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Glucocorticoid receptor expression after the return of spontaneous circulation in patients who experienced cardiac arrest: A prospective observational study

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Abstract

Objectives: Rapid changes in glucocorticoid (GC) levels and adrenal insufficiency are related to the development of post-cardiac arrest (CA) syndrome. However, changes in GC receptor (GR) expression have not been studied. Hence, the aim of this study was to investigate the association of early changes in GR expression and prognosis and immune response in patients who experienced CA.

Design: Prospective observational study.

Setting: Emergency department.

Participants: Patients (85) who were in the early period of return of spontaneous circulation (ROSC) after CA and were admitted between October 2018 and October 2019. Age- and sex-matched healthy individuals (40) were recruited for the control group after a physical examination.

Primary and secondary outcome measures: GR expression and cell counts of circulatory T and B lymphocytes, natural killer cells, and regulatory T (Treg) cells were assessed. Plasma total cortisol and adrenocorticotrophic hormone (ACTH) levels were also tested.

Results: All cell counts were lower, and plasma total cortisol levels were higher ($P<0.001$), in patients who experienced CA than in the healthy control group. GR expression in Treg cells and $CD3^{+}CD4^{+}$ T lymphocytes was not significantly different, but the mean fluorescence intensity and GR expression in other cells were lower in patients who experienced CA ($P<0.05$) than in the healthy control group. ACTH levels were not different. There were no significant differences between survivors and non-

survivors.

Conclusions: This study revealed that GR expression and cell counts rapidly decreased, whereas plasma total cortisol levels increased, in the early period after ROSC among patients who experienced CA. Our findings provide insights into GC sensitivity and immunosuppressive status in these patients, and a new perspective for GC targeted treatment.

Strengths and limitations of this study

1. Explore whether controversy over glucocorticoid use is associated with different levels of glucocorticoid receptor expression in cardiac arrest patients for the first time.
2. Glucocorticoid receptor expression rapidly decreased in the early period following restoration of spontaneous circulation among patients who experienced cardiac arrest .
3. We only observed changes in glucocorticoid receptor expression of cardiac arrest patients at the early period following restoration of spontaneous circulation, and long-term dynamic observation would be helpful to understand the significance of clinical steroid therapy.

Introduction

Cardiac arrest (CA) is an important health problem globally; about 356,500 people experience medical emergencies due to CA in the United States, and over 544,000 people die from sudden CA in China annually. [1, 2] The systemic ischemia-reperfusion response in patients who have experienced CA can present as post-CA syndrome (PCAS) or systematic inflammatory response syndrome (SIRS), which increases the

risk of multiple organ failure and infection and affects the inflammatory response and prognosis of patients after the return of spontaneous circulation (ROSC). [3-6]

CA is the most intense among acute stress events, which seriously affect the function of the pituitary and adrenal axis. [7] Studies have shown that abnormal cortisol levels and relative adrenocortical insufficiency after ROSC in patients who experienced CA are related to their prognosis. [8-11] However, the clinical application of glucocorticoids (GCs) is controversial. In the 2015 International Cardiopulmonary Resuscitation Guidelines, the routine use of GCs is not recommended for the resuscitation of patients with in-hospital or out-of-hospital CA. [12] Recent clinical studies have shown that early administration of corticosteroids after CA can improve the success rate of ROSC, nervous system functional outcome, and prognosis, which is speculated to be related to its influence on hemodynamics, SIRS response, and other mechanisms. [12-17] Therefore, the role of GCs in the occurrence and development of PCAS needs to be studied further.

GCs combine with intracellular GC receptors (GRs) to exert anti-inflammatory and immunosuppressive effects and reduce the production as well as release of inflammatory cytokines. [18, 19] The affinity of GRs to GCs in circulating monocytes is decreased in patients with acquired immunodeficiency syndrome. [20] The expression of GR is decreased in patients with critical illness, [21] pediatric septic shock, and high serum cortisol level. [22] However, hitherto, no study has reported the GR expression after ROSC in patients who experienced CA. Previous studies have found that the counts of circulating B and T lymphocytes, regulatory T (Treg) cells, and

monocytes and expression of human leukocyte antigen DR (HLA-DR) on circulatory monocytes and B and T lymphocytes are reduced. [23, 24] Hence, the aim of this study was to investigate the relationship between GR expression and immune alteration in the early period after ROSC in patients who experienced CA by observing GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells, their cell counts, and plasma total cortisol and adrenocorticotrophic hormone (ACTH) levels.

MATERIALS AND METHODS

Study participants

This was an observational study conducted in the Emergency Department (ED). Following the 2015 International Cardiopulmonary Resuscitation Guidelines, [25] we enrolled patients who were in the early period of ROSC after CA and were admitted to the ED between October 2018 and October 2019. The inclusion criteria were (a) ROSC 6 h after CA and (b) Glasgow Coma Scale score <8 after ROSC. The exclusion criteria were (a) <18 years of age, (b) terminal stage of disease (such as cancer of any type, acquired immunodeficiency syndrome), (c) corticosteroid treatment within the past 3 months, (d) administration of corticosteroids, and (e) adrenal insufficiency. All patients were treated according to the 2015 International Cardiopulmonary Resuscitation Consensus. [13] Age- and sex-matched healthy individuals were recruited for the control group after a physical examination.

Data collection

We collected data on demographics, resuscitation (initial heart rhythm, ROSC time, and cumulative adrenaline epinephrine dose), and laboratory findings (routine blood cell counts, blood gas analysis, and blood biochemical tests performed 6 h after ROSC). Acute Physiology and Chronic Health Evaluation (APACHE) II and the Sequential Organ Failure Assessment (SOFA) were used to determine disease severity. Residual samples of blood, with heparin anticoagulant, from routine clinical tests or physical health examinations were collected, maintained at 4 °C during transport and storage, and used to determine GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells and their cell counts. The plasma was maintained at -80 °C during storage and used to determine total cortisol and ACTH levels. During follow-up, 28-day survival data were also collected. Supplemental Figure 1 shows the workflow of this study.

Flow cytometry

GR expression in T and B lymphocytes, NK cells, and Treg cells was measured. Briefly, a 100-μL peripheral blood sample was stained for 20 min with surface antibodies (CD3, CD4, CD8, CD19, CD16, CD56, CD25, and CD127) in a dark place. Erythrocytes were lysed for 15 min, and the debris was washed away. Before intracellular GR staining, surface-stained cells were fixed and permeabilized using the BD Transcription Factor Buffer Set (BD Pharmingen, San Diego, USA, Catalogue No. 562574). Monoclonal antibodies and their isotype controls were all purchased from BD Biosciences (San Jose, CA, USA). Details of all antibodies are shown in Supplemental Table 1.

