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Glucocorticoid Receptor Expression in Peripheral WBCs of Critically Ill Children*

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Objectives: To characterize glucocorticoid receptor expression in peripheral WBCs of critically ill children using flow cytometry.

Design: Prospective observational cohort.

Setting: A university-affiliated, tertiary PICU.

Patients: Fifty-two critically ill children.

Interventions: Samples collected for measurement of glucocorticoid receptor expression and parallel cortisol levels.

Measurements and Main Results: Subjects with cardiovascular failure had significantly lower glucocorticoid receptor expression both in CD4 lymphocytes (mean fluorescence intensity, 522 [354–787] vs 830 [511–1,219]; $p = 0.036$) and CD8 lymphocytes (mean fluorescence intensity, 686 [350–835] vs 946 [558–1,511]; $p = 0.019$) compared with subjects without cardiovascular failure. Subjects in the upper 50th percentile of Pediatric Risk of Mortality III scores and organ failure also had significantly lower glucocorticoid receptor expression in CD4 and CD8 lymphocytes. There was no linear correlation between cortisol concentrations and glucocorticoid receptor expression.

Conclusions: Our study suggests that patients with shock and increased severity of illness have lower glucocorticoid receptor expression in CD4 and CD8 lymphocytes. Glucocorticoid receptor expression does not correlate well with cortisol levels. Future studies could focus on studying glucocorticoid receptor expression variability and isoform distribution in the pediatric critically ill population as well as on different strategies to optimize glucocorticoid response. (*Pediatr Crit Care Med* 2015; 16:e132–e140)

Key Words: adolescent; child; critical care; flow cytometry; glucocorticoid receptors; infant

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Severe sepsis and septic shock are a significant cause of morbidity and mortality in patients admitted to ICUs (1–3). Adjunctive systemic corticosteroids can be used in the presence of shock with cardiovascular failure (CV Failure) in an attempt to improve hemodynamics, but there is still controversy regarding its efficacy and indications (4–6), and even when the pediatric septic shock population was stratified by mortality risk, the analysis did not show benefit from systemic steroids administration (7). Two large, randomized, controlled trials of cortisol replacement therapy in patients with septic shock have shown opposite results regarding survival benefit (8, 9). Currently, the recommendation from the Surviving Sepsis Campaign 2012 is to administer stress doses of hydrocortisone for patients with catecholamine-resistant shock or patients with suspected or proven absolute adrenal insufficiency (10).

One of the possible causes for the conflicting results found in these trials may lie in the fact that the individual patient response to steroids is variable, and there is no consensus regarding who would benefit from its use and how to properly identify these patients (11).

Over a decade ago, Marik et al (12) introduced the concept of critical illness–related corticosteroid insufficiency (CIRCI) as a condition in which the level of endogenous cortisol is thought to be low relative to the degree of illness severity. It has

been described in association with a broad spectrum of critical illnesses, including septic shock (8), acute respiratory distress syndrome (13), traumatic brain injury (14), liver failure (15), burns (16), pancreatitis (17), and following cardiopulmonary bypass (18). With the new CIRCI definition, the field of critical care medicine aimed to enter an era of personalized medicine through the use of a simple and quick method, the adrenocorticotropic hormone (ACTH) stimulation test, to identify individuals who would specifically have a better response to the use of adjunctive steroid therapy (8, 19), but subsequent studies failed to demonstrate its efficacy at identifying a subpopulation that would clearly benefit from systemic steroids culminating in the most recent surviving sepsis campaign guidelines not recommending routine use of ACTH stimulation tests (9, 10).

Therefore, investigators have more recently sought alternative mechanisms that may account for interpatient corticosteroid response variability and perhaps develop new diagnostic tools by evaluating peripheral steroid resistance and cortisol metabolism (20–23).

In order to act, circulating cortisol has to diffuse across the cell membrane and bind to the intracellular cytosolic glucocorticoid receptor (GCR)-α. The cortisol-GCR complex then migrates to the nucleus where it inhibits the transcription of inflammatory genes by nuclear factor κ-light-chain-enhancer of activated B cells or activator protein-1, thus inhibiting the production of inflammatory cytokines and intracellular adhesion molecule-1 (24, 25). This could imply that the extent of its effect is proportional to the GCR expression, subtype, and affinity in a determined target cell (26).

Using genome-wide expression profiling, we recently reported a subclass of children with septic shock characterized by decreased expression of a group of genes corresponding to the GCR signaling pathway (27–29). This subclass of patients had a higher level of illness severity and a higher mortality rate compared with two other identified gene expression–based subclasses. We hypothesized that a subset of critically ill patients with cardiovascular dysfunction is characterized by decreased expression of the GCR and that this group has worse severity of illness measured by Pediatric Risk of Mortality (PRISM) III (30) and multiple organ failure (OF) burden (31). We performed a prospective, observational cohort study to characterize GCR expression in peripheral WBCs of critically ill pediatric patients.

