.03    |
| In hospital  | 6 (13%)        | 7 (19%)       | 10 (30%)      | 10 (50%)      | NA            | 0.01    |
free cortisol was below this value (Table 2). This threshold of 1·096 µg/dl for plasma free cortisol reflected ±25 µg/dl of plasma total cortisol and a free cortisol fraction of ±12%. Patients who had been treated with hydrocortisone within a window of 48 hours (h) prior to blood and tissue sampling were also allocated to one of the above dichotomised categories based on their concentration of plasma free cortisol. Patients who had received synthetic glucocorticoid treatment within a window of 48 h prior to blood and tissue sampling were all given high doses (cumulative over 48 h always ≥30mg) of (6α-)methylprednisolone which qualified them all for allocation to the category ‘with very high systemic glucocorticoid availability’.

Quantification of GR expression and actions in immune cells, skeletal muscle, and adipose tissue.

RNA was isolated with the RNeasy kit (Qiagen) from the enriched cell suspensions and from +30mg of frozen skeletal muscle and adipose tissue samples. Genomic DNA was removed with DNase treatment. Total RNA (immune cells: 500ng, skeletal muscle: 800ng, adipose tissue: 250ng) was reverse-transcribed with the use of random hexamers (Invitrogen). Commercial (11β-HSD1, FKBP51, GILZ) and custom-made TaqMan probes (GRα and GRβ) (Applied Biosystems) were used for quantitative PCR experiments (Supplementary table S1). All PCR experiments were performed with the use of a QuantStudio3 Real Time PCR system (Applied Biosystems, Thermo Fisher Scientific) as per manufacturer’s instructions. Relative gene expressions were always calculated with the 2−ΔΔCT method with internally validated stable housekeeping genes: for neutrophils, monocytes, subcutaneous adipose tissue: iHS ribosomal RNA (Rn18s); and for human skeletal muscle tissue: CASC3 exon Junction Complex Subunit (CASC3). Genes were considered ‘undetected’ if >50% of all samples were not amplified before PCR cycle 35.

Mouse studies

Study design. To study the impact of 1) duration of critical illness and 2) the level of systemic glucocorticoid availability on the expression and actions of the GR in vital tissues (heart, lung, diaphragm, liver and kidney), which are not accessible in living critically ill human patients, we performed secondary analyses of two previously performed mouse studies of sepsis-induced, fluid-resuscitated, antibiotics- and analgetic-treated and parenterally fed critical illness.25,26 In brief, healthy male, 24-week old C57BL/6J mice (Janvier SAS) were randomly allocated to a ‘critical illness’ or a ‘healthy control’ group. Mice randomised to a ‘critical illness group’ were anesthetised, the left internal jugular vein was cannulated with a catheter, followed by a median laparotomy and cecal ligation and puncture to induce sepsis and subsequently sepsis-induced critical illness. During the first 24h after the procedure, mice were resuscitated with a 4/1 crystalloid/colloid mixture (Plasmalyte, Baxter). Hereafter, septic mice received intravenous parenteral nutrition (Oliclinomel N7E, Baxter). All critically ill mice received twice daily a subcutaneous injection with broad-spectrum antibiotics (Imipenem/Cilastin (Aurobindo Pharma)) and opioid-analgetics (Buprenorphine (Vetergesic)). Animals randomised to the ‘healthy control group’ did not underwent any procedure, were transferred to individual cages and received ad libitum standard chow (ssniff R/M-H, ssniff Spezialdiäten GmbH) and tap water. All animal cages were kept in an animal cabinet under controlled temperature (27°C) and 12 hours light and dark cycles until sacrifice. The animal model is further in detail described in.27

To study the impact of duration of illness (mouse study 1), critically ill mice were further allocated to 1 of 4 predefined time cohorts, corresponding to a duration of illness of one, three, five or seven days until sacrifice and tissue harvesting. Comparisons were done between each critical illness time cohort group and healthy control mice. The allocation procedure was randomised...
and done prior to the sepsis-inducing surgical procedure.