According to the manufacturer's recommendations, all antibodies and their isotype controls were used at a concentration of 1 μL per 100 μL of whole blood. Samples were measured using the Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed using Gallios Software version 1.0 (Beckman Coulter). The flow cytometer was periodically calibrated by an engineer. Cells were stained for 20 min; thresholds were defined using the manufacturer's recommended isotype controls. T cells were gated by $CD3^+CD4^+$ or $CD3^+CD8^+$, B cells were gated by $CD3^-CD19^+$, NK cells were gated by $CD16^+CD56^+$, and Tregs were gated by $CD4^+CD25^{high}CD127^{low}$. At least 10,000 events were collected in the lymphocyte cell gate for each sample. Results are expressed as percentages and mean fluorescence intensity (MFI) values.

Absolute $CD3^+$ and $CD4^+$ lymphocyte, NK cell, and Treg cell counts were obtained using Flow-Count fluorospheres (Beckman Coulter, Catalogue No. 7547053), according to the manufacturer's instructions. B, $CD3^+CD4^+$T, $CD3^+CD8^+$T, and Treg cell counts were calculated by their percentages in $CD3^+$ or $CD4^+$ lymphocytes multiplied by $CD3^+$ or $CD4^+$ lymphocyte counts.

Determination of plasma total cortisol and ACTH levels after ROSC

Venous blood samples were collected in ethylenediaminetetraacetic acid tubes, centrifuged 10 min at 3000 rpm, and then stored at -80 °C. Plasma total cortisol (IMMULITE 2000 Cortisol, L2KCO2, UK) and ACTH (IMMULITE 2000 ACTH, L2KAC2, UK) levels were assayed using a chemiluminescent immunoassay on a Siemens automated analyzer (IMMULITE 2000 XPi; Siemens Healthcare Diagnostics,

Erlangen, Germany). The equipment and reagents were calibrated by engineers before use. The lower detection limit of total cortisol was 2.00 ng/mL and that of ACTH was 5.00 pg/mL.

Statistical analyses

All data were analyzed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). For normally distributed data, continuous variables are expressed as means with standard deviations. Since the data for total cortisol and ACTH levels had a skewed distribution, we compared our results with the natural logarithmic conversion values after adding 1 (ln [total cortisol+ 1], ln [ACTH+ 1]). Measurement data with a skewed distribution are expressed as medians (25th and 75th percentiles). The Mann–Whitney U test was used to compare variables between groups. The qualitative parameters in the 2 × 2 contingency table were used for analysis. All statistical tests were two-tailed, and a P-value of <0.05 was considered statistically significant.

Follow-up

Patients who experienced CA were classified into survivor and non-survivor groups according to the 28-day survival endpoint. Those with all-cause mortality within the follow-up period were considered non-survivors. If data were lost, the corresponding candidate was excluded.

Patient and public involvement

This study was approved by the Medical Ethics Committee (2013-KE-1). Patient consent to participate was obtained prior to enrolment in this study.

Results

Patient characteristics

In total, 40 healthy individuals and 85 patients who experienced CA were analyzed. The demographics and clinical characteristics of both groups are shown in Table 1. In this study, acute cardiac and brain events were the main causes of CA. Other causes of CA included poisoning (including carbon monoxide poisoning) and hypokalemia. Sex and age were not significantly different between the CA and healthy control groups. The comparisons of clinical characteristics of the survivor and non-survivor groups based on 28-day survival are shown in Supplemental Table 2. The APACHE II and SOFA scores were significantly different between the CA and healthy control groups (P<0.001 for all) and survivor and non-survivor groups (P<0.001 and P=0.011, respectively).

Table 1. Patient Characteristics at Admission

Characteristics	Healthy Control Group (n=40)	Successful Resuscitation Group (n=85)	*P*-value
Age (years), median [IQR]	64.0 (54.3, 69.8)	65.0 (55.0, 74.0)	0.209
Male/Female (n)	23/17	58/27	0.241
Previous medical history, n (%)			

Hypertension	5 (12.5%)	38 (44.7%)	<0.001
Diabetes	3 (7.5%)	27 (31.8%)	0.003
Coronary heart disease	2 (5.0%)	29 (34.1%)	<0.001
Chronic lung disease	1 (2.5%)	9 (10.6%)	0.230
Chronic kidney disease	0	9 (10.6%)	0.077
Cardiac arrest cause (n, %)			
Cardiac		34 (40.0%)	
Respiratory		20 (23.5%)	
Cerebral		23 (27.1%)	
Others		7 (8.2%)	
Unknow		1 (1.2%)	
Initial resuscitation			
Time to ROSC (min), median [IQR]		20.0 (10.0, 30.0)	
Adrenaline (mg), median [IQR]		2.0 (0.0, 5.0)	
Initial rhythm VF/VT, n (%)		30 (35.3%)	
MAP (mmHg), median [IQR]	95.7 (86.0, 103.2)	74.3 (56.2, 97.2)	<0.001
White cell count ($\times 10^9$/L), median [IQR]	5.81 (4.85, 6.53)	13.56 (10.84, 18.29)	<0.001
APACHE Ⅱ score, mean±SD	0	32.9±6.5	<0.001
SOFA score, median [IQR]	0	11.5 (8.5, 14.0)	<0.001
28-day mortality, n (%)		65（76.5%）	
28-day CPC 1–2, n (%)		14 (16.5%)	

Abbreviations: IQR: interquartile range; ROSC: return of spontaneous circulation; VF: ventricular fibrillation; VT: ventricular tachycardia; MAP: mean arterial pressure; APACHE Ⅱ: acute physiology and chronic health evaluation; SOFA: sequential organ failure assessment; SD: standard deviation; CPC: cerebral performance category.

Changes in circulatory T and B lymphocyte, NK cell, and Treg cell counts after ROSC

The T and B lymphocyte, NK cell, and Treg cell counts were significantly lower after ROSC in patients who experienced CA than in healthy controls ($P<0.001$ for all). Additionally, the $CD3^+CD4^+$/T lymphocyte, $CD3^+CD8^+$/T lymphocyte, and Treg cell/$CD4^+$ T lymphocyte ratios were significantly lower after ROSC in patients who experienced CA than in healthy controls ($P<0.001$ for all) (Fig. 1; Supplemental Table 3). However, there were no significant differences in these cell counts and ratios between survivors (n=20) and non-survivors (n=65) ($P>0.05$ for all) (Supplemental Table 4).

GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells after ROSC

The MFI and percentages of GR expression in B and T lymphocytes, NK cells, and $CD3^+CD8^+$ T lymphocytes were significantly lower after ROSC in patients who experienced CA than in healthy individuals ($P<0.01$ for all) (Fig. 2A–D, G, H, K, L). There were also significant reductions in the MFI in Treg cells and $CD3^+CD4^+$ T lymphocytes ($P<0.05$ for all) (Figs. 2E, I) but not in the percentages of GR expression ($P>0.05$ for all) (Figs. 2F, J; Supplemental Table 5). However, there were no significant differences in the MFI and percentages of GR expression in these cells between survivors and non-survivors ($P>0.05$ for all) (Supplemental Table 6).