METHODS

Patients and Data Collection

The study protocol was approved by Cincinnati Children's Hospital Medical Center (CCHMC) Institutional Review Board and written informed consent was obtained from a parent or legal guardian for each enrolled patient. Subjects were eligible if they were admitted to the PICU at CCHMC and had an indwelling catheter (central venous catheter or an arterial catheter) from which blood samples could be obtained. Patients were excluded from the study if informed consent was not obtained or if the attending physician did not approve enrollment.

After initial enrollment, blood samples were obtained in the first 24 hours of admission to the PICU for GCR flow cytometry analysis and random serum cortisol levels. Clinical and laboratory data were prospectively collected daily until discharge from PICU using a standardized paper based collection form. The following variables were evaluated initially: CV Failure (defined as the need for vasoactive or inotropic drug support at the admission day), use of steroids (defined as administration of any dose of steroids during the present admission prior to sample collection either in the emergency department or PICU), and chronic steroids use (above 14 d of steroids preceding admission). Severity of illness (PRISM III) was evaluated at admission, maximum number of OF was followed up daily from admission until the seventh day of PICU stay, and 28-day mortality was evaluated.

Laboratory Procedure

A single random total cortisol level was collected simultaneously with the GCR expression samples. Serum was sent to the main laboratory at CCHMC for cortisol level analysis using a chemiluminiscent microparticle immunoassay and the Architect i200 SR Analyzer (Abbot Laboratories, Abbot Park, IL). GCR receptor expression was measured using flow cytometry using surface antibodies to determine cell type (Pacific Blue Mouse Anti-Human CD4—BD Pharmingen (Franklin Lakes, NJ) for CD4 lymphocytes; Alexa Fluor 700 Mouse Anti-Human CD8—BD Pharmingen for CD8 lymphocytes; Phycoerytrin Mouse Anti-Human CD14—BD Pharmingen for monocytes; Alexa Flour 647 Mouse Anti-human CD66b—BD Pharmingen for neutrophils). After surface staining, cells were permeabilized to detect intracellular GCR receptors (anti-glucocorticoid receptor [FITC] Mouse, clone 5E4 MA1-81793—Thermo Scientific, Waltham, MA). Isotype (Mouse IgG1 [HyblgG1] [fluorescein isothiocyanate]—Abcam, Cambridge Science Park, Cambridge, UK) and fluorescence minus one (FMO) controls were used. Fluorescence samples were fixed in paraformaldehyde and read within a maximum of 5 days on a BD LSR II machine (BD biosciences, Franklin Lakes, NJ). The results were then analyzed using FACSDiva software (BD biosciences). The lymphocyte population (P1) and monocyte and neutrophil populations (P2) were discriminated, and gates were generated for cell type (*x*-axis) and GCR expression (*y*-axis). The results were gated in four areas, and mean fluorescence was measured for 10,000 events on the area expressing both the surface antigen and the GCR antigen. FMO sample values were subtracted from GCR values resulting in a mean fluorescence intensity (MFI), and those values were used for statistical analysis. Isotype controls were used for experimental control (to check if blocking was appropriate).

Statistical Analysis

Data were analyzed using SigmaStat Software (Systat Software, San Jose, CA). For the primary analysis, the patient population was initially divided into patients with CV Failure and hemodynamically stable (No CV Failure) and both groups had their GCR expression compared. For the secondary analysis, the patient population was first divided in two groups according to PRISM III scores less than or equal to 7 (the lower 50th

TABLE 1. Demographic Characteristics and Comparison Between Cardiovascular Failure and Non-Cardiovascular Failure Groups

Demographic Factor	All Subjects (*n* = 52)	No CV Failure (*n* = 28)	CV Failure (*n* = 24)
Age (median, IQR, yr)	7.2 (2.3–11.9)	5.4 (1.9–9.7)	9.2 (3.0–13.3)
Gender (no. of males, %)	30 (58)	17 (61)	13 (54)
Sepsis, *n* (%)	21 (40)	6 (21)	15 (63)[a]
Pediatric Risk of Mortality III (median, IQR)	7.5 (3–12)	4.5 (2–10)	11 (7.3–13.5)[a]
Maximum no. of organ failure (median, IQR)	2 (1–3)	1 (0–2)	3 (2–3)[a]
Cortisol (median, IQR, μg/dL)	8.4 (2.2–18.1)	2.4 (0.9–8.5)	15.4 (9.1–20.2)[a]
Received steroids, *n* (%)	25 (48)	16 (57)	9 (38)
Chronic steroids (> 14 d), *n* (%)	8 (15)	5 (18)	3 (13)
Mortality (no. of deaths, %)	1 (2)	0 (0)	1 (4)

CV = cardiovascular, IQR = interquartile range.
[a]$p < 0.05$ CV failure versus no CV failure on rank-sum test.

percentile) and PRISM III scores greater than or equal to 8 (the upper 50th percentile), and secondarily, the population was divided into two groups according to maximum number of OF (admission to day 7 using Goldstein et al [31] criteria): no OF or one system (the lower 50th percentile) and two or more systems (the higher 50th percentile).

