



Use of IFN γ /IL10 Ratio for Stratification of Hydrocortisone Therapy in Patients With Septic Shock

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Large clinical trials testing hydrocortisone therapy in septic shock have produced conflicting results. Subgroups may benefit of hydrocortisone treatment depending on their individual immune response. We performed an exploratory analysis of the database from the international randomized controlled clinical trial Corticosteroid Therapy of Septic Shock (CORTICUS) employing machine learning to a panel of 137 variables collected from the Berlin subcohort comprising 83 patients including demographic and clinical measures, organ failure scores, leukocyte counts and levels of circulating cytokines. The identified theranostic marker was validated against data from a cohort of the Hellenic Sepsis Study Group (HSSG) ($n = 246$), patients enrolled in the clinical trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis (SISPCT, $n = 118$), and another, smaller clinical trial (Crossover study, $n = 20$). In addition, *in vitro* blood culture experiments and *in vivo* experiments in mouse models were performed to assess biological plausibility. A low serum IFN γ /IL10 ratio predicted increased survival in the hydrocortisone group whereas a high ratio predicted better survival in the placebo group. Using this marker for a decision rule, we applied it to three validation sets and observed the same trend. Experimental studies *in vitro* revealed that IFN γ /IL10 was

negatively associated with the load of (heat inactivated) pathogens in spiked human blood and in septic mouse models. Accordingly, an *in silico* analysis of published IFN γ and IL10 values in bacteremic and non-bacteremic patients with the Systemic Inflammatory Response Syndrome supported this association between the ratio and pathogen burden. We propose IFN γ /IL10 as a molecular marker supporting the decision to administer hydrocortisone to patients in septic shock. Prospective clinical studies are necessary and standard operating procedures need to be implemented, particularly to define a generic threshold. If confirmed, IFN γ /IL10 may become a suitable theranostic marker for an urging clinical need.

Keywords: sepsis, hydrocortisone, immune therapy, machine learning, theranostics, steroids, IFN γ /IL10

INTRODUCTION

Though prospective, randomized, controlled multicenter trials have consistently reported faster shock resolution (1, 2), the utility of “low-dose” hydrocortisone (HC) in patients with septic shock remains controversial. Whereas, two French studies reported outcome benefit from a combination of hydrocortisone plus oral fludrocortisone (3, 4), the pan-European CORTICUS trial and the 5-country ADRENAL trial found no survival effect from hydrocortisone alone (2, 5). Possible explanations for this disparity included differences in mortality risk in the populations with a two-fold higher risk of mortality in the French control group [of ref. (1)] compared to CORTICUS (61 vs. 31%, respectively), and an increase in superinfections, variations in other aspects of clinical management, and genetic variations. Of note, a subset analysis of the ADRENAL trial indicated survival benefit from hydrocortisone in Australasian patients, no effect in British and Danish patients, and a trend to harm in patients enrolled in Saudi Arabia (2). It is increasingly recognized that patients presenting in septic shock are hyper-inflamed yet at the same time immunosuppressed (6–8). Corticosteroids are traditionally considered to induce immune suppression *via* the glucocorticoid receptor (GR) and its repressive effect on pro-inflammatory transcription factors such as AP-1 and NF κ B (9). Thus, patients in an overall state of immunosuppression may be potentially compromised by administration of an immunosuppressive drug. This argument is, however, complicated by increasing evidence implicating corticosteroids and GRs in immune-reconstitutive processes (10, 11).

In human monocytes corticosteroid treatment induced expression of innate immune-related genes, such as Toll-like Receptors and anti-inflammatory genes (10, 11). In macrophages and derived cell lines glucocorticoids induced a central component of the inflammasome (NLRP3) and,

upon stimulation with endotoxin (lipopolysaccharide, LPS), induced secretion of pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNF α) (11). Furthermore, glucocorticoid-dependent NLRP3 induction resulted in sensitization of innate immune cells to extracellular ATP and, thus, in an enhanced ATP-mediated secretion of pro-inflammatory cytokines following endotoxin stimulation (12). This immune-activating role of corticosteroids has been described as a response to acute stress enhancing the peripheral immune response, whereas chronic corticosteroid exposure leads to immune suppression (13, 14). These diverging effects of corticosteroids support the need for biomarkers to guide their application.

We applied machine learning to physiological and laboratory data from patients enrolled within a sub-population of the CORTICUS study to search for a potential theranostic marker for hydrocortisone treatment. Using the ratio of serum interferon gamma (IFN γ) to interleukin 10 (IL10), we were able to identify specific sub-cohorts with increased and decreased survival upon treatment. Furthermore, we explored the predictive utility of this biomarker in further datasets of septic shock patients and performed experimental studies regarding this marker as a function of pathogen challenge.

MATERIALS AND METHODS

Included Cohorts and Studies

The discovery dataset based on the Berlin subcohort of the CORTICUS trial. The CORTICUS study was approved by local Ethics Committees (No: 153/2001). Written informed consent was obtained from patients, proxies or their legal representatives. Eligible patients were enrolled if they met the following inclusion criteria: clinical evidence of infection, evidence of a systemic response to infection, the onset of shock within the previous 72 h and hypoperfusion or organ dysfunction attributable to sepsis. Notable exclusion criteria included an underlying disease process with a poor prognosis, life expectancy <24 h, long-term immunosuppression and treatment with long-term corticosteroids within the past 6 months or with short-term corticosteroids within the past 4 weeks. Detailed eligibility criteria are given in **Supplementary Table 1** and in the original study (5). Patients were randomized to receive either *placebo* or 200 mg hydrocortisone (HC) daily for 5

Abbreviations: ACTH, Adrenocorticotrophic hormone; ADRENAL, Adjunctive corticosteroid treatment in critically ill patients with septic shock; CI, Confidence interval; CORTICUS, Corticosteroid therapy of septic shock; HC, Hydrocortisone; HSSG, Hellenic sepsis study group; NE, Norepinephrine; PSM, Propensity score matching; OR, Odds ratio; SISPECT, Sodium selenite and procalcitonin guided antimicrobial therapy in severe sepsis; SOFA, Sequential organ failure assessment; VASST, Vasopressin and septic shock trial.

days, followed by a tapering dose until day 11. Demographic, baseline and progression characteristics were extracted from the CORTICUS database.