Changes in plasma total cortisol and ACTH levels after ROSC

We measured the plasma total cortisol and ACTH levels of the 40 healthy individuals and 85 patients who experienced CA (two samples were excluded because their total cortisol levels were not measured). Plasma total cortisol levels were significantly higher in patients who experienced CA than in healthy controls ($P<0.001$) but ACTH levels were not (Figs. 3A, C). No significant differences in ln (total cortisol+1) and ln (ACTH+1) values were observed between survivors and non-survivors ($P>0.05$ for all) (Fig. 3B, D).

Discussion

In this study, the relationship between GR expression and immune alteration in the early period after ROSC in patients who experienced CA was explored by observing GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells and changes in cell counts and plasma total cortisol and ACTH levels. We found that GR expression, cell counts, and ratios rapidly decreased, and plasma total cortisol levels increased, in these patients.

After ROSC, the immune response of patients who experience CA is impaired, and the systemic inflammatory response is increased. [6, 26] In this study, the T and B lymphocyte, NK cell, and Treg cell counts as well as $CD3^+CD4^+$/T, $CD3^+CD8^+$/T, and Treg cell/$CD4^+$ T lymphocyte ratios were significantly reduced after ROSC. NK cells, which are special innate immune cells that have cytotoxic functions similar to

$CD3^+CD8^+$ T lymphocytes, mainly distinguish infected and stressed cells from healthy cells and eliminate intracellular infection as well as dysfunctional cells. [27, 28] T lymphocytes are also important because of their function as adaptive immune cells for the control and elimination of infection. [27] Moreover, B and T lymphocytes mediate humoral and cellular immunity, respectively. This study was performed at an earlier period and involved a more comprehensive assessment of the immune system of patients who experienced CA, and our findings more substantially supported the rapid emergence of immune dysfunction in these patients after ROSC than previous reports.

The effectiveness of GC use in these patients during and after resuscitation has been controversial due to insufficient evidence. However, the use of GCs during resuscitation improves the survival rate of patients who experience CA due to its direct anti-inflammatory, immunosuppressive effects, hemodynamics, and positive inotropic effects. All of this ultimately leads to an increased stress capacity of the body. [18, 19] GCs can activate GRs in cells when the body is under stress, thereby increasing both the effectiveness of resuscitation and discharge survival rate. This study is the first to explore GR expression in circulating immune cells in patients who experienced CA after ROSC. We observed that GR expression in B and T lymphocytes, NK cells, and $CD3^+CD8^+$ T lymphocytes decreased significantly in patients who experienced CA, whereas the percentage of GR^+ Treg cells and $CD3^+CD4^+$ T lymphocytes showed a slight decrease. Moreover, we observed a more significant decrease in the MFI of GR expression in Treg cells and $CD3^+CD4^+$ T lymphocytes but not in the percentage of GR

expression. Previous studies have found decreased expression of GRs in peripheral polymorphonuclear cells in critically ill patients, [21] and antagonism to GRs aggravates viral and bacterial infections. [29] The results of this study suggest that the decrease in intracellular GR expression in patients who experienced CA is one of the causes of GC resistance, due to insufficient binding of GRs and GCs, GC insensitivity, and the inability of GCs to effectively exert anti-inflammatory and immunosuppressive effects. These findings may also explain why different results regarding the clinical application of GCs have been reported previously and support the possibility of using GCs in the clinical treatment of patients who experienced CA.

We also found that the total plasma cortisol levels were significantly higher in patients who experienced CA, but ACTH levels were not. High levels of inflammatory cytokines inhibit ACTH release. [18] During critical illness, the body does not sufficiently metabolize cortisol. [30] In addition, the continuous increase in plasma cortisol levels may trigger the negative feedback pathway of the hypothalamic-pituitary-adrenal axis, inhibiting the release of ACTH and cortisol and eventually leading to adrenal insufficiency. These factors may explain the opposite trends of plasma ACTH and cortisol levels in the patients who were included in this study and experienced CA. Notably, this result suggests that low GR expression levels are not matched with high plasma total cortisol levels. Previous studies have found that GC use during resuscitation may benefit patients who experience CA. [13-16] The benefits, such as direct anti-inflammatory and anti-shock effects, improvement of vascular endothelial permeability, and other mechanisms may be related to the effects of using

a high dose of GCs, or GCs may work through other non-GR pathways. It is also possible that the immune function of patients who experience CA is suppressed due to ischemia-reperfusion injury, which requires a large dose of GCs to stimulate GRs to function. This study did not provide data on plasma GC levels and GR expression in a group of patients who were administered GCs and successfully resuscitated; therefore, further studies are required to explore the exact mechanisms of GCs.

Limitations

Our study has several limitations. First, to assess changes, we only enrolled patients who experienced CA and had signs of systemic ischemic hypoxia, such as GCs <8 after ROSC. The patients were not stratified by age, sex, and occurrence of comorbidities or mild systemic ischemic hypoxia. Second, since this was a preliminary observational study, we observed only early changes. A dynamic observation for a longer duration would be helpful to understand the significance of GR expression in evolving immunity during the clinical course of CA after ROSC. Third, the samples used in this study were from the clinical laboratory; thus, plasma total cortisol and ACTH in the samples were at a risk of degradation before we collected the samples. Finally, we did not discuss the changes in and roles of GR isoforms, free cortisol, and corticosteroid-binding globulin. Therefore, future studies on these aspects are warranted to better understand the immunosuppressive effects of ROSC among patients who experienced CA.

In conclusion, this study revealed that GR expression, cell counts, and ratios rapidly decreased, whereas plasma total cortisol levels increased, in the early period after ROSC among CA patients. These findings may provide important information

about GC sensitivity and immunosuppressive status in these patients. In addition, this study provides a new perspective for clinical targeted treatment using GCs and high-quality prognosis in CA patients.

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Ethics Approval: This study was approved by the Medical Ethics Committee of Beijing Chaoyang Hospital (2013-KE-1). Patient consent to participate was obtained prior to enrolment in this study.

Contributorship statement: CL designed the study and reviewed the manuscript. YNY searched the literature and contributed to the experimental studies, data analysis, and writing of the manuscript. ZRT, CCH, and LA collected and analyzed data. JBL and MRX helped with the statistical analyses. All authors have read and approved the final manuscript.

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Data sharing statement: All data relevant to the study are included in the article or uploaded as supplementary information. Due to privacy and ethical concerns, data can not be shared.