For nonnormally distributed variables, Mann-Whitney rank-sum test was performed, Fisher exact test was used for categorical data, *t* test was used to compare the groups for primary and secondary analysis, and linear regression was used to evaluate the relationship between MFI values (dependent variable) and cortisol levels (independent variable).

RESULTS

A cohort of 52 critically ill children were studied with 28 subjects in the No CV Failure group and 24 in the CV Failure group.

The demographic characteristics of the subjects enrolled are shown in **Table 1**. The CV Failure group had a higher proportion of patients with sepsis, higher cortisol levels, higher PRISM III scores, and a higher number of OFs compared with the no CV Failure group. No other differences were noted. Forty-eight percent of the patients received steroids for various indications, including oncologic therapy, management of cerebral edema after neurosurgery, active immunosuppression, and airway edema (**Supplementary Table 1**, Supplemental Digital Content 1, http://links.lww.com/PCC/A154).

GCR expression showed a large range of variability between different individuals (CD4 from 12 to 1,784 MFI, CD8 from 671 to 2,305 MFI, CD14 from 260 to 4,212 MFI, and CD66b from 126 to 8,853 MFI).

Primary Analysis

When comparing the CV Failure and No CV Failure groups, the subjects with shock had a significantly lower GCR expression both in CD4 lymphocytes ($p = 0.036$) and CD8 lymphocytes ($p = 0.019$) (**Fig. 1**). GCR expression in monocytes and neutrophils showed a trend to be lower in the subjects with CV Failure, but this association did not reach statistical significance.

Secondary Analysis

A secondary analysis was performed to further determine if decreased GCR expression is associated with greater illness severity. Accordingly, the study cohort was divided into two groups, representing the lower and upper 50th percentile of PRISM III scores or maximum number of OF.

When PRISM III score groups were compared, subjects within the upper 50th percentile of PRISM III values had a significantly lower GCR expression in CD4 lymphocytes ($p = 0.008$) and CD8 lymphocytes ($p = 0.010$) when compared with patients within the lower 50th percentile of PRISM III values. Subjects in the upper 50th percentile and subjects in the lower 50th percentile of PRISM values did not show a different level of GCR expression in monocytes ($p = 0.098$) or neutrophils ($p = 0.124$) (**Fig. 2**).

When OF score groups were compared, subjects within the higher 50th percentile of maximum number of OF had a significantly lower GCR expression in CD4 lymphocytes ($p = 0.010$) and CD8 lymphocytes ($p = 0.009$) when compared with patients within the lower 50th percentile of maximum OF. Patients with the higher 50th percentile of maximum number of OF showed a trend to have lower GCR expression in monocytes ($p = 0.053$) than patients in the lower maximum OF number group. Subjects within the upper 50th percentile of maximum number of OF did not show a statistically different GCR expression in neutrophils ($p = 0.132$) when compared with patients within the lower 50th percentile number of maximum OF (**Fig. 3**).

A linear regression analysis was performed using random cortisol levels as the independent variable and GCR expression as