In addition to the standard protocol, the Berlin sub-group of CORTICUS (consisting of 13 of the 52 study centers) sampled blood for subsequent measurement of cytokines and other circulating inflammatory mediators from all 84 patients. Seventy-nine out of these patients received norepinephrine at baseline while none received epinephrine. Blood samples were taken directly before an ACTH stimulation test and administration of the study medication. **Supplementary Table 2** shows the timing of blood sampling relative to the onset of shock. The average time period between onset of septic shock and blood sampling was 29.4 h (standard deviation: 16.6 h). Blood samples were collected on day 0, on day 2, on the morning of day 5 (end of full dose HC application), on day 12 (day after HC cessation), day 17 and day 27. The soluble mediators interleukin (IL) 6, 8, 10, 12p70, IFN γ , TNF α , soluble TNF-receptor-I (sTNF-RI), soluble FAS (all OptEIATM Set Human, BD Biosciences, New Jersey, USA), and E-selectin (R&D, Minnesota, USA) were measured in serum, plasma, or culture supernatant by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions. This included calculating calibration and standard curves. All measurements were performed in duplicate. One patient was removed due to lack of cytokine data. Of the remaining 83 patients, serum lactate values (pre-treatment) and serial values were available in 53 and 41 patients, respectively. For 10 patients either IFN γ or IL10 were below the detection limit. Excluding these patients did not change the overall results. For details, see **Supplementary Text 1**.

The first validation set was obtained by the Hellenic Sepsis Study Group from septic shock patients with community-acquired pneumonia or intraabdominal infection. This study included a prospective collection of clinical data and biosamples from patients admitted to 45 study sites in Greece. Patients were enrolled after written informed consent provided by themselves or their legal representatives. Eligibility criteria are given in **Supplementary Table 1**. All enrolled patients had been reclassified into infection and sepsis using the Sepsis-3 classification criteria (15, 16). In HC-treated patients 200 mg/day HC had been administered for 7 days followed by gradual tapering. Start of administration of hydrocortisone and sampling of blood was later than 3 h after onset of septic shock. After discarding data from patients dying or being discharged on the day of admission, a total of 342 shock patients were selected. Secreted cytokines were measured using the LEGENDplex Human Inflammation Panel (13-plex, BioLegend, San Diego, USA) according to the manufacturer's instructions with half of the reagents volume and sample incubation at 4°C overnight. After removing all specimens with <8 of 13 successful cytokine measurements or without IFN γ and IL10 measurements, a total of 246 eligible shock patients (HC treatment: $n = 93$, No HC treatment: $n = 153$) were selected. If only one of the cytokines (IFN γ or IL10) was below the detection limit, the respective value was set as the detection limit. Excluding these samples from the analysis did not alter the findings (**Supplementary Text 7**). Propensity score matching (PSM) was

not necessary as the available dataset did not consist of unbiased baseline variables. For more details and the patient characteristics see **Supplementary Text 2** and **Supplementary Table 3**. As a further validation cohort, we investigated serum IFN γ /IL10 of patients in the *placebo* arm of the randomized and *placebo*-controlled trial of Sodium Selenite and Procalcitonin-guided antimicrobial therapy in Severe Sepsis (SISPCT) (17). SISPCT was a multicenter, randomized, clinical, 2 \times 2 factorial trial performed in 33 intensive care units in Germany. It was conducted from November 6, 2009, to June 6, 2013, including a 90-day follow-up period. In this study patients were randomly assigned to receive sodium selenite or placebo. In addition, patients were randomized to receive anti-infectious therapy guided by a procalcitonin algorithm or without procalcitonin guidance. Using the same protocol as for HSSG blood samples, secreted cytokines were measured for our study using the LEGENDplex Human Inflammation Panel (13-plex, BioLegend, San Diego, USA) according to the manufacturer's instructions with half of the reagents volume and sample incubation at 4°C overnight. We selected septic shock patients from the SISPCT trial randomized to the placebo (non-selenite) treated arm with or without procalcitonin guidance. Here, administration of HC was at the discretion of the treating physician. Patients were included for our study only, if they were either not treated with HC (arm not treated with HC), or treated with at least 50 mg daily hydrocortisone for at least the first 3 days (HC treated arm). Patients were excluded if IFN γ and IL10 both were below the detection limits. If only one of these cytokines (IFN γ , IL10) was below the detection limit, the respective value was set at the detection limit. After removing all specimens with <8 out of 13 successful cytokine measurements or without IFN γ and IL10 measurements, a total of 254 eligible shock patients (HC treatment: $n = 77$, No HC treatment: $n = 177$) were selected. We observed considerable differences in their clinical variables (**Supplementary Table 4a**), hence we needed to select a balanced cohort employing propensity score matching (see below, **Supplementary Table 4b**). In addition, we analyzed the serum IFN γ /IL10 ratio of patients from an earlier small crossover study of patients in septic shock (18). Details about this study and the crossover scheme is given in **Table 2g**, **Supplementary Text 3**. In this study, the early arm got a comparable HC application as the/verum arm of CORTICUS, and was also used for validating our marker. The cytokine values of the crossover study were taken from the original publication.