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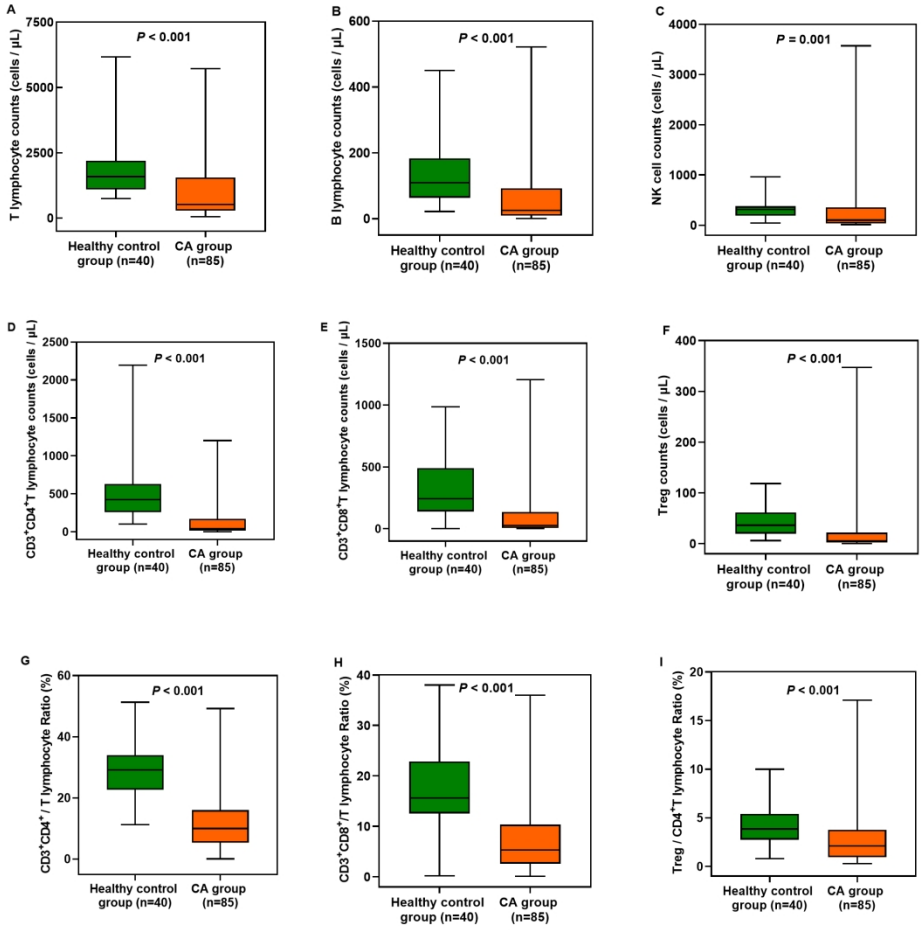
Figure legends

Fig. 1. Changes in circulatory T and B lymphocyte, NK cell, and Treg cell counts, and $CD3^+CD4^+/T$, $CD3^+CD8^+/T$, and $Treg/CD4^+T$ lymphocyte ratios between the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group ($P<0.001$). CA, cardiac arrest; CD, cluster-of-differentiation; NK, natural killer; Treg, regulatory T.

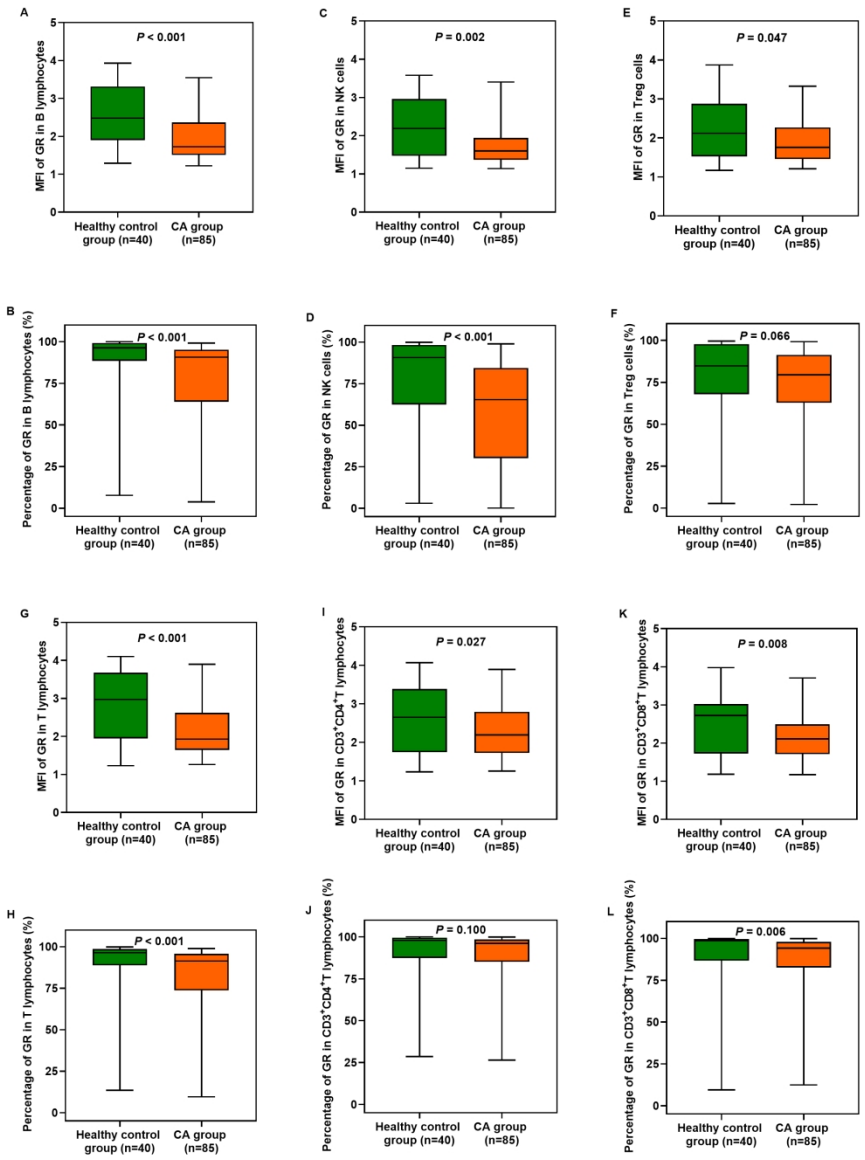
Fig. 2. Expression of GRs in circulatory T and B lymphocytes, NK cells, and Treg cells in the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group ($P<0.05$). CA, cardiac arrest; CD, cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; ROSC, return of spontaneous circulation; Treg, regulatory T.

Fig. 3. (A, B) Plasma total cortisol and ACTH levels (the natural logarithmic conversion values after adding 1) after ROSC in the healthy control group and CA group. (C, D) Plasma total cortisol and ACTH levels in survivors and non-survivors after ROSC. The CA group showed significant differences compared with the healthy control group ($P<0.05$). ACTH, adrenocorticotrophic hormone; CA, cardiac arrest; ROSC, return of spontaneous circulation.