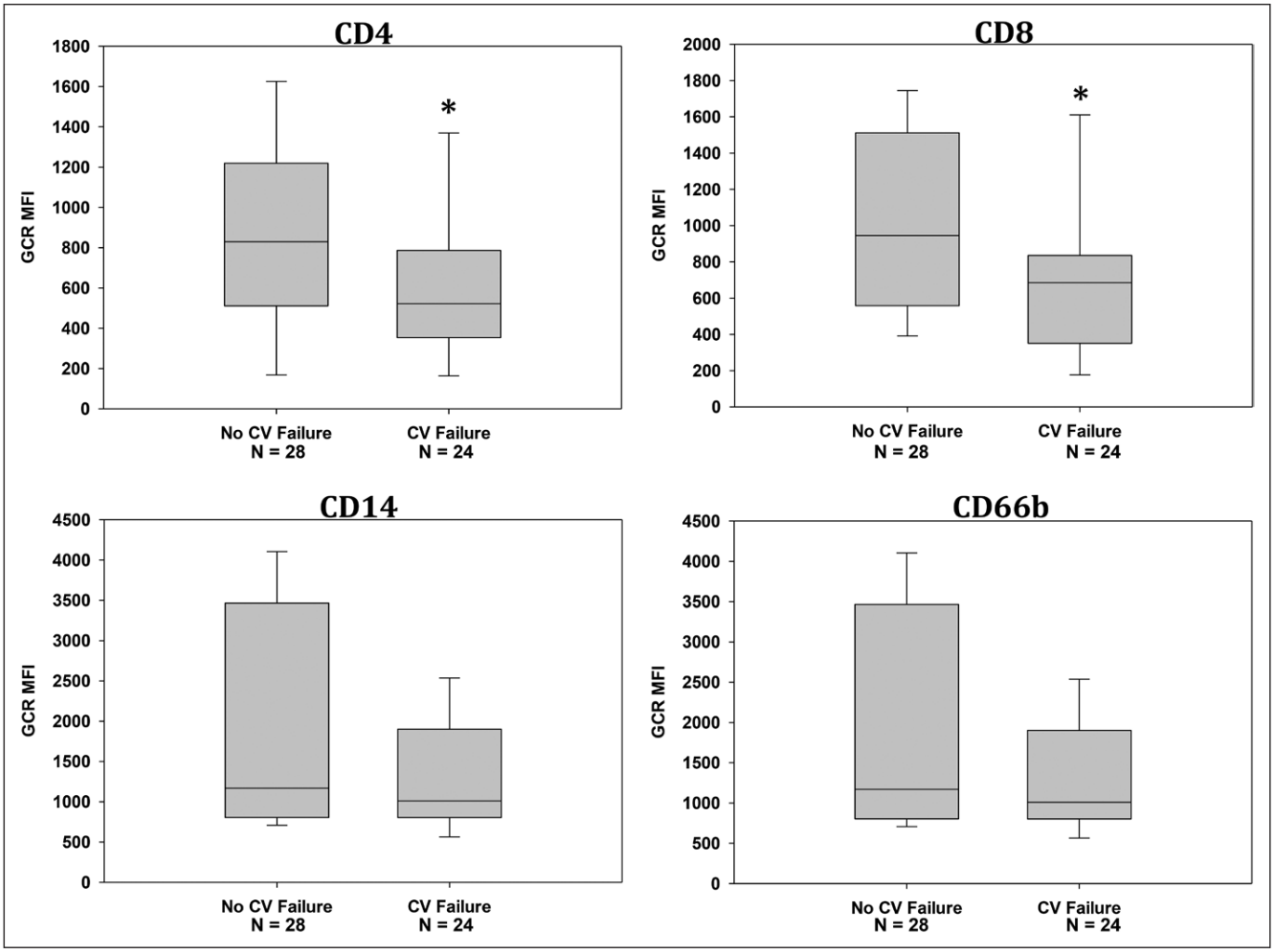

Figure 1. Glucocorticoid receptor (GCR) expression in CD4 lymphocytes, CD8 lymphocytes, monocytes (CD14), and neutrophils (CD66b) in patients with cardiovascular (CV) failure and without CV failure. GCR expression was lower in patients with CV failure when compared with patients without CV failure, $p < 0.05$ by rank-sum test. MFI = mean fluorescence intensity.

the dependent variable. This analysis showed no linear correlation between the variables in all cell types (CD4 Rsqr = 0.06, CD8 Rsqr = 0.06, CD14 Rsqr = 0.03, and CD66b Rsqr = 0.06) (**Fig. 4**). When a nonparametric statistical test was applied, there was a weak, but statistically significant, inverse correlation between cortisol levels and GCR expression in CD4 (Spearman rank correlation coefficient, –0.277; $p = 0.047$) and CD8 (Spearman rank correlation coefficient, –0.288; $p = 0.038$) lymphocytes.

DISCUSSION

Our primary observation is that GCR expression is decreased in CD4 and CD8 lymphocytes of critically ill children with cardiovascular dysfunction in the first 24 hours of admission. Our secondary observation is that GCR expression is decreased in patients with greater severity of illness measured by PRISM III scores as well as in patients with greater OF burden. Both results are consistent with our gene expression studies that showed repression of genes associated with the GCR signaling pathway in the cohort of children with septic shock who had the worst outcome (27). These findings suggest the need to evaluate PICU patients at a pharmacogenomic level for GCR expression in lymphocytes as a risk factor associated with worse severity of illness and the presence of CV Failure.

In general, within the same patient, GCR expression was decreased in all cell types (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/PCC/A154).

There was a significant overlap between the CV failure group and the higher 50th percentile of PRISM III and OF levels (**Fig. 5**), which could account for the similar statistical findings between the three groups.