To study the impact of further increasing systemic glucocorticoid availability (mouse study 2), critically ill mice were allocated to a treatment or control group, receiving either a 7-day treatment with hydrocortisone [HC, daily dose of 1-2mg/day, corresponding to a human equivalent dose of 3-25 mg per kg body weight/day,26 which is in the range of the recommended human “stress doses”8,9] or 7-day treatment with placebo. The study drug was administered for 7 days via a subcutaneous osmotic pump (ALZET Osmotic Pumps), implanted just below the right scapula during the sepsis-inducing surgical procedure. Randomization for treatment was blinded until the end of the study (data analysis). Comparisons were done between hydrocortisone-treated and placebo-treated critically ill mice. Healthy controls were included for methodological purpose (normalization of gene expression).

All animals were regularly checked for the presence of humane endpoints. At any time point, when animals got into irreversible metabolic disorders, were in a bad condition (i.e. lack of mobility, eyes closed behavior, no reaction on stimuli and breathing difficulties), they were excluded from the study and euthanised by intraperitoneal injection of Dolethal (min 0.03ml/30g). At the end of each of the study periods, all surviving animals were sacrificed and whole blood and tissue samples were collected, snap frozen and stored at -80°C until analysis. Total numbers of animals per group for mouse study 1 were as follows: Healthy controls: n=15, Sepsis 1-day: n=15, Sepsis 3-days: n=16, Sepsis 5-days: n=16, Sepsis 7-days: n=15; and for mouse study 2: healthy controls: n=11, HC-treated critical illness: n=12, Placebo-treated critical illness: n=14.

The animal studies were designed and performed to investigate two separate research questions. First, the impact of duration of illness (study 1) and the impact of further increasing systemic glucocorticoid availability during critical illness (study 2) on the functioning of the hypothalamic-pituitary-adrenocortical axis during critical illness. Second, the impact of duration of illness (study 1) and the impact of further augmenting systemic glucocorticoid availability during critical illness (study 2) on the expression and signaling of the glucocorticoid receptor during critical illness. The needed sample size was calculated for the first research question. For the second research question, which is investigated in this paper, we used all available samples. Given these sample sizes, calculated for the first research question (animal study 1: 15 per group; animal study 2: 12-14 per group),25,26 the minimum effect size (Cohen’s d) that yields a significant result (α=0.05) with a power of 0.8 (β=0.2) is 1.09 and 1.18, indicating that any significant findings will be rather large in effect size.

Quantification of GR expression and actions in vital tissue
After sacrifice, heart, lungs, diaphragm, liver, and kidneys were harvested and immediately snap-frozen in liquid nitrogen and stored at -80°C for analysis. Total RNA (500ng) was reverse-transcribed with the use of random hexamers (Invitrogen). Commercial TaqMan probes (11β-HSD1, FKBP51, GILZ) for Taqman quantitative PCR experiments (Applied Biosystems) and customised forward and reverse primers (GRα and GRβ) for SYBR Green quantitative PCR experiments (Eurogentec) were used (in detail: supplemental table S2). All mouse PCR experiments were performed as described above, with validated stable house keeping genes: for skeletal muscle, epididymal adipose tissue, heart, lung, kidney, liver: 18S ribosomal RNA (Rn18s) and for diaphragm muscle: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Ethics
The human study protocol was in accordance with the 1964 Declaration of Helsinki and its later amendments and was approved by the Institutional Ethical Review Board of UZ Leuven (S58533). Written informed consent was obtained from all participants.

For the animal studies, all animals were treated according to the Principles of Laboratory Animal Care (U.S. National Society of Medical Research) and to the European Union Directive 2010/63/EU concerning the welfare of laboratory animals. The studies were designed and prepared before the start of the study and approved by the KU Leuven Institutional Ethical Committee for Animal Experimentation of KU Leuven (P134-2013 and P181-2018) and complied with the essential 10 ARRIVE guidelines.29

Statistical analyses
Data are presented as numbers (proportions), medians (IQR), or mean (SD) and detailed in the figure legends and table footnotes. For univariable analysis, continuous data were compared with non-parametric Mann-Whitney U or Kruskal-Wallis test, as appropriate and categorical data with Chi-square or Fisher exact test, as appropriate. Multivariable linear regression models were constructed to assess the independent association between the level of systemic glucocorticoid availability and expression of the studied genes, and the association between sepsis at admission and expression of the studied genes. The models are adjusted for a priori selected confounders as co-variates (prior knowledge). The confounders were identified following a systematic literature search. The Medline database was searched through PubMed for full-text, original human research articles (prospective, retrospective observational studies, randomised controlled trials, reviews
and meta-analyses) reporting on the association between demographic factors and (tissue-specific) glucocorticoid receptor expression, in healthy controls or patients, as well between illness characteristics and glucocorticoid receptor expression, in ICU patients, published in the last ten years (up to December 2021).