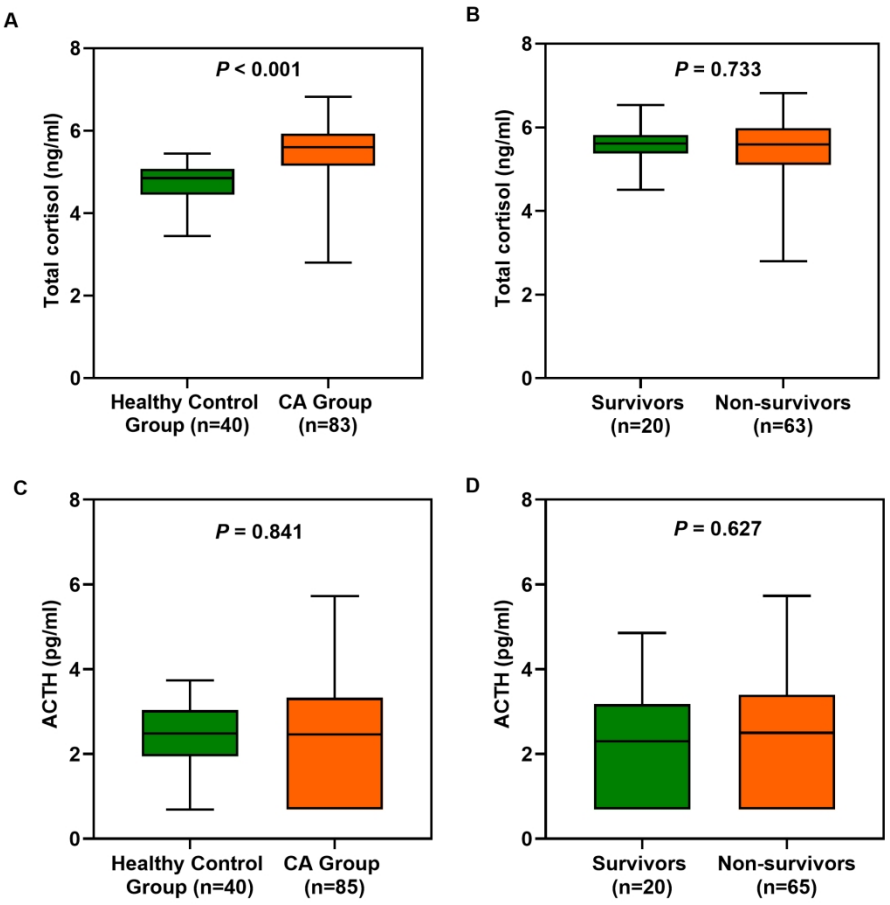
Changes in circulatory T and B lymphocyte, NK cell, and Treg cell counts, and CD3+CD4+/T, CD3+CD8+/T, and Treg/CD4+T lymphocyte ratios between the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group (P<0.001). CA, cardiac arrest; CD, cluster-of-differentiation; NK, natural killer; Treg, regulatory T.

187x183mm (300 x 300 DPI)



Expression of GRs in circulatory T and B lymphocytes, NK cells, and Treg cells in the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group (P<0.05). CA, cardiac arrest; CD, cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; ROSC, return of spontaneous circulation; Treg, regulatory T.

199x256mm (300 x 300 DPI)



(A, B) Plasma total cortisol and ACTH levels (the natural logarithmic conversion values after adding 1) after ROSC in the healthy control group and CA group. (C, D) Plasma total cortisol and ACTH levels in survivors and non-survivors after ROSC. The CA group showed significant differences compared with the healthy control group (P<0.05). ACTH, adrenocorticotrophic hormone; CA, cardiac arrest; ROSC, return of spontaneous circulation.

185x178mm (300 x 300 DPI)

Electronic supplemental material

Expression of glucocorticoid receptors early after the return of spontaneous circulation in patients who experienced cardiac arrest: A prospective observational study

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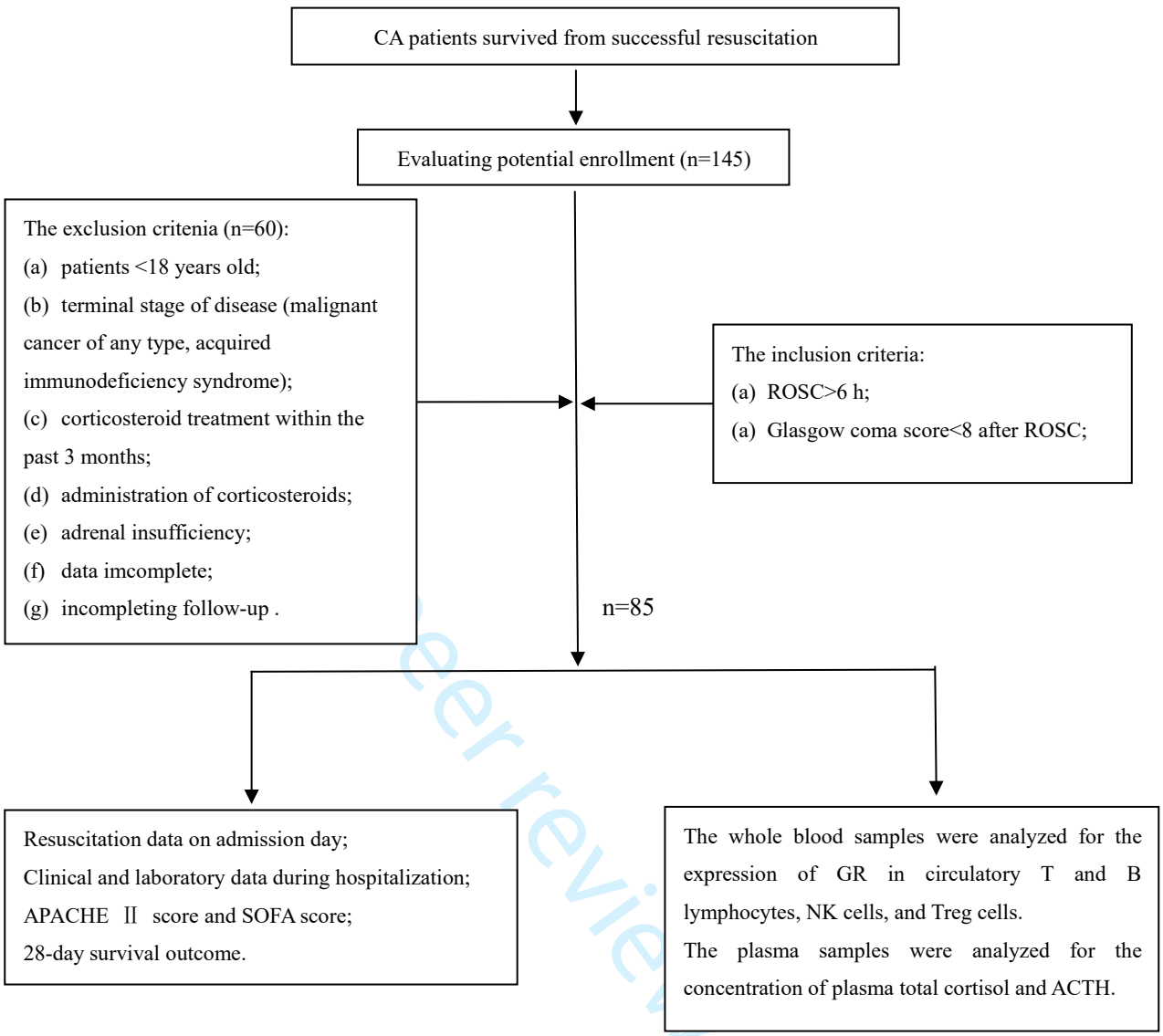

Supplemental Figure 1. The flow chart of the study.