It is known that GCR-α, when not bound to a ligand, is located in the cytoplasm and that binding of cortisol to GCR-α leads to receptor translocation to the nucleus to affect gene expression (32, 33). GCR-β, on the other hand, is predominantly found in the nucleus, and it inhibits the GCR-α-mediated gene activation (32). Other splice variants, such as GCRγ (GCRP), GCRτ1, and GCRτ2, have been reported in association with glucocorticoid resistance (32–34). The antibody and methodology used in our study allowed us to

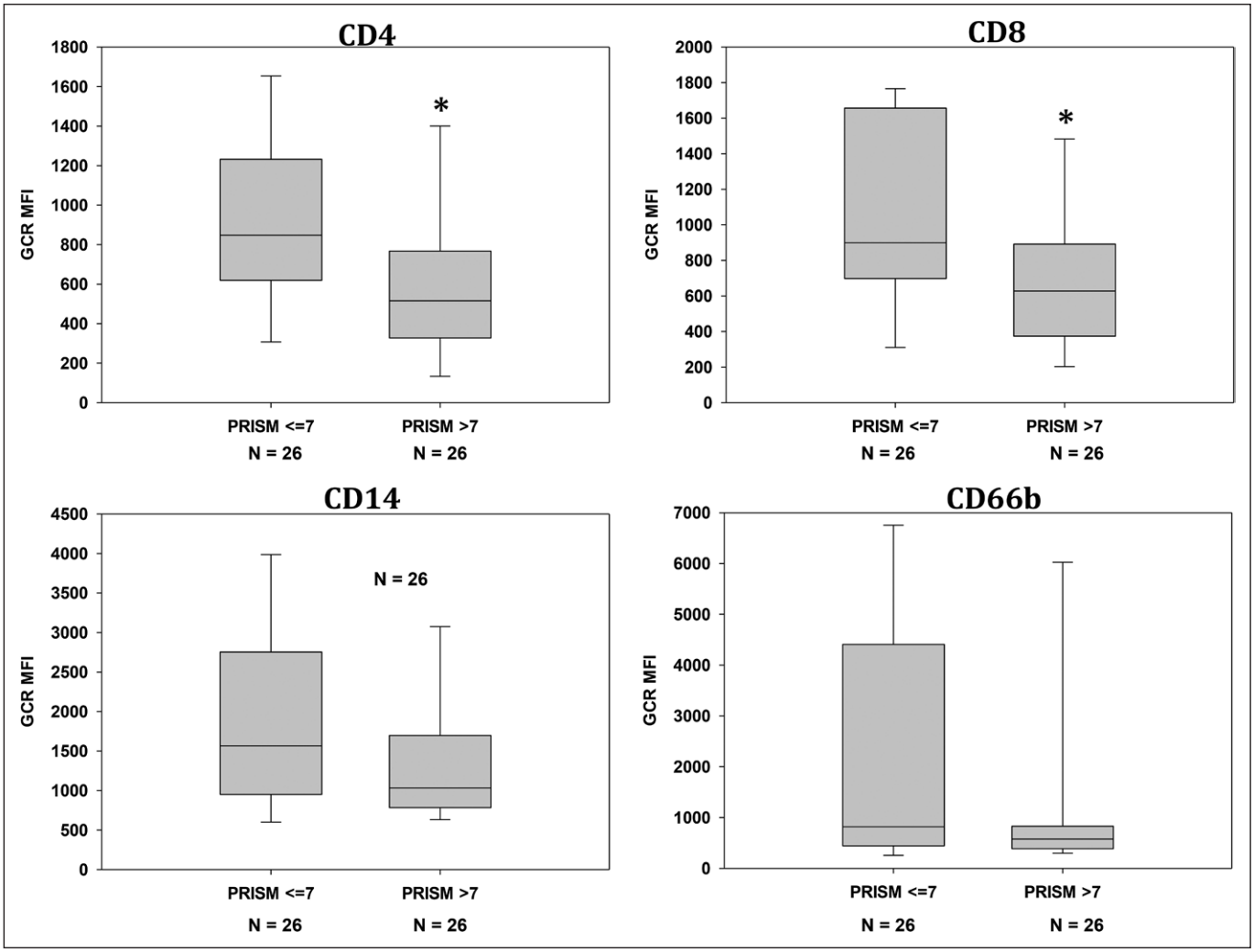

Figure 2. Glucocorticoid receptor (GCR) expression in CD4 lymphocytes, CD8 lymphocytes, monocytes (CD14), and neutrophils (CD66b) in patients with admission Pediatric Risk of Mortality (PRISM) III score ≤ 7 and admission PRISM III score ≥ 8. *GCR expression was lower in patients with PRISM III score ≥ 8 when compared with patients with PRISM III score ≤ 7, $p < 0.05$ by rank-sum test.

measure total GCR levels without distinction of subtypes and separation for location of expression, which constitutes a limitation of the current study. It is not possible to speculate if the lower total GCR levels presented here correlate with lower nuclear GCR-α levels and decreased anti-inflammatory effect, but GCR-β has been reportedly expressed in lower concentrations than GCR-α (33).