Propensity Score Matching

To account for the non-random application of hydrocortisone within the HSSG and SISPCT studies, we performed a PSM procedure using the 24 and 182 available baseline variables of these studies. According to (19), features are unbalanced if they show an absolute standardized mean difference $d > 0.1$ between HC-treated and untreated patients, and are thus candidates for PSM. Furthermore, to identify true confounding variables, we applied the method described by Austin (20) and determined features which were significantly correlated with treatment (HC) and outcome (28-days survival). Within HSSG, no variables fulfilled these criteria, and thus patient balancing using PSM

was not applied. Within SISPECT, 11 out of 182 variables were significantly correlated with treatment and outcome, and showed $d > 0.1$. These variables were regarded as true confounding and comprised the variables minimal value of thrombocytes (<24 h before inclusion), distorted metabolism of glucose (yes/no), deregulated body temperature (yes/no), known location of infection (yes/no), sodium minima (<24 h before inclusion), gram positive infections (yes/no), SOFA subscore of renal function, paO₂/FIO₂ ratio, amount of urine, potassium maxima (<24 h before inclusion and arterial hypotension (yes/no)). This set of true confounding features was used for PSM at both leaves of IFN γ /IL10 ratio high and low using the R package matchIt (method “nearest,” caliper value of 0.2). We repeated this PSM analysis 99 times (with different random seeds) and calculated a robust significance value by calculating the median p -value of the corresponding Fisher’s exact-tests. By counting the re-occurrence of patient numbers within 100,000 Propensity Score Matching runs, we determined the consensus cohort comprising patients with the maximal number of selections ($n = 86,273$). By this, a balanced cohort of $n = 118$ patients (HC treatment: $n = 42$, No HC treatment: $n = 76$) was determined and used for the following analysis. **Supplementary Tables 7a, 7b** show the patients’ characteristics before and after balancing.

***In vitro* Whole Blood Culture Experiments and *in vivo* Studies on Septic Mouse Models**

To assess biological plausibility of the data-driven biomarker IFN γ /IL10, we performed *in vitro* whole blood culture experiments in which blood of healthy donors was challenged with bacterial lysates or LPS (endotoxin of *E. coli* B4:O111, Sigma Aldrich) to simulate pathogen burden. Two hundred microliter of diluted (HBSS, 1:1, V/V) heparinized whole blood obtained from healthy volunteers (male, 20–25 years, $N = 2$ –5) was stimulated with serial dilutions either of LPS, lysates from two *E. coli*, two *S. aureus*, one *E. faecalis* or one *E. faecium* isolates obtained from septic patients. In addition, lysates from lab strain *S. aureus* USA300 were obtained. Bacterial lysates were created by heat inactivation, following sonification and a serial dilution of the obtained fragment stock. Following incubation (37°C, 18 h, gentle agitation at 2 rpm) plasma supernatant was prepared by centrifugation (2,500 g at room temperature for 10 min). Secreted cytokine levels were measured using the LEGENDplex Human Inflammation Panel (13-plex, BioLegend), as described above. Comparison was made to vehicle controls. The data of the *in vivo* septic mouse model [cecal ligation and puncture (CLP)] for IFN γ and IL10 was provided from Dahlke et al. (21) and the log₂-ratio of IFN γ and IL10 was calculated. Additionally, a second group of mice were infected by peritoneal contamination and infection (PCI). For the study, male C57BL/6J mice had been used (untreated controls: $n = 7$, sham: $n = 3$, CLP septic mice: $n = 5$, PCI septic mice: $n = 6$). Throughout all experiments, animals were kept under standardized conditions with access to food and water *ad libitum*. Cecal ligation and puncture and PCI were performed as described elsewhere (22). Whole blood was taken from CLP, PCI, sham, and control mice, for CLP, PCI, and

sham mice 6 h post-intervention. For plasma preparation whole blood was centrifuged at 2,000 g for 10 min. Afterwards samples were frozen and stored at -80°C for further analysis. Plasma cytokine levels were measured using the CBA assay (Cytometric Bead Array, CBA; Mouse Inflammation Kit, Becton Dickinson, East Rutherford, USA) by FACS according to product manuals.

Strategy for Data Analysis

We used data consisting of 137 features (patient variables, potential predictors) from 83 patients of a subcohort of the CORTICUS study population to perform an exploratory data analysis. Only data which was available at baseline was used for propensity matching and machine learning. This table also included measured cytokine levels and ratios of all possible cytokine combinations. We aimed to find a theranostic marker distinguishing HC responders (i.e., survivors) from non-responders (non-survivors). The complete list of these features is shown in **Supplementary Table 5** and the workflow is depicted in **Supplementary Figure 1**. From this data, we sought the best predictor of 28-day survival for the placebo arm using one-level decision trees (stumps) calculated by a leave-one-out cross-validation scheme. The best stumps were chosen by enumeration of all available variables. To apply the marker to other datasets than the CORTICUS subset, the threshold was newly calculated by selecting the one with best significance of improved survival (employing a Fisher’s exact-test). All analyses were carried out in R (www.r-project.org) using custom scripts.

The results from the CORTICUS, the HSSG, the SISPECT and the Crossover study cohorts were integrated by using a Mantel-Haenszel test implementing the R stats function `mantelhaen.test`.

RESULTS

IFN γ /IL10 Stratifies CORTICUS Patients

The CORTICUS study included 499 patients (5). The Berlin sub-study (Immune sub-study) comprised of 84 patients, and only for these cytokine measurements were available (see section Materials and Methods). Hence, our analyses focused on this sub-cohort. Its patients’ characteristics are summarized in **Table 1**. Median plasma concentrations of soluble mediators and leukocytes were not significantly different between the arms at baseline (**Table 1**). Age differed but was not a confounder (see **Supplementary Text 4**). Analysis of baseline characteristics was performed on 137 variables including demographic and clinical variables, Sepsis-related Organ Failure Assessment (SOFA) scores, lymphocyte counts, plasma protein concentrations of cytokines and patient blood stimulation experiments. We performed a leave-one-out cross-validation with one-level decision trees (using only one predictor at a time) to the *placebo* arm to discriminate between 28-day-survivors and non-survivors. In 95% of the cross-validation runs, the serum IFN γ /IL10 ratio (referred to as IFN γ /IL10 or ratio in the following) was selected as the best predictor with a stable threshold of 0.95. Patients in which IFN γ /IL10 was below 0.95 were denoted “low-ratio patients,” the others “high-ratio patients.” Untreated high-ratio patients showed a higher survival rate than such with a low ratio (95 vs. 45%, **Table 2a**).

TABLE 1 | Patient characteristics, initial cytokine and blood counts of the studied CORTICUS sub-cohort.