Abbreviations: CA, cardiac arrest; ROSC, return of spontaneous circulation; APACHE II, acute physiology and chronic health evaluation II; SOFA, sequential organ failure assessment; GR, glucocorticoid receptor; Treg, regulatory T; ACTH, adrenocorticotrophic hormone.

Supplemental Table 1. Details of antibodies for flow cytometry.

Antigen	Catalog Number	Fluorescein Conjugate	Source
CD3	558117	Pacific Blue	BD Pharmingen[a]
CD4	555347	PE	BD Pharmingen
CD4	560345	Horizon V450	BD Pharmingen
CD8	557746	PE-Cy7	BD Pharmingen
CD19	557835	PE-Cy7	BD Pharmingen
CD16	558122	Pacific Blue	BD Pharmingen
CD56	557747	PE-Cy7	BD Pharmingen
CD25	557741	PE-Cy7	BD Pharmingen
CD127	557938	PE	BD Pharmingen
GR	MCA2469F	FITC	Bio-Rad[b]
Mouse IgG1 Isotype	MCA928F	FITC	Bio-Rad
Mouse IgG1,κ Isotype	557872	PE-Cy7	BD Pharmingen
Mouse IgG1,κ Isotype	554680	PE	BD Pharmingen
Mouse IgG1,κ Isotype	558120	Pacific Blue	BD Pharmingen

[a] BD Pharmingen, San Diego, USA; [b] Bio-Rad AbD Serotec, Oxford, UK.

Abbreviations: CD, cluster-of-differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate; GR, glucocorticoid receptor; Ig: immunoglobulin.

Supplemental Table 2. Characteristics of CA survivors and non-survivors on admission.

	Survivors (n=20)	**Non-survivors (n=65)**	***P*-value**
Age (years), median [IQR]	59.0 (53.3, 72.8)	66.0 (59.0, 75.5)	0.070
Male/Female (n)	12/8	46/19	0.366
Cardiac arrest cause (n, %)			
Cardiac	10 (50.0%)	24 (36.9%)	0.297
Non-Cardiac	10 (50.0%)	41 (63.1%)	0.297
Initial resuscitation			
Time to ROSC (min), median [IQR]	15.0 (7.3, 26.0)	20.0 (15.0, 30.0)	0.032
Adrenaline (mg), median [IQR]	1.0 (0.0, 3.0)	2.0 (0.0, 5.0)	0.091
Initial rhythm VF/VT, n (%)	11 (55.0%)	19 (29.2%)	0.035
MAP (mmHg), median [IQR]	89.9 (70.5, 104.9)	70.7 (50.0, 93.5)	0.033
White cell count ($\times 10^9$/L), median [IQR]	12.40 (6.98, 18.76)	13.80 (11.67, 18.20)	0.286
Lactate (mmol/L), median [IQR]	3.50 (1.33, 7.05)	7.50 (3.80, 11.20)	0.008
APACHE II score, mean±SD	27.8±6.6	34.4±5.6	<0.001
SOFA score, median [IQR]	9.0 (7.3, 11.8)	12.0 (9.0, 15.0)	0.011

Data are presented as mean±SD or interquartile range (IQR) as appropriate. The *P*-value represents comparison between groups. Abbreviations: ROSC: return of spontaneous circulation; VF: ventricular fibrillation; VT: ventricular tachycardia; MAP: mean arterial pressure; APACHE II: acute physiology and chronic health evaluation; SOFA: sequential organ failure assessment.

Supplemental Table 3. The flow cytometry results of cell counts and ratios of healthy control group and successful resuscitation group

	Healthy Control Group （n=40）	**Successful Resuscitation Group (n=85)**	***Z*-value**	***P*-value**
T lymphocyte count (cells /μL)	1586.0 (1101.5, 2192.5)	514.0 (287.5, 1555.0)	-4.515	<0.001
NK cell count (/μL)	311.5 (191.0, 378.8)	101.0 (36.0, 351.5)	-3.332	0.001
B lymphocyte count (/μL)	109.3 (63.7, 183.3)	25.7 (9.4, 92.3)	-5.076	<0.001
Treg count (/μL)	0.259 (0.095, 0.516)	0.233 (0.135, 0.488)	-5.518	<0.001
Treg / $CD4^+$T lymphocyte Ratio	0.039 (0.028, 0.054)	0.021 (0.010, 0.038)	-4.418	<0.001
$CD3^+CD4^+$ T lymphocyte count (/μL)	421.7 (258.6, 627.4)	38.9 (17.6, 168.3)	-6.256	<0.001
$CD3^+CD4^+$ / T lymphocyte Ratio	0.292 (0.227, 0.340)	0.100 (0.054, 0.160)	-7.066	<0.001
$CD3^+CD8^+$ T lymphocyte count (/μL)	241.1 (139.5, 488.6)	26.3 (7.2, 135.9)	-5.287	<0.001
$CD3^+CD8^+$ / T lymphocyte Ratio	0.157 (0.126, 0.229)	0.053 (0.026, 0.104)	-5.719	<0.001

All the data in Supplemental table 3 are represented as the median [IQR]; IQR: Interquartile Range; CD: cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; Treg, regulatory T.

Supplemental Table 4. The flow cytometry results of cell counts and ratios of the CA patients on admission based on 28-day survival

	Survivors (n=20)	**Non-survivors (n=65)**	***Z*-value**	***P*-value**
T lymphocyte count (/μL)	502.0 (353.8, 1199.8)	514.0 (282.5, 1891.0)	-0.186	0.852
NK cell count (/μL)	167.0 (29.8, 309.3)	100.0 (36.0, 404.0)	-0.218	0.828
B lymphocyte count (/μL)	38.6 (15.7, 103.5)	19.2 (7.1, 65.7)	-0.632	0.527
Tregs count (/μL)	0.318 (0.145, 0.552)	0.212 (0.128, 0.479)	-0.611	0.396
Treg / $CD4^+$ T lymphocyte Ratio	0.025 (0.009, 0.043)	0.021 (0.010, 0.034)	-0.498	0.619
$CD3^+CD4^+$ T lymphocyte count (/μL)	55.1 (32.4, 228.0)	38.0 (16.0, 168.1)	-0.850	0.396
$CD3^+CD4^+$ / T lymphocyte Ratio	0.118 (0.070, 0.236)	0.097 (0.049, 0.142)	-1.565	0.118
$CD3^+CD8^+$ T lymphocyte count (/μL)	25.4 (12.5, 96.2)	26.3 (6.3, 138.8)	-0.021	0.983
$CD3^+CD8^+$ / T lymphocyte Ratio	0.054 (0.033, 0.104)	0.053 (0.025, 0.104)	-0.187	0.852

All the data in Supplemental table 4 are represented as the median [IQR]; IQR: Interquartile Range; CD: cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; Treg, regulatory T.