Previous studies have reported total and cytoplasmic decrease in GCR levels in peripheral blood mononuclear cells from critically ill children (35), decreased GCR-receptor binding in cytosolic extracts of peripheral WBCs from critically ill adult patients (36), and down-regulation of GCR-α in adults with sepsis or septic shock, findings that are supportive of our results. van den Akker et al (37) also showed that GCR-α and GCRP messenger RNA expression was transiently decreased in neutrophils of children with septic shock compared with the same patients 3 months after the episode.

In agreement with the fact that a lower expression of GCR was associated with shock and worse severity of illness, in vitro studies have previously shown that an increased GCR expression is protective for sepsis (38) and sepsis-induced acute lung injury (39). van den Akker et al (37) reported different findings with GCR levels not showing correlation with PRISM III scores or the presence of shock. The smaller number of patients enrolled in their study may account for a difference in power between both studies that could explain the difference between our results and their findings.

Our findings suggest that patients who have lower GCR expression have higher severity of illness scores. Both in vitro studies (40, 41) and in vivo studies (42–44) suggest the existence of a phenomenon characterized by peripheral resistance mechanism of cortisol insufficiency that could be happening in the lower GCR expression individuals.

In a healthy individual, cortisol is secreted in a diurnal pattern under the influence of corticotropin with a circadian rhythm throughout the day and 90% of the circulating cortisol is bound to corticosteroid-binding globulin with only less than 10% in the bioavailable free form that can be measured in saliva and urine (45, 46). This diurnal variation is lost in severe illness, and the percentage of circulating free cortisol