Used search terms: Search 1 (demographics): ((demographic OR anthropometric OR age OR gender OR sex OR BMI) AND ("Glucocorticoid Receptors"[Mesh] OR GR OR (GR alpha)) AND ((expression) OR (gene

---

**Figure 1.** Plasma glucocorticoid concentrations (cortisol/corticosterone) in the human and two mouse studies.

Panel a: plasma free cortisol concentrations (y-axis) of the healthy controls and human patients of each time cohort (x-axis). Each dot represents a sample. White dots are healthy controls, green dots are patients who did not receive glucocorticoids (“No GC treatment”), red dots are patients who received treatment with natural glucocorticoids (“Treatment with hydrocortisone”) and blue dots are patients who received treatment with synthetic glucocorticoids (“Treatment with methylprednisolone”). The a priori defined cut-off to discriminate between patients with and patients without very high systemic glucocorticoid availability (3.096 µg/dl) is displayed as a solid black horizontal line; all patients treated with synthetic glucocorticoids are categorized into the ‘with very high systemic glucocorticoid availability’ group. Panel b: plasma total corticosterone concentrations (y-axis) of 1) (left side, mouse study 1) healthy control mice and critically ill mice per time cohort (x-axis) and 2) (right side, mouse study 2) of placebo-treated critically ill mice and hydrocortisone-treated critically ill mice (x-axis).
regulation) OR (gene signaling)) AND (determinants OR confounder OR variables OR association)) ; and Search 2 (illness characteristics): ("Critical Illness"[Mesh] OR "Critical Care"[Mesh] OR "Intensive Care Units"[Mesh] OR "Shock"[Mesh] OR "Systemic Inflammatory Response Syndrome"[Mesh] OR "Multiple organ failure"[Mesh] OR critical-illness* OR critical-care* OR intensive-care* OR shock* OR Systemic-Inflammatory-Response-Syndrome* OR multiple-organ-failure*) AND ("Glucocorticoid Receptors"[Mesh] OR GR OR (GR alpha)) AND ((expression) OR (gene regulation) OR (gene signaling)) AND (determinants OR confounder OR variables OR association)). A confounder was defined as a factor identified from literature or prior knowledge to be associated with altered expression or signaling of one of the studied parameters (11β-HSD1, GR isoforms, GILZ, FKBP51). The identified and selected confounders are baseline characteristics: age, gender, BMI and illness characteristics: APACHE II, sepsis and diagnostic category at admission and duration of illness at the time of sampling (~time cohorts).17,20,30-39 All analyses were performed with JMP 16.0 (SAS). Two-sided p values ≤0.05 were considered statistically significant.

Role of funders
The funders of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or the decision to submit for publication. The corresponding author had full access to all data and had final responsibility for the decision to submit for publication. Authors were not precluded from accessing data in the study.

Results

Human study
From January 11, 2017 to September 3, 2020, a total of 137 patients, clustered in four distinct time-cohorts, and 20 age-, sex- and BMI-matched healthy controls, were included in the human study. Demographics and markers of illness severity are summarised in Table 1. Plasma concentrations of free cortisol (human study) and plasma total corticosterone (mouse studies) are displayed in Figure 1, respectively panel a and panel b. The proportion of patients receiving glucocorticoid treatment within a time window of 48h prior to sampling as well as the proportion of patients with and patients without very high systemic glucocorticoid availability on the day of sampling was comparable across all time-cohorts (Table 2).

In peripheral blood neutrophils and monocytes of both patients and healthy controls, mRNA of 11β-HSD1 was undetectable.