Supplemental Table 5. The flow cytometry results of GR expression in the CA group and successful resuscitation group.

	Healthy Control Group （n=40）	**Successful Resuscitation Group (n=85)**	***Z*-value**	***P*-value**
Percentage of GR on B lymphocytes	0.963 (0.885, 0.992)	0.896 (0.605, 0.949)	-3.742	<0.001
MFI of GR on B lymphocytes	2.48 (1.91, 3.31)	1.73 (1.50, 2.37)	-3.980	<0.001
Percentage of GR on T lymphocytes	0.964 (0.889, 0.986)	0.900 (0.703, 0.955)	-3.755	<0.001
MFI of GR on T lymphocytes	2.98(1.95, 3.68)	1.92 (1.36, 1.99)	-3.853	<0.001
Percentage of GR on NK cells	0.907 (0.624, 0.983)	0.611 (0.306, 0.840)	-3.792	<0.001
MFI of GR on NK cells	2.19 (1.48, 2.96)	1.60 (1.36, 1.99)	-3.171	0.002
Percentage of GR on Treg cells	0.848 (0.680, 0.978)	0.784 (0.589, 0.911)	-1.837	0.066
MFI of GR on Treg cells	2.12 (1.53, 2.88)	1.76 (1.44, 2.30)	-1.990	0.047
Percentage of GR on $CD3^+CD4^+$ T lymphocytes	0.980 (0.874, 0.996)	0.957 (0.824, 0.985)	-2.204	0.100
MFI of GR on $CD3^+CD4^+$ T lymphocytes	2.65 (1.75, 3.38)	2.17 (1.70, 2.92)	-1.646	0.027
Percentage of GR on $CD3^+CD8^+$T lymphocytes	0.986 (0.868, 0.996)	0.938 (0.823, 0.979)	-2.758	0.006
MFI of GR on $CD3^+CD8^+$ T lymphocytes	2.73 (1.73, 3.02)	2.10 (1.68, 2.54)	-2.668	0.008

All the data in Supplemental table 5 are represented as the median [IQR]. Abbreviations: IQR, interquartile Range; CD, cluster-of-differentiation; NK, natural killer; Treg, regulatory T; GR, Glucocorticoid receptor; MFI, mean fluorescence intensity.

Supplemental Table 6. The flow cytometry results of GR expression in the survivors and non-survivors.

	Survivors (n=20)	**Non-survivors (n=65)**	***Z*-value**	***P*-value**
Percentage of GR on B lymphocytes	0.904 (0.595, 0.976)	0.906 (0.657, 0.946)	-0.787	0.431
MFI of GR on B lymphocytes	1.92 (1.52, 2.54)	1.72 (1.51, 2.31)	-0.881	0.378
Percentage of GR on T lymphocytes	0.899 (0.778, 0.969)	0.913 (0.692, 0.951)	-1.057	0.291
MFI of GR on T lymphocytes	2.05 (1.67, 2.83)	1.91 (1.64, 2.46)	-1.031	0.303
Percentage of GR on NK cells	0.717 (0.292, 0.886)	0.556 (0.302, 0.823)	-0.756	0.449
MFI of GR on NK cells	1.54 (1.37, 2.09)	1.61 (1.34, 1.87)	-0.565	0.572
Percentage of GR on Tregs	0.780 (0.667, 0.849)	0.799 (0.576, 0.923)	-0.440	0.660
MFI of GR on Tregs	1.61 (1.48, 2.30)	1.77 (1.45, 2.27)	-0.005	0.996
Percentage of GR on $CD3^+CD4^+$ T lymphocytes	0.975 (0.876, 0.985)	0.957 (0.845, 0.987)	-0.617	0.538
MFI of GR on $CD3^+CD4^+$ T lymphocytes	2.08 (1.72, 3.35)	2.22 (1.71, 2.69)	-0.865	0.387
Percentage of GR on $CD3^+CD8^+$ T lymphocytes	0.963 (0.816, 0.977)	0.938 (0.834, 0.980)	-0.254	0.800
MFI of GR on $CD3^+CD8^+$ T lymphocytes	2.08 (1.68, 3.10)	2.11(1.71, 2.46)	-0.653	0.514

All the data in Supplemental table 6 are represented as the median [IQR]. Abbreviations: IQR, Interquartile Range; CD, Cluster-of-differentiation; NK, natural killer; Treg, regulatory T; GR, glucocorticoid receptor; MFI, mean fluorescence intensity.

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3-5
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	Supplemental Figure 1
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5-9, Supplemental Figure 1
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5,6,8,9
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5, 6, 8, Supplemental Figure 1
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-8
Bias	9	Describe any efforts to address potential sources of bias	6-8
Study size	10	Explain how the study size was arrived at	Supplemental Figure 1
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8, 11

(*b*) Describe any methods used to examine subgroups and interactions	N/A
(*c*) Explain how missing data were addressed	8, 11
(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	11
(*e*) Describe any sensitivity analyses	

Continued on next page

Results			
Participants	13 *	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	9, Supplemental Figure 1
		(b) Give reasons for non-participation at each stage	11, Supplemental Figure 1
		(c) Consider use of a flow diagram	Supplemental Figure 1
Descriptive data	14 *	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9
		(b) Indicate number of participants with missing data for each variable of interest	9-11
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	8
Outcome data	15 *	*Cohort study*—Report numbers of outcome events or summary measures over time	9-11
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-11, Electronic supplemental material
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N/A
Discussion			
Key results	18	Summarise key results with reference to study objectives	12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12-14
Generalisability	21	Discuss the generalisability (external validity) of the study results	15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely

available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Glucocorticoid receptor expression in patients with cardiac arrest in the early period after the return of spontaneous circulation: A prospective observational study

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Glucocorticoid receptor expression in patients with cardiac arrest in the early period after the return of spontaneous circulation: A prospective observational study

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Keywords: Cardiac arrest, glucocorticoid receptor, immunosuppression, cortisol

Word count of the main text: 3,335 words

Abstract

Objectives: Rapid changes in glucocorticoid (GC) levels and adrenal insufficiency are related to the development of post-cardiac arrest (CA) syndrome. However, GC receptor (GR) expression changes have not been studied. Hence, this study aimed to investigate the association of early changes in GR expression and prognosis and immune response in patients who experienced CA.

Design: Prospective observational study.

Setting: Emergency department.

Participants: Patients (85) in the early period of return of spontaneous circulation (ROSC) after CA were admitted between October 2018 and October 2019. After a physical examination, age- and sex-matched healthy individuals (40) were recruited for the control group.

Primary and secondary outcome measures: GR expression and cell counts of circulatory T and B lymphocytes, natural killer cells, and regulatory T (Treg) cells were assessed. Plasma total cortisol and adrenocorticotrophic hormone (ACTH) levels were also tested.