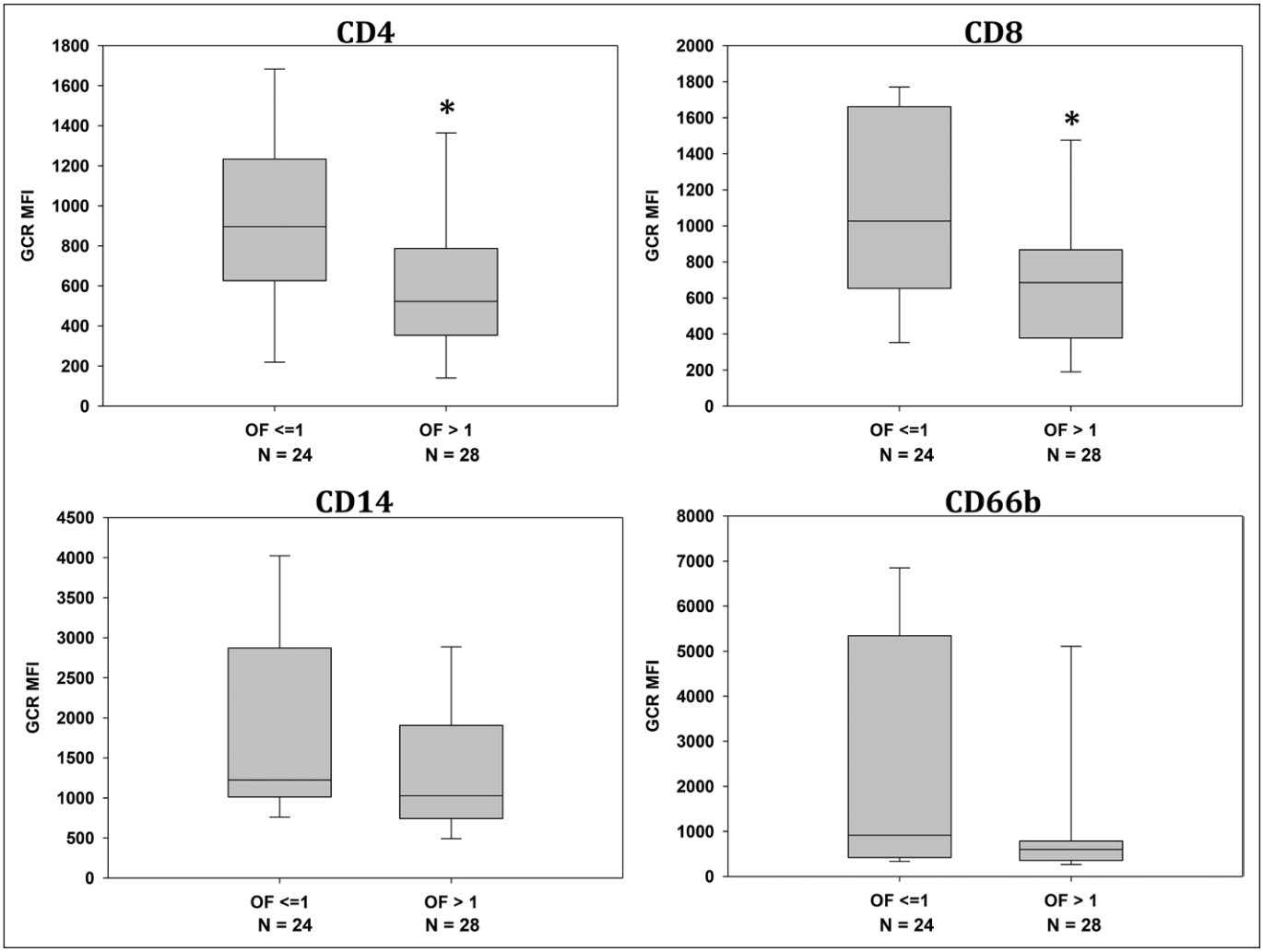

Figure 3. Glucocorticoid receptor (GCR) expression in CD4 lymphocytes, CD8 lymphocytes, monocytes (CD14), and neutrophils (CD66b) in patients with organ failure (OF) ≤ 1 and OF ≥ 2. *GCR expression lower in patients with OF ≥ 2 when compared with patients with OF ≤ 1, $p < 0.05$ by rank-sum test.

increases due to a decrease in cortisol-binding globulin levels, and inflammatory cytokines can change cortisol metabolism, increasing cortisol levels (45). All these issues illustrate the challenges associated with the interpretation of a single random total cortisol level and may account for the finding that cortisol levels, albeit collected at the same time as GCR samples, were not correlated to them.

The lack of a linear correlation between random total cortisol level and GCR expression illustrates that it would not be possible to infer if the individual GCR expression is increased or decreased based solely on circulating random cortisol level. These findings emphasize the importance of measuring GCR expression. Similar findings were previously described by Imamura et al (47) in cord blood from term newborns where GCR-α expression did not correlate with cortisol level or GCR-β expression and by Indyk et al (35) who showed that nuclear GCR levels reflecting ongoing cortisol activity did not correlate to total, free, salivary, and urinary-free cortisol levels.

There were a large amount of patients with a cortisol level lower than 10 μg/dL (Fig. 4), the majority of these patients were postoperative patients, but some CV failure patients also showed a low cortisol level. These findings are compatible with data reported by Menon et al (48) (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/PCC/A154).

Our findings do not suggest that the evaluation of the hypothalamic-pituitary-adrenal axis should be regarded as not useful. Rather, we suggest that the mechanisms of action of glucocorticoids in critical illness are complex and the evaluation of GCR expression could add information to the assessment of the presence of peripheral resistance to corticosteroids.

Future studies could focus on further investigating if a population with a decreased GCR expression has a good response to cortisol therapy or if they may not benefit from its use but could still suffer from side effects of hyperglycemia, myopathy, and immune suppression (49–51). For future protocols studying the effectiveness and indications of the use of steroids in critically ill patients, our study suggests that solely analyzing cortisol levels and ACTH stimulation response may not completely predict response to steroid therapy and that the individual GCR expression has to be taken into account when designing these studies. Further relevant investigation could also evaluate if there are drugs previously studied in vitro (41, 52)