In neutrophils of critically ill patients, gene expression of GRα was suppressed in all time cohorts as compared with healthy controls (figures 2 and 3). Gene expression of the glucocorticoid-induced GRα-affinity reducing GR-co-chaperone FKBP51 was increased in neutrophils of patients in time cohorts 1 and 2 but not of patients in time cohorts 3 and 4. Gene expression of the GR-target gene and marker of glucocorticoid activity, GILZ, was always suppressed in neutrophils of patients.

In monocytes, GRα mRNA was suppressed in patients of time cohort 2 and 3, but not in time cohort 1 and 4 (figures 2 and 3). Monocyte FKBP51 mRNA was always increased in patients of all time cohorts, which was also the case for GILZ mRNA.

Adjusted for confounders including the time cohort, the multivariable regression analysis revealed that patients with very high systemic glucocorticoid availability (n=80), as compared with patients without such very high systemic glucocorticoid availability (n=47), had comparable expression levels of GRα and GILZ but higher expression of FKBP51 in both white blood cell types (Figures 2 and 3; Table 3).

In skeletal muscle of critically ill patients, gene expression of 11β-HSD1, GRα, FKBP51 or GILZ in neutrophils or circulating monocytes (Table 4).

In subcutaneous adipose tissue of patients, 11β-HSD1 and GRα mRNA gene expression was suppressed in all time cohorts, whereas FKBP51 and GILZ mRNA were always normal (Figures 3 and 4).

Adjusted for risk factors, skeletal muscle of patients with very high systemic glucocorticoid availability, as compared with those without such very high systemic glucocorticoid availability, showed a further suppression of 11β-HSD1 and GRα mRNA, while FKBP51 mRNA was increased and GILZ mRNA was unaltered (Figures 3 and 4; Table 3). In adipose tissue of patients, such very high systemic glucocorticoid availability did not further suppress 11β-HSD1 and GRα mRNA, whereas FKBP51 and GILZ mRNA were increased (Figures 3 and 4; Table 3).

Adjusted for risk factors, sepsis at admission, as compared with other diagnoses, was not independently associated with any difference in gene expression of 11β-HSD1, GRα, FKBP51 or GILZ in skeletal muscle or adipose tissue (Table 4). In all cells and tissues from patients and controls, GRβ mRNA was undetectable.
Figure 2. Gene expression of glucocorticoid receptor expression and action in circulating cells.
Mouse studies
In skeletal muscle of critically ill septic mice, 11β-HSD1, GRα and FKBP51 mRNA was always normal or increased. As in the human patients, muscle GILZ mRNA was increased in all septic mice without an additional enhancing effect of further increasing systemic glucocorticoid availability (Figure 3, Supplemental-Figure 2).

In adipose tissue of septic mice, gene expression of 11β-HSD1 and GRα was decreased or normal, while FKBP51 mRNA was always increased. As in the human patients, adipose GILZ mRNA in septic mice was only increased when the systemic glucocorticoid availability was further increased (hydrocortisone-treated septic mice).

In heart of critically ill mice, gene expression of 11β-HSD1 and FKBP51 was always several-fold upregulated, GRα mRNA was suppressed and GILZ mRNA was increased, without a clear impact of duration of illness (Figure 3, Supplemental-Figure 3). Seven-days infusion of stress dose of hydrocortisone during critical illness further increased heart FKBP51 mRNA, without any effect on 11β-HSD1 mRNA, GRα mRNA or GILZ mRNA.

In lung of critically ill mice, 11β-HSD1 mRNA was always increased, while GRα mRNA was unaltered in the 3 first time cohorts and increased in time cohort 4 (Figure 3, Supplemental-Figure 3). Lung FKBP51 mRNA and GILZ mRNA levels were always increased in critically ill mice. Hydrocortisone treatment did not affect lung 11β-HSD1 or GRα mRNA but further increased FKBP51 and GILZ mRNA.

In diaphragm of critically ill mice, 11β-HSD1 mRNA was normal or upregulated (time cohort 2 only) and GRα mRNA was normal (time cohort 1 and 4) or downregulated (Figure 3, Supplemental-Figure 4). Both FKBP51 and GILZ mRNA were always upregulated during critical illness. Hydrocortisone treatment did not affect diaphragm 11β-HSD1 or GRα mRNA, but further increased FKBP51 and GILZ mRNA.