	HC (N = 42, 49.4%)	Placebo (N = 41, 50.6%)
Sex (female)	13 (31%)	11 (27%)
Age (years)	59 \pm 15*	69 \pm 11*
Admission category		
- Medical	1 (2%)	0 (0%)
- Emergency surgery	39 (93%)	37 (90%)
- Elective surgery	2 (5%)	4 (10%)
Temperature ($^{\circ}$ C)	38.5 \pm 1.2	38.6 \pm 0.8
Heart rate (bpm)	121 \pm 18	123 \pm 24
Systolic blood pressure (mmHg)	83 \pm 17	90 \pm 22
SOFA score at inclusion	10.4 \pm 3.2	9.8 \pm 2.7
SAPS II score	43.7 \pm 15.6	48.0 \pm 14.9
Leukocytes (thousands/mm 2)	13.8 \pm 7.3	15.1 \pm 8.8
Platelets (thousands/mm 2)	187 \pm 121	165 \pm 98
Arterial lactate (mmol/L)	3.0 \pm 2.3	3.9 \pm 4.0
Survival		
- Day 28 (survivors)	31 (74%)	29 (71%)
- ICU (survivors/all)	27/42 (64%)	28/41 (68%)
- Hospital (survivors/all)	25/42 (60%)	26/41 (63%)
- One year (survivors/all)	20/41 (49%)	17/40 (42%)
ICU-stay (days) all patients	28 \pm 27	24 \pm 19
Hospital stay (days) all patients	51 \pm 42	46 \pm 31
Cytokines**	Median (Q1–Q3)	Median (Q1–Q3)
IFN γ (pg/ml)	35.05 (15.79–152.13)	30.16 (17.93–57.80)
IL10 (pg/ml)	31.21 (13.30–76.22)	31.66 (15.68–54.76)
IL12 (pg/ml)	10.03 (3.90–43.60)	13.00 (3.90–43.27)
IL6 (pg/ml)	388.02 (177.80–487.27)	377.21 (237.17–509.01)
IL8 (pg/ml)	130.95 (56.63–226.28)	86.81 (53.81–244.44)
sFas (pg/ml)	2137.17 (1544.84–3335.26)	2155.38 (1551.38–3196.85)
sTNF-R1 (pg/ml)	25,806 (13,322–40,599)	18,809 (12,009–28,885)
White blood cell counts**		
Lymphocytes (/nl)	0.71 (0.34–0.93)	0.75 (0.41–1.05)
Monocytes (/nl)	0.53 (0.36–0.80)	0.56 (0.33–0.92)
NK cells (/nl)	0.04 (0.02–0.07)	0.05 (0.03–0.12)
PMNs (/nl)	11.69 (7.17–14.36)	11.06 (7.75–15.21)
B-lymphocytes (/nl)	0.09 (0.04–0.20)	0.08 (0.05–0.13)
T-Helper lymphocytes (/nl)	0.30 (0.13–0.43)	0.26 (0.14–0.50)
T-Suppressor lymphocytes (/nl)	0.10 (0.05–0.14)	0.07 (0.04–0.17)
Thrombocytes (/nl)	170.00 (99.50–250.00)	146.50 (92.00–203.25)

*Values are given in means \pm SD for all continuous variables of the patient characteristics.

**All cytokine and blood count values did not significantly differ between verum and placebo treated patients.

Upon applying this predictor to HC-treated patients, the reverse behavior was observed i.e., a low IFN γ /IL10 indicated a high likelihood of survival (85 vs. 69%, **Table 2b**).

Performing a logistic regression with HC treatment, IFN γ /IL10 ratio and their interaction as independent variables

TABLE 2 | Survival rates according to high and low IFN γ /IL10.

	Non-survivors	Survivors	% Survivors
(a) CORTICUS patients treated with placebo			
IFN γ /IL10 high	1	20	95% <input checked="" type="checkbox"/>
IFN γ /IL10 low	11	9	45% <input type="checkbox"/>
(b) CORTICUS patients treated with verum (HC, hydrocortisone)			
IFN γ /IL10 high	9	20	69% <input type="checkbox"/>
IFN γ /IL10 low	2	11	85% <input checked="" type="checkbox"/>
(c) HSSG patients, not treated with HC			
IFN γ /IL10 high	43	36	46% <input checked="" type="checkbox"/>
IFN γ /IL10 low	53	21	28% <input type="checkbox"/>
(d) HSSG patients, treated with HC			
IFN γ /IL10 high	31	14	31% <input type="checkbox"/>
IFN γ /IL10 low	29	19	40% <input checked="" type="checkbox"/>
(e) SISPCT patients, not treated with HC			
IFN γ /IL10 high	0	15	100% <input checked="" type="checkbox"/>
IFN γ /IL10 low	13	48	79% <input type="checkbox"/>
(f) SISPCT patients, treated with HC			
IFN γ /IL10 high	2	7	78% <input type="checkbox"/>
IFN γ /IL10 low	5	28	85% <input checked="" type="checkbox"/>
(g) Patients from the early arm of the crossover study (treated with HC)			
IFN γ /IL10 high	5	6	55% <input type="checkbox"/>
IFN γ /IL10 low	1	8	89% <input checked="" type="checkbox"/>

* Compliant to the rule; Non-compliant to the rule.

and 28-day survival as the dependent variable, we observed a significant effect of the interaction term ($p = 0.042$, **Supplementary Text 5** and **Supplementary Figure 2A**). These observations suggested a new decision rule, that is, no HC treatment if the ratio is high, and HC treatment if it is low. This decision rule yielded an odds ratio of survival of OR= 6.97 (95% CI: 1.79–40.50), $p = 0.0013$ (**Figure 1**). Notably, the identified rule was valid also when regarding an earlier or later onset of septic shock before inclusion (0–14, 24–48, and 48–72 h, see **Supplementary Figure 6F**). Regarding white blood cell (WBC) counts of different leukocytes did not reveal a clear separation of IFN γ and IL10 producers. The IFN γ /IL10 ratio did not significantly correlate with any of the investigated leukocyte subtypes. Notably, IL-10 negatively correlated with most of the leukocyte subtypes (**Supplementary Table 6**). However, neither IFN γ , IL10 nor any WBC count alone could be used for a treatment rule.