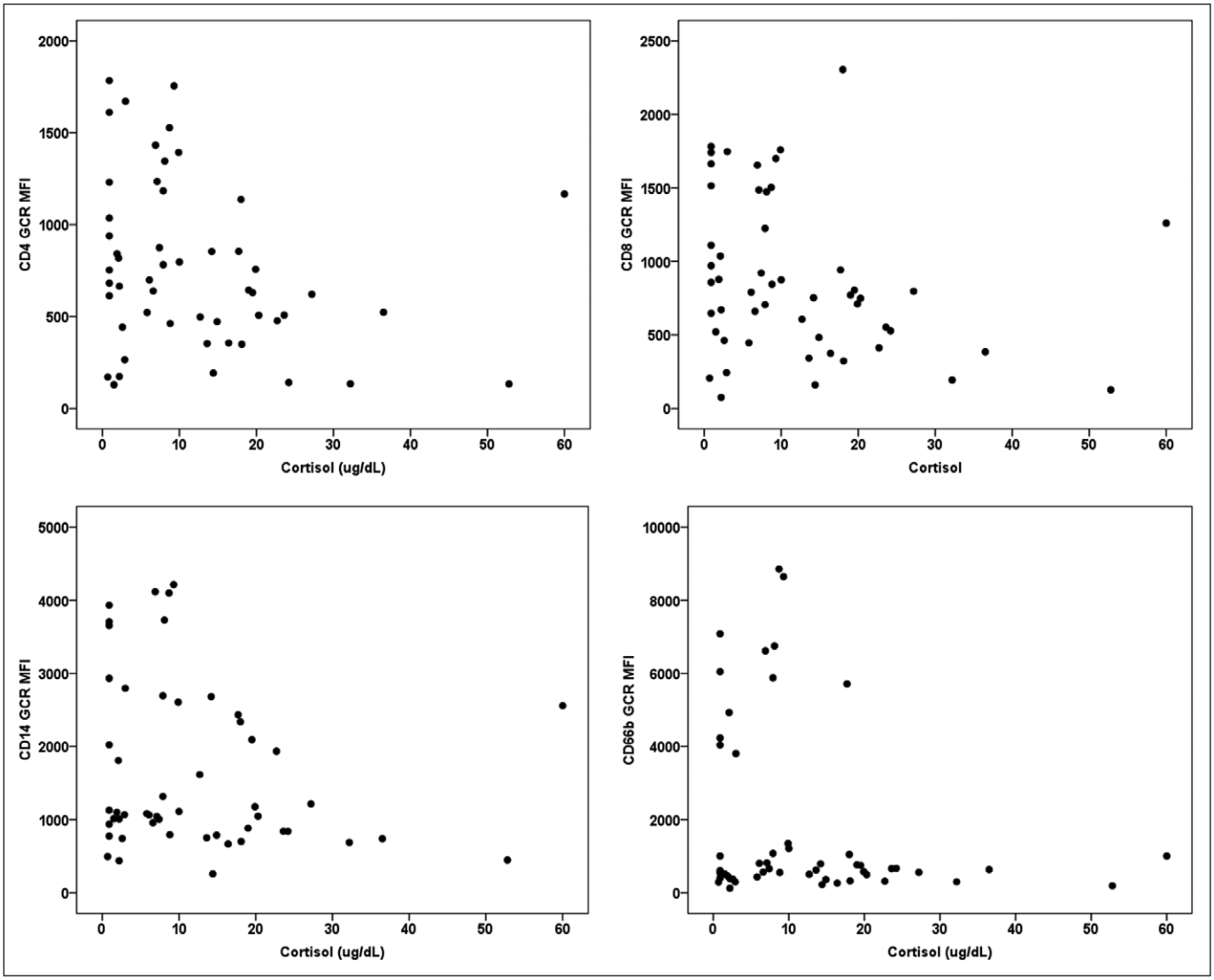

Figure 4. Linear regression analysis of cortisol levels and glucocorticoid receptor (GCR) expression correlation. MFI = mean fluorescence intensity.

that could modulate the GCR expression in vivo and if that timely modulation could impact on the inflammation severity, morbidity, and mortality of study subjects.

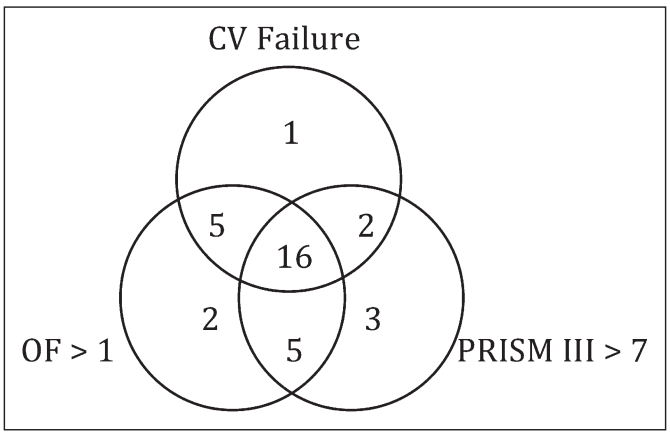

Figure 5. Venn diagram representing the patient distribution in cardiovascular (CV) failure group, organ failure (OF) > 1 group, and Pediatric Risk of Mortality (PRISM) III > 7 group.

It would be most optimal to analyze tissue/end-organ GCR expression as this is probably where its level will reliably be clinically relevant, but we were restricted to analyzing peripheral blood cells' GCR expression since it was easily and less invasively available. It is not known if GCR expression in WBCs correlates with GCR expression in tissues and end organs. Our protocol did not allow us to separate nuclear and cytoplasmatic GCR expression levels; instead, we measured total GCR expression. We could not separately evaluate the different GCR isoforms because the antibody used did not allow us to do that. We measured random cortisol but not salivary, urinary, free cortisol, or cortisol-binding globulin levels. The current laboratory procedure we followed is a technology- and hardware-dependent, time-consuming process that would be difficult to be done at the bedside. Lastly, we divided our patient sample by their median PRISM III scores and OF values for convenience to be able to have two groups with the same number of individuals to run the statistical analysis.

CONCLUSIONS

This study suggests that patients with shock and increased illness severity have lower GCR expression in both CD4 and CD8 lymphocytes, consistent with gene expression studies (27–29). We speculate that this difference may have conceptual implications, identifying a subset population of critically ill children that may present a peripheral resistance form of critical illness–related cortisol insufficiency. GCR expression does not seem to correlate with random serum cortisol concentrations. Future studies could focus on studying GCR expression variability and isoform distribution in the pediatric critically ill population as well as on different strategies to optimize glucocorticoid response.

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