In kidney of critically ill mice, 11β-HSD1 mRNA was increased in time cohort 1 but suppressed in time cohorts 2, 3 and 4 (Figure 3, Supplemental-Figure 4).

While kidney GRα mRNA was always suppressed, FKBP51 and GILZ mRNA was always increased in critically ill mice. Hydrocortisone treatment only further increased kidney FKBP51 mRNA.

In liver of critically ill mice, 11β-HSD1 mRNA was always suppressed, while GRα mRNA was always normal and FKBP51 always increased (Figure 3, Supplemental-Figure 5). Liver GILZ mRNA was transiently suppressed in time cohort 1 followed by an increase in time cohort 2, 3 and 4. Hydrocortisone treatment only further increased kidney FKBP51 mRNA.

In all vital tissues GRβ mRNA was always similar between critically ill mice and healthy controls and between hydrocortisone-treated and placebo-treated critically ill mice.

Discussion
The prospective, observational, cross-sectional human and the two translational mouse studies together revealed that during critical illness, expression of regulators of local glucocorticoid availability and GR-action is altered in a tissue-specific, largely time-independent manner. GR-action during critical illness was found to be clearly suppressed in neutrophils, likely due to the substantial suppression of the GRα. In contrast, most tissues showed higher than normal GR-action, kept in balance by a tissue-specific regulation of local GR-ligand availability and GRα expression. Very high systemic glucocorticoid availability and hydrocortisone treatment further increased GR-action only in human adipose tissue and murine adipose tissue, lung and diaphragm, respectively. These results argue against a generalised critical illness-induced state of glucocorticoid resistance which can be overcome by stress doses of hydrocortisone. Instead, the observed tissue specific alterations can be interpreted as adaptively guiding vital actions of glucocorticoids to tissues with increased need during critical illness, such as the heart, kidneys, liver and even more so the lungs, whereas neutrophils are fully protected and vulnerable tissues such as skeletal muscle partially protected against harmful effects of high systemic glucocorticoid availability.

In the left panels, gene expression of GRα, FKBP51 and GILZ in neutrophils of patients and controls. In the right panels, gene expression of GRα, FKBP51 and GILZ in monocytes of patients and controls. Box-and-whiskers represent median, interquartile range (IQR), and the furthest points within 1.5 times the IQR. Gray bars represent IQR of the matched-healthy controls. P-values above each box-and-whiskers plot indicates significance between the respective time cohort group of critically ill patients (1, 2, 3 and 4 from light blue to darker blue) and the matched-healthy controls. P-values above GC+++ indicates significance between patients with and patients without very high systemic glucocorticoid availability (univariate / multivariate*). For the comparison between patients with and patients without very high systemic glucocorticoid availability, time cohorts were pooled together. Number of patients/samples per group, for the time cohorts: time cohort 1 (admission to ICU day 3) n=47, time cohort 2 (ICU day 4 to 7) n=37, time cohort 3 (ICU day 8 to 14) n=33, time cohort 4 (ICU day 15 to 28) n=20, healthy control n=20; for impact of systemic glucocorticoid availability: without very high systemic glucocorticoid availability n=80, with very high systemic glucocorticoid availability n=57; ADM: admission; AU: arbitrary unit; D: day; GC+: patients without very high systemic glucocorticoid availability, GC+++ patients with very high systemic glucocorticoid availability; IQR: interquartile range.
First, we corroborated findings of earlier experimental (in vitro), animal and small observational human studies revealing a critical illness-induced decrease in GRa expression in peripheral blood cells, and we further identified that mainly neutrophils and to a lesser extent monocytes drive this finding.17,18,40,41 We found that GRa expression was also suppressed in most other glucocorticoid-target tissues, including skeletal muscle, adipose tissue, heart, diaphragm and kidney, but not in lung or liver. In contrast to previous studies,17,39 we did not find a substantial effect of illness duration on GRa gene expression in any of the studied cells or tissues.