Validation Based on Patients From the Hellenic Sepsis Study Group, a Published Crossover Study and the “Trial of Sodium Selenite and Procalcitonin-Guided Antimicrobial Therapy in Severe Sepsis”

Supplementary Table 3 summarizes the demographics of the HSSG validation cohort. Applying IFN γ /IL10 to HSSG patients, we observed a similar pattern as for the CORTICUS subgroup (**Tables 2c, d**). High IFN γ /IL10 indicated distinct higher survival

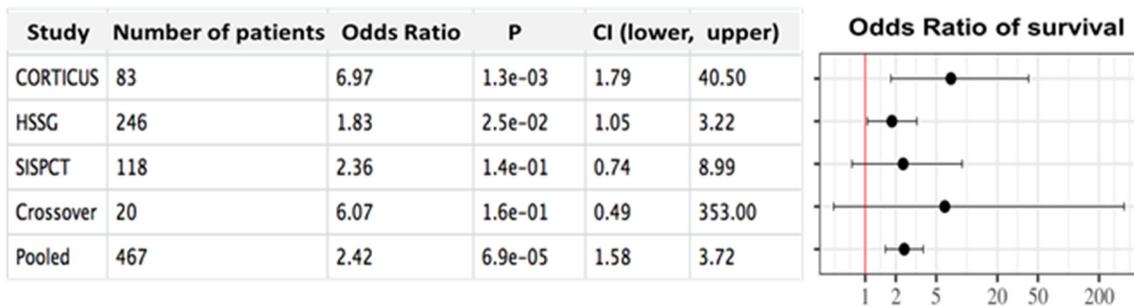


FIGURE 1 | Forest plot showing the outcome in the investigated cohorts depending on hydrocortisone administration according to the theranostic marker. Odds ratios including confidence intervals and thresholds between low and high ratios are shown regarding the IFN γ /IL10 prediction rule for the sub-cohorts of CORTICUS (discovery set), HSSG (validation set), SISPCT (validation set) and the crossover study (validation set).

of the HC non-treated patients (46 vs. 28%). In contrast, in the HC treated group we observed the opposite behavior (31 vs. 40%), yielding an odds ratio of 1.83 (95% CI: 1.05–3.22), $p = 0.025$. As the means to measure the cytokines differed, we had to adjust the IFN γ /IL10 ratio threshold for the HSSG (threshold = 1.01). For the HSSG study, days of survival were available. Thus, we assembled survival curves for patients treated according and against our rule (**Supplementary Figure 3**). During the first 10 days, patients with a high IFN γ /IL10 ratio showed a higher rate of survival, suggesting an initial better condition of patients with higher ratios (this is evidenced also by their lactate levels, see next section). After 10 days, the HC treatment (during the first 7 days following by gradual tapering) seems to impact the outcome, as the survival rate of the low ratio patients with HC-treatment (treatment in compliance with our rule) crossed the high ratio patients with HC-treatment (treatment not in compliance with our rule). The HSSG cohort consisted of patients in sepsis either caused by community acquired pneumonia (CAP) or intra-abdominal infection. To note, our rule performed better for the subcohort of patients with intra-abdominal infection [$n = 98$ patients, OR = 2.55 (95% CI: 1.03–6.49), $p = 0.038$], compared to patients with CAP [$n = 148$ patients, OR = 1.73 (95% CI: 0.82–3.73), not significant].

Next, we analyzed serum IFN γ /IL10 of patients from an earlier, smaller crossover study (18) in which the early arm got a comparable HC application as the HC arm of CORTICUS (details about this study and the crossover scheme is given in **Supplementary Text 3**). In line to the results from CORTICUS and HSSG, low IFN γ /IL10 indicated better survival (89% survivors), whereas high IFN γ /IL10 was an indicator for considerably worse outcome (55% survivors), yielding an odds ratio of 6.07 (95% CI: 0.49–353), $p = 0.16$. Due to the small sample size the result from the crossover study failed to achieve significance.

Furthermore, we investigated serum IFN γ /IL10 of propensity score matched patients from the *placebo* arm of the randomized *placebo*-controlled trial of Sodium Selenite and Procalcitonin-guided antimicrobial therapy in Severe Sepsis (SISPCT) (17). For details about the study and the selection of patients, see Methods and **Supplementary Text 6**. In contrast to the

former patient populations, the 28-day-survival rate of SISPCT patients receiving or not receiving HC differed considerably (68 vs. 84%, respectively, $p = 0.0041$, Fisher's exact-test, see **Supplementary Figure 4A**). We evaluated if there was a variable which may elucidate such an uneven distribution and identified the time of septic shock before inclusion (and HC treatment) to be strongly correlated with the survival rate of the HC-treated patients. This variable was not correlated with the survival of the non-HC-treated patients (**Supplementary Figure 4B**). Thus, we excluded patients with shorter time intervals between shock and study inclusion (<7 h), and balanced the remaining patients after separation into IFN γ /IL10 ratio low and high. The cohort comprised of $n=118$ patients and showed comparable survival rates of HC- and non-HC-treated patients (83 vs. 84%).

In summary, and comparable results when applying our treatment rule (**Tables 2e,f**), the investigated patients from all investigated validation studies evidenced IFN γ /IL10 as a potential theranostic marker for HC application in septic shock. **Figure 1** shows the forest plot including all investigated studies.

CORTICUS Time Courses of Serum Lactate and Norepinephrine Requirement Reflect Hemodynamic Stabilization in Patients Treated in Compliance With the Decision Rule

High serum lactate levels have been demonstrated to indicate severity of metabolic derangements and increased mortality in sepsis (15). In all studied subpopulations where it was available, median initial lactate was distinctly lower in patients with high compared to patients with low IFN γ /IL10 ratio (CORTICUS: 1.90 vs. 2.89 mmol/L, SISPCT: 2.20 vs. 3.00 mmol/L, Crossover study: 1.49 vs. 2.24 mmol/L). Together, lactate values were significantly different between low ratio and high ratio patients ($p = 0.0029$, $n = 330$, Yuen's bootstrap-test), suggesting that IFN γ /IL10 associated with disease severity. Of note, although the lactate levels correlate inversely with IFN γ /IL10 at baseline, serum lactate itself performed worse as a theranostic marker. Notably, we identified a significantly stronger decrease of the lactate values of patients which were treated in compliance

with the decision rule in comparison to patients which were treated against the rule (**Figure 2A**). Hemodynamic stabilization was also supported by a visible stronger trend of reduction of norepinephrine (NE) requirement specifically in the group of patients in compliance with the decision rule (**Figure 2B**). In summary, time series of serum lactate and NE requirement reflect better recovery of septic shock in patients treated in compliance with the proposed theranostic marker.

IFN γ /IL10 as an Indicator of Pathogen Challenge

In light of published evidence associating IFN γ and IL10 with the severity of parasitic infection and tuberculosis (23,

24), we investigated whether IFN γ /IL10 reflects the pathogen burden of immune cells when challenged with typical pathogens found in sepsis. We spiked blood from healthy donors with fragments of clinical isolates (*Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*) or endotoxin (LPS) across a wide range of concentrations. As expected, the higher the pathogen challenge, the higher the immune response and hence the concentration of IFN γ and IL10 in the supernatant. Remarkably, we observed the inverse behavior for the ratio, i.e., a high IFN γ /IL10 ratio for low pathogen concentrations and *vice versa*. **Figure 3** shows the results exemplarily for one *E. coli* isolate. Results for the second *E. coli* isolate, *S. aureus*, *E. faecalis* and *E. faecium* isolates and LPS are shown in the **Supplementary Material (Supplementary Figure 5)**. To note, testing also a lab strain of *S. aureus* (USA 300) showed not this tendency (**Supplementary Figure 5**, see section Discussion). To validate the obtained results in an *in vivo* sepsis model we challenged mice by cecal ligation and puncture (CLP sepsis model) as well as peritoneal contamination and infection (PCI), and compared them to untreated controls and sham treated mice (sham-operated mice underwent an identical operation except for the actual cecal ligation and puncture). Indeed, we observed the same behavior: the ratio of IFN γ /IL10 was distinctively higher in the controls (negative and sham treated mice) and lower in septic mice (**Supplementary Figure 6**).

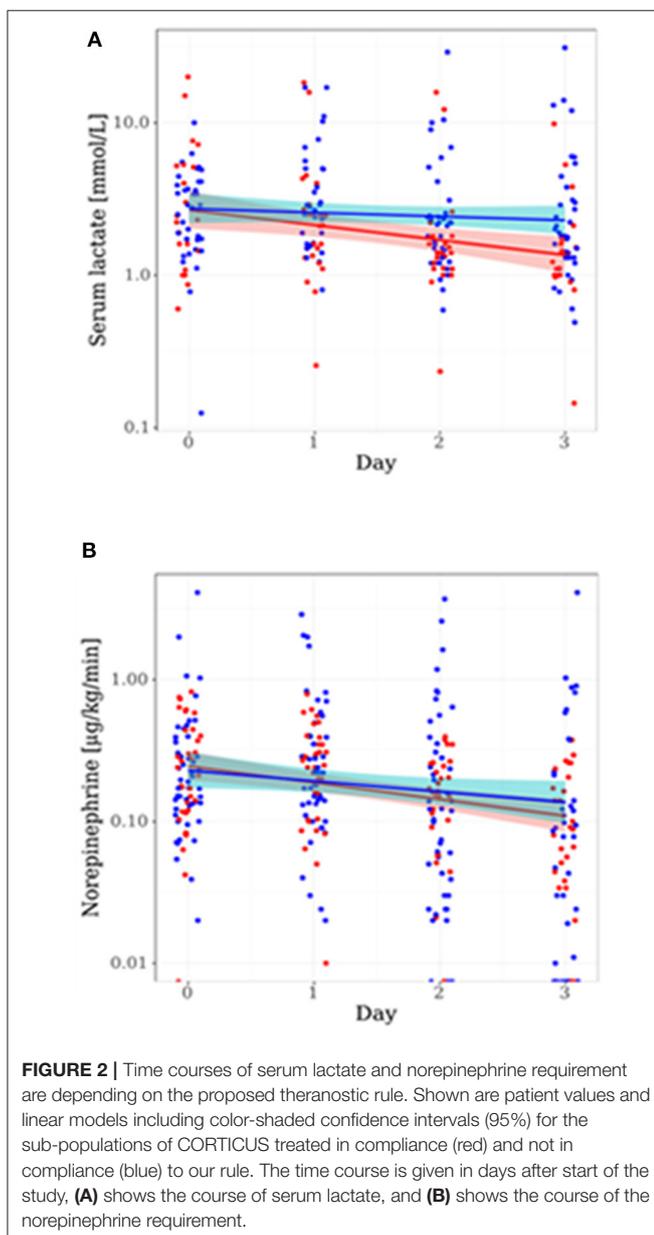
Furthermore, we associated IFN γ /IL10 to the bacterial load in critical ill patients, and compared publically available data of patients with and without bacteremia. Matera et al. (25) investigated 52 patients (39 survivors and 13 non-survivors) with signs of SIRS at hospital admission. Only 4% of them were in septic shock. Twenty-eight of 52 were diagnosed with bacteremia. In line with our experimental observations, a distinctively higher ratio (1.6 ± 0.77) for IFN/IL10 in SIRS patients was observed compared to patients with bacteremia (0.8 ± 0.88 , details: **Supplementary Text 7**). Healthy volunteers showed the highest IFN γ /IL10 ratio (2.80 ± 1.22) of all groups investigated by Matera et al.