Next, we found that very high systemic glucocorticoid availability in critically ill patients and mice, because of high endogenous plasma free glucocorticoid levels or because of exogenous treatment with glucocorticoids, was independently associated with further lowered GRa expression in skeletal muscle, but not in any other cell or tissue type. The relation between systemic glucocorticoid availability and GRa gene expression during critical illness thus appears to be tissue-specific, which may explain the discrepancies in observed associations between plasma cortisol and GRa gene expression across other studies, as these studies each investigated either only a single or a different type of cell or tissue.17,39,42-44 Whereas in our human study, in each cell and tissue type and in both patients and controls, GRb mRNA was not detectable, in the mouse studies the expression of the GRb isoform was found to be normal in all tissues. Together, these data do not support an important role for the dominant negative GRb isoform in altering glucocorticoid action during critical illness.

In contrast with the GRa, expression of GILZ, a well-known GR target gene and marker of genomic action hereof,45 was only robustly suppressed in neutrophils but not in other cell or tissue types. In contrast, GILZ expression was upregulated during critical illness in monocytes, skeletal muscle and diaphragm, heart, lung, kidney and liver, a finding that strongly advocates...
against presence of total glucocorticoid resistance within these target tissues. Interestingly, in lung, diaphragm and adipose tissues, but not in other cells or tissues, further increasing glucocorticoid availability also further increased GILZ mRNA. In lung, the combination of normal to elevated GRα mRNA, an increased GILZ mRNA and the responsiveness of GILZ to very high systemic glucocorticoid availability, suggests that especially lung tissue could benefit from increasing systemic glucocorticoid availability during critical illness. These findings are supported by the clinical use and recommendation regarding the use of glucocorticoids in critically ill patients suffering from acute respiratory distress syndrome (ARDS).4,46 In contrast, in skeletal muscle, a tissue that appeared ‘not responsive’ to a further increase in circulating abundance of the GR-ligand, gene expression of the regulators of local glucocorticoid availability and GR-action, 11β-HSD1 and GRα, was substantially suppressed and even further downregulated in patients with very high systemic glucocorticoid availability. This suggests that skeletal muscle could be harmed by too much GR-action and that these counteracting mechanisms in response to further increasing systemic glucocorticoid availability are activated to prevent such harm. In addition, FKBP51, which is upregulated by glucocorticoids but lowers GR ligand-binding capacity and nuclear translocation (ultra-short feedback-loop), was always further upregulated in response to a further increase in systemic glucocorticoid availability. Together, these data indicate that not only GRα gene expression, but also regulators of local glucocorticoid availability and GR-action are altered in a tissue-specific manner in response to the magnitude of the systemic glucocorticoid availability.

Our studies have several limitations to highlight. First, although GILZ is highly regulated by ligand-bound GRα (GR target gene) and may be considered as a surrogate of GR-action, we did not investigate the functional impact of the altered GR-expression and -action profiles, such as altered activation of catabolic pathways within the target cells and tissues or alterations in the expression and function of inflammatory and immune pathways. Second, despite the well-balanced demographics and illness characteristics across the time cohorts of the patients and the carefully selected co-variates for adjustment in the multivariable regression models, the observational nature of the human study does not allow to draw firm conclusions regarding causality. In addition,
we did not perform additional exploratory analyses, such as investigating the impact of age, gender, ethnicities or co-morbidities on GR-expression or -action as this was not part of the a priori defined statistical protocol. Third, despite the large number of human patients and controls, the predefined sample size per time cohort could not be reached as a result of the COVID-19 pandemic. The larger size of detectable effects may have resulted in a higher risk of type 2 errors. Fourth, despite the clinical relevance and earlier validation of the mouse model of sepsis-induced, fluid-resuscitated, antibiotics- and analgetics-treated and parenterally fed critical illness27, we were unable to investigate in the mice subcutaneous adipose tissue (epididymal adipose tissue was used instead) or additional important vital tissues including the various compartments of the vasculature.

In conclusion, throughout critical illness, GR-action was found to be clearly suppressed in neutrophils, likely due to near-maximal suppression of GRα expression. GR resistance that could not be overcome by further increasing glucocorticoid availability. In contrast, in most other vital tissues, GR-action in the critically ill was higher than normal. In adipose tissue, lung and diaphragm, further increasing systemic glucocorticoid availability resulted in increased GR-action, while in other tissues, most clearly shown for skeletal muscle, counter-regulatory mechanisms (11β-HSD, GRα and/or FKBP51 expression) were activated to prevent a further increase in GR-action. Together, these findings argue against a state of generalised glucocorticoid resistance that would necessitate treatment with stress doses of glucocorticoids, as proposed in the CIRCI guidelines34. Instead, the data provide evidence for tissue-specific adaptations that guide glucocorticoid action to sites of need while protecting at least partially against collateral undesirable effects.

Figure 4. Gene expression of glucocorticoid receptor expression and action in peripheral tissue.

In the left panels, gene expression of 11β-HSD1, GRα, FKBP51 and GILZ in skeletal muscle of patients and controls. In the right panels, gene expression of 11β-HSD1, GRα, FKBP51 and GILZ in subcutaneous adipose tissue of patients and controls. Box-and-whiskers represent median, interquartile range (IQR), and the furthest points within 1.5 times the IQR. Gray bars represent IQR of the matched-healthy controls. P-values above each box-and-whiskers plot indicates significance between the respective time cohort group of critically ill patients (1, 2, 3 and 4 from light blue to darker blue) and the matched-healthy controls. P-values above GC+++ indicates significance between patients with and patients without very high systemic glucocorticoid availability (univariate / multivariate*). For the comparison between patients with and patients without very high systemic glucocorticoid availability, time cohorts were pooled together. Number of patients/samples per group, for the time cohorts: time cohort 1 (admission to ICU day 3) n=47, time cohort 2 (ICU day 4 to 7) n=37, time cohort 3 (ICU day 8 to 14) n=33, time cohort 4 (ICU day 15 to 28) n=20, healthy control n=20; for impact of systemic glucocorticoid availability: without very high systemic glucocorticoid availability n=57; ADM: admission; AU: arbitrary unit; D: day; GC+: patients without very high systemic glucocorticoid availability, GC+++: with very high systemic glucocorticoid availability; IQR: interquartile range.

Contributors
AT, LVD, IV, LL and GVdB conceptualised the study. AT, LVD, NVA, SVP, LP, ID, YD did the data curation. PJW was responsible for project administration. AT, LL and GVdB did the formal analysis, investigation and wrote the original draft. All authors wrote, reviewed and edited the manuscript. IV, LL, GVdB acquired the funding. AT, LL and GVdB had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final version of the manuscript.

Data sharing statement
The individual-level data used in this study cannot be publicly shared due to ethical approval. Data sets and other documents may be shared under the format of future collaborative projects that further elaborate on this specific research topic. Proposals for collaborative projects must be directed to the corresponding author and will be considered for approval.

Declaration of interests
We declare no competing interests.

Acknowledgements
The authors acknowledge the support of the European Respiratory Society (ERS Gold Medal in ARDS to GVdB), the Research Foundation-Flanders (FWO Grant G091918N to GVdB), the Methusalem Program of the Flemish Government (METH/14/06 to GVdB, LL and IV via KU Leuven), and a European Research Council Advanced Grant (AdvG-2017-785809 to GVdB), from the European Union’s Horizon 2020 research and innovation programme. The authors thank the patients and healthy controls for their willingness to participate in the study.

Supplementary materials
Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2022.104057.
References

1. Teblick A, Peeters B, Langouche L, Van den Bergh G. Adrenal function and dysfunction in critically ill patients. Nat Rev Endoci- 
   nol. 2019;15(7):477–487.

2. Mark PE. Critical illness-related corticosteroid insufficiency. Chem. 
   2000;9(3):181–193.

3. Annane D, Pastores SM, Arlt W, et al. Critical illness-related corti-
   costeroid insufficiency (CIRCI): a narrative review from a Multispe-
   cial Task Force of the Society of Critical Care Medicine (SCCM) 
   and the European Society of Intensive Care Medicine (ESICM). 
   Intensive Care Med. 2017;43(12):1751–1762.

4. Annane D, Pastores SM, Rochwerg B, et al. Guidelines for the diag-
   nosis and management of critical illness-related corticosteroid insufficiency (CIRCI) in critically ill patients (Part I): Society of 
   Critical Care Medicine (SCCM) and European Society of Intensive Care Medicine (ESICM) 2017. Intensive Care Med. 
   2017;43(12):1751–1762.