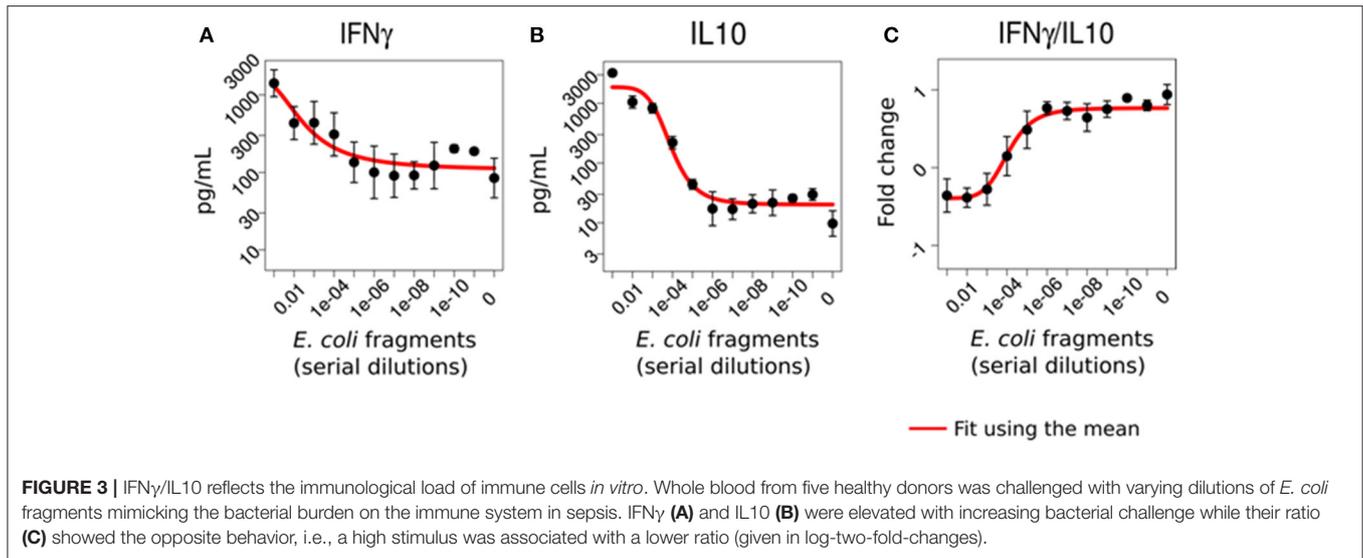
In summary, IFN γ /IL10 inversely correlates with the immunological load of infection in an *in vitro* system, in septic mice and when comparing bacteremic and non-bacteremic critically ill patients.

DISCUSSION

Starting from a larger panel of 137 predictors derived from septic shock patients ($n = 83$) in CORTICUS, we identified the ratio of serum IFN γ and IL10 as a promising biomarker to guide the treatment decision for or against HC. We validated our results by applying the marker to three other datasets.

Metabolically, high serum lactate indicates severity of cellular disturbances (15) and is associated with poor outcome (26). Consistent with this, patients with low IFN γ /IL10 showed high serum lactate. Furthermore, stratifying patients in compliance with the proposed decision rule, we observed a considerable decrease over time in serum lactate specifically in the group of





patients, which were treated in compliance with the theranostic marker. This further extends the concept that HC is generally able to stabilize cardiovascular function in septic shock and suggests that this effect might also be restricted to patients with a high burden of the disease, and compares to the different beneficial effects of steroids in septic shock observed in the trials mentioned in the introduction (French studies with higher mortality showed a beneficial effect compared to no beneficial effect in the studies ADRENAL and CORTICUS).

IFN γ and IL10 have been suggested as biomarkers for parasitic (23) and other infections, such as tuberculosis (24). E.g. McIlleron et al. reported the time course of IFN γ indicating response to treatment in pulmonary tuberculosis (27). However, for diagnosis of pulmonary tuberculosis, IFN γ alone has limitations relating to sensitivity (28). In turn, high IL10 was observed with mycobacterial persistence (29) suggesting that in particular the ratio of these cytokines might reflect the immunological burden of infection. In line with this, it has been shown that the ratio of IFN γ and IL10 correlates with disease severity of tuberculosis and may differentiate pulmonary from extra-pulmonary forms (30). To elucidate the functional implications of low and high IFN γ /IL10, we performed blood culture assays challenging the immune cells with fragments of bacterial isolates from septic patients. Indeed, we observed the ratio to be negatively correlated with the load of pathogen fragments. Similarly, we observed a significant lower ratio in untreated and sham controls compared to septic mice. This was supported by clinical data from Matera et al. (25) when regarding critically ill patients with bacteraemia (higher bacterial load) and without bacteraemia (no or non-detectable bacterial load). Thus, IFN γ /IL10 is supposedly (negatively) associated with the pathogen load.

IFN γ has been consistently documented to activate cells of adaptive immunity (31), and IL10 as a suppressor of innate immune responses and inflammation (32, 33). Hence, high

IL-10 may be a response to a high load to prevent and limit over-whelming immune reactions and, in consequence, tissue damage, whereas low IFN γ reflects a low adaptive immune response.

Interestingly, Sommerfeld et al. observed recently that mouse mutants lacking the receptor of complement factor C5a showed increased survival in mild septic mouse models, and, IFN γ /IL10 was increased compared to wildtype mice under the same conditions (34). The mutants also showed a similar survival benefit when mutant and wildtype mice were treated with antibiotics. Sommerfeld et al. explained the effect by an increased adaptive immune response in the mutants. It is reasonable that a high pathogen load causes repression or even disruption of the adaptive immune system or/and induces an overwhelming innate immune response monitored by the ratio of IFN γ /IL10. Nevertheless, this needs exploration in future studies. We observed *in vitro*, *in vivo* and in patients that blood challenged with bacteria/bacterial fragments displayed lower IFN γ /IL10 at higher loads, and *vice versa*. Transferring these observations to septic shock patients with low IFN γ /IL10, we speculate that HC treatment yielded a better outcome in these HC-responders because HC may allow the highly loaded immune system more pace for recovery. This is evidenced by the reduction of serum lactate and norepinephrine requirements as measures of recovery particularly in the subgroup of very sick patients.

To mention, we also challenged the blood culture with the lab strain *S. aureus* USA300, for which this behavior was not observed. We speculate that such a pathogen causing rather skin and soft tissue inflammation than bacteraemia and sepsis may evoke other cytokine responses and suggest follow up analyses on this aspect in more detail.