5. Annane D, Sebbini V, Charpentier C, et al. Effect of treatment with 
   low doses of hydrocortisone and fludrocortisone on mortality in 
   patients with septic shock. JAMA. 2003;289(7):862–871.

6. Sprung CL, Annane D, Keh D, et al. Hydrocortisone therapy for 
   patients with septic shock. N Engl J Med. 2008;358(23):2114–2124.

7. Annane D, Renaud A, Brun-Buisson C, et al. Hydrocortisone plus 
   fludrocortisone for adults with septic shock. N Engl J Med. 
   2018;378(9):897–908.

8. Venkatsh E, Finder S, Cohen J, et al. Adjunctive glucocorticoid 
   therapy in patients with septic shock. N Engl J Med. 2018;378(9):
   797–808.

9. Evans L, Rhodes A, Alhazzani W, et al. Surviving sepsis campaign: 
   international guidelines for management of sepsis and septic shock 2021. Intensive Care Med. 2021.

10. Iglesias J, Vassallo AV, Patel VV, Sullivan JB, Cavanaugh J, Elbaga 
    Y. Outcomes of metabolic resuscitation using ascorbic acid, thia-
    mine, and glucocorticoids in the early treatment of sepsis: the 
    ORANGES Trial. Cht. 2020;78(3):164–173.

11. Sevransky JE, Rothman RE, Hager DN, et al. Effect of vitamin C,
    thiamine, and glucocorticoids, and vitamin B1 on longer-term mortality in adults 
    with septic shock. Crit Care Med. 2012;40(1):141–144.

12. Fujii T, Salanti G, Agapitidis E, et al. Adrenal aging and its 
    implications on stress responsiveness in humans. Front Endocrinol 
    (Lausanne). 2019;10:34.

13. Quinn M, Ramamoorthy S, Cidlowski JA. Sexually dimorphic 
    actions of glucocorticoids: beyond chromosmes and sex hor-
    mones. Ann N Y Acad Sci. 2014;1317:1–6.

14. Bale TL, Epperson CN. Sex differences and stress across the life-
    span. Nat Neurosci. 2015;18(1):141–1420.

15. Moore SR, Hallborsdottir T, Martins J, et al. Sex differences in the 
    genetic regulation of the blood transcriptome response to glucocor-
    ticoi receptor activation. Transl Psychiatry. 2021;11(1):63.

16. Woods CP, Corrigian M, Gathercole I, et al. Tissue specific regula-
    tion of glucocorticoids in severe obesity and the response to signifi-
    cant weight loss following bariatric surgery (BARI2CORT). J Clin Endocrinol Metab. 2015;100(4):1414–1444.

17. Cheng T, Dimitrov S, Pruitt C, Hong S. Glucocorticoid mediated 
    regulation of inflammation in human monocytes is associated with 
    depressive mood and obesity. Psychoneuroendocrinology. 2016;69:105–204.

18. Morratis AG, Block T, Nguyen D, Belanoff JK. The role of glucocor-
    ticoi receptors in metabolic syndrome and psychiatric illness. J 
    Steroid Biochem Mol Biol. 2017;165:114–120. Pt A.

19. Vassilou AG, Stamouguouros G, Jalal E, et al. Longitudinal evalua-
    tion of glucocorticoid receptor alpha/beta expression and signal-
    ing, adrenocortical function and cytokines in critically ill steroid-
    free patients. Mol Cell Endocrinol. 2015;391:1126–1136.

20. Mohsin GJ, Koper JW, van Uffenen CJ, et al. Temperature-induced down-regulation of glucocorticoid receptor in peripheral 
    blood mononuclear leucocyte in patients with sepsis or septic shock. Clin Endocrinol (Oxf). 1999;51(3):279–280.

21. Abraham MW, Jimenez DM, Fernandes TD, Deutschman CS. 
    Cecal ligation and puncture alters glucocorticoid receptor expres-
    sion in mice: a promising marker for monitoring and treating sepsis. Front Immunol. 2020;11:66649.

22. Bereszhchenko O, Migliorati G, Bruscoli S, Riccardi C. Glucocorti-
    coid-induced leucine zipper: a novel anti-inflammatory molecule. 
    Front Pharmacol. 2015;6:1038.