The concern about side effects of corticosteroids such as infections in patients with less severe septic shock stipulated more restrictive recommendations by the Surviving Sepsis

Campaign. HC application is currently recommended only for patients not responding to adequate fluid resuscitation and vasopressor therapy (35, 36). Considering an immune biomarker, such as IFN γ /IL10 reflecting the status of the patients' immune system status may intuitively be a promising path to improve the treatment decision. Consistent with this concept, Bentzer et al. studied data from patients of the "Vasopressin vs. norepinephrine in Septic Shock Trial" (VASST) investigating corticosteroid treated vs. non-treated patients (only vasopressin or catecholamine vasopressors). They identified a signature of three cytokines (IL3, IL6, CCL4) suggesting response to corticosteroid treatment (37). However, these results based on a study, which was not randomized, blinded or protocolized according to corticosteroid treatment, and Bentzer et al. did not elaborate how these cytokines interact with corticosteroid treatment.

In a very recent randomized controlled trial, patients who were hospitalized with Covid-19 were randomly assigned to receive dexamethasone (RECOVERY) or placebo. This trial elucidated that dexamethasone treatment is beneficial for patients receiving either invasive mechanical ventilation or oxygen alone at randomization but not among those receiving no respiratory support (38). Here the supportive measurement of the affected organ was sufficient for predictive enrichment of steroids treatment. In our case (septic shock), predictive enrichment basing solely on the supportive measurement (vasopressor requirement) was not sufficient and we needed to monitor the immune status (by the cytokines IFN γ and IL10). As an intriguing project for the future, we propose developing a generic rule distinguishing different disease/syndrome entities employing such categories.

Limitations and Strengths

We excluded patients from the very early window of septic shock of SISPECT (<7 h) to balance the survival between HC-treated and HC-untreated patients. This difference was not surprising, as the HC treatment was applied to patients in more severe conditions, as suggested by guidelines. This is consistent with the CORTICUS data where inclusion criteria were broader (up to 72 h) and the onset of sepsis in CORTICUS occurred on average 29 h before inclusion. In the light of the current recommendations to use HC restrictively, it might indicate, that HC treatment could be supported by a theragnostic biomarker. In addition, we were not able to outbalance every variable in SISPECT. Another limitation of our study is the variability of thresholds to distinguish between high and low IFN γ /IL10 ratio. For CORTICUS it was 0.95, for HSSG 1.01, for SISPECT 0.48, and for the crossover study it was 0.81. DeJager et al. (33) reported that timing of sampling, sample storage, the choice of the platform and the blood collection tubes are crucial issues when measuring cytokines from blood. Cytokines can be stored below -80°C for a longer time, but even at -80°C degradation was observed, in particular of IL10. Most of these parameters varied between the different studies that we included in our analysis making it difficult to compare the ratios between the studies. Our cytokine measurements seemed to depend on the assay and sample handling described by DeJager et al. The discrepancies of the optimal threshold

between the studies may also be due to the rather older time point when Corticus and the Crossover study were performed. Hence, when implementing the rule into the clinics, well defined standard operating procedures need to be developed to obtain an appropriate, center independent threshold. A limitation of the *in vitro* blood culture study is that we used only blood of younger male volunteers.

A strength of our study is that our rule seems to be valid across studies of very heterogenous survival rates (CORTICUS: 72%, Crossover: 70%, HSSG: 37%, SISPECT: 84%). Applying our decision rule successfully across a broad range of these studies suggests the marker to be generic. The analyzed Berlin cohort of CORTICUS contained a high number of surgical patients and a rather low number of medical patients. Also the averaged ICU and hospital stay was longer as usual for septic shock patients [when compared e.g. to the prospective INSEP study (39)]. The validation cohorts didn't show this notable discrepancy suggesting a good generalizability of our rule. A randomized controlled trial also better representing these variables is, thus, in every case inevitable to confirm the identified rule. Furthermore, strengths of our study relates to the use of (i) well-phenotyped cohorts, (ii) that our marker showed remarkably similar results across all studies, and (iii) that we provide clinical, *in vivo* and *in vitro* data relating it to the pathogen challenge of the immune system.

CONCLUSIONS

We identified the ratio of serum cytokines IFN γ and IL10 as a potential theragnostic marker for hydrocortisone treatment in septic shock. An accompanying study into the mechanism suggests that this ratio monitors the immune response challenged across distinct pathogen loads. The development of a standard operating procedure for obtaining the cytokine concentrations in serum followed by a clinical trial is necessary confirming our rule before implementing it into the clinical routine.

DATA AVAILABILITY STATEMENT

The original contributions generated in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The CORTICUS trial was a multicenter study, the protocol was approved by the Ethics Committee at each of the 52 participating intensive care units. In addition to the standard CORTICUS protocol, the Berlin study group sampled blood for subsequent measurement of cytokines and other circulating inflammatory mediators. This was approved by the Local Ethics Committee (No: 153/2001). Written informed consent was obtained from patients, proxies or their legal representatives. The

HSSG cohort represents a prospective collection of clinical data and biosamples in 45 study sites in Greece. The study protocol was approved from the Ethics Committees of all participating hospitals. Patients were enrolled after written informed consent provided by themselves or by first-degree relatives if patients were unable to consent. The Placebo-Controlled Trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis (SISPCT) was a multicenter clinical trial. It was conducted in 33 multidisciplinary intensive care units across Germany. The study protocol was approved by the ethics board of Jena University Hospital. Written informed consent was obtained from all patients or their legal representatives. The study protocol for the crossover study was approved by the institutional (Charité, Berlin) Ethics Committee. All animal experiments were in accordance with the German legislation on protection of animals and with permission of the regional animal welfare committee.

AUTHOR CONTRIBUTIONS

RK, AK, MOS, DK, and MB conceptualized and designed the study. MOS, RK, AK, and VK developed the methodology. AK, VK, MB, MS, JB, DK, DR, and RK analyzed and interpreted the data. Animal experiments were performed by OS. Administrative, technical, or material support was given by OA, DR, GD, IT, EA, HB, ML, CS, MS, FB, MOP, HG, RC, SC, and JB. The study was supervised by RK, DK, and MB, and they are the guarantor of the article. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.607217/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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