Results: All cell counts were lower, and plasma total cortisol levels were higher ($P<0.001$) in patients who experienced CA than in the healthy control group. GR expression in Treg cells and $CD3^+CD4^+$ T lymphocytes were not significantly different, but the mean fluorescence intensity and GR expression in other cells were lower in patients who experienced CA ($P<0.05$) than in the healthy control group. ACTH levels were not different. There were no significant differences between survivors and non-

survivors.

Conclusions: This study revealed that GR expression and cell counts rapidly decreased, whereas plasma total cortisol levels increased in the early period after ROSC among patients who experienced CA. Our findings provide important information about GR level and function, and immunosuppressive status in these patients. Assessing GR expression in CA patients may help screening for those who are more sensitive to glucocorticoid therapy.

Strengths and limitations of this study

1. The study design will be single-center, prospective.
2. This is the first study to evaluate the GR expression in the early period following ROSC among CA patients.
3. Only CA patients in the early period following ROSC will be included, limiting the generalisability of the results.
4. Decreased GR expression may affect the sensitivity of CA patients to GCs.
5. Decreased GR expression may affect potential immune consequences of CA patients.

Introduction

Cardiac arrest (CA) is a significant health problem globally; about 356,500 people experience medical emergencies due to CA in the United States, and over 544,000 people die from sudden CA in China annually. [1, 2] The systemic ischemia-reperfusion

response in patients who have experienced CA can present as post-cardiac arrest syndrome (PCAS) or systematic inflammatory response syndrome (SIRS), which increases the risk of multiple organ failure and infection and affects the inflammatory response and prognosis of patients after the return of spontaneous circulation (ROSC). [3-6]

CA is the most intense among acute stress events, which seriously affect the pituitary and adrenal axis function. [7] Studies have shown that abnormal cortisol levels and relative adrenocortical insufficiency after ROSC in patients who experienced CA are related to their prognosis. [8-11] However, the clinical application of glucocorticoids (GCs) is controversial. In the 2015 International Cardiopulmonary Resuscitation Guidelines, the routine use of GCs is not recommended for the resuscitation of patients with in-hospital or out-of-hospital CA. [12] Recent clinical studies have shown that early administration of corticosteroids after CA can improve the success rate of ROSC, nervous system functional outcome, and prognosis, which is speculated to be related to its influence on hemodynamics, and SIRS response, and other mechanisms. [12-17] Therefore, the role of GCs in the occurrence and development of PCAS needs to be studied further.

GCs combine with intracellular GC receptors (GRs) to exert anti-inflammatory and immunosuppressive effects and reduce the production and the release of inflammatory cytokines. [18, 19] The affinity of GRs to GCs in circulating monocytes is decreased in patients with acquired immunodeficiency syndrome. [20] The expression of GR alpha and beta in peripheral polymorphonuclear cells is decreased in patients with critical

illness, [21] pediatric septic shock, and high serum cortisol levels. [22] However, no study has reported the GR expression after ROSC in patients who experienced CA. Previous studies have found that the counts of circulating B and T lymphocytes, regulatory T (Treg) cells, and monocytes and expression of human leukocyte antigen DR (HLA-DR) on circulatory monocytes and B and T lymphocytes are reduced. [23, 24] Hence, this study aimed to investigate the relationship between GR expression and immune alteration in the early period after ROSC in patients who experienced CA by observing GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells, their cell counts, and total plasma cortisol and adrenocorticotrophic hormone (ACTH) levels.

Materials and methods

Study participants

This was an observational study conducted in the Emergency Department (ED). According to the 2015 International Cardiopulmonary Resuscitation Guidelines, [25] we enrolled patients in the early ROSC period after CA (both in-hospital and out-of-hospital CA) and were admitted to the ED between October 2018 and October 2019. The inclusion criteria were patients with CA > 6 and < 24 hours after ROSC, with a Glasgow coma score < 8. The exclusion criteria were (a) <18 years of age, (b) terminal stage of disease (such as cancer of any type, acquired immunodeficiency syndrome), (c) corticosteroid treatment within the past three months, (d) administration of corticosteroids, and (e) adrenal insufficiency. All patients were treated according to the

2015 International Cardiopulmonary Resuscitation Consensus. [13] After a physical examination, age- and sex-matched healthy individuals were recruited for the control group.

Data collection

Data collection was performed according to the 2004 guidelines of the Utstein Style template. [26] We collected data on demographics, resuscitation (initial heart rhythm, ROSC time, and cumulative adrenaline [epinephrine] dose, and laboratory findings routine blood cell counts, blood gas analysis, and blood biochemical tests performed > 6 h and < 24 h after ROSC). Acute Physiology and Chronic Health Evaluation (APACHE) II and the Sequential Organ Failure Assessment (SOFA) were used to determine disease severity. Residual blood samples from routine clinical tests or physical health examinations in the morning were collected, maintained at 4 °C during transport and storage, and used to determine GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells and their cell counts. The plasma was maintained at -80 °C during storage and used to determine total cortisol and ACTH levels. During follow-up, 28-day survival data were also collected. Supplemental Figure 1 shows the workflow of this study.

Outcome measures

The primary outcomes of this study were GR expression and cell counts of T and B cells, NK cells, and Treg cells, measured by flow cytometry. Venous blood samples collected in ethylenediaminetetraacetic acid tubes, then used to measure GR expression in T and B lymphocytes, NK cells, and Treg cells. Briefly, a 100-μL peripheral blood

sample was stained for 20 min with surface antibodies (CD3, CD4, CD8, CD19, CD16, CD56, CD25, and CD127) in a dark place. Erythrocytes were lysed for 15 min, and the debris was washed away. Before intracellular GR staining, surface-stained cells were fixed and permeabilized using the BD Transcription Factor Buffer Set (BD Pharmingen, San Diego, USA, Catalogue No. 562574). Monoclonal antibodies and their isotype controls were all purchased from BD Biosciences (San Jose, CA, USA). Details of all antibodies are shown in Supplemental Table 1. According to the manufacturer's recommendations, all antibodies and their isotype controls were used at a concentration of 1 μL per 100 μL of whole blood. Samples were measured using the Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed using Gallios Software version 1.0 (Beckman Coulter). The flow cytometer was periodically calibrated by an engineer. Cells were stained for 20 min; thresholds were defined using the manufacturer's recommended isotype controls. Representative plots and gating strategy from a single sample are shown in Supplemental Figure 2. T cells were gated by $CD3^+CD4^+$ or $CD3^+CD8^+$, B cells were gated by $CD3^-CD19^+$, NK cells were gated by $CD16^+CD56^+$, and Tregs were gated by $CD4^+CD25^{high}CD127^{low}$. At least 10,000 events were collected in the lymphocyte cell gate for each sample. Results are expressed as percentages and mean fluorescence intensity (MFI) values.

Absolute $CD3^+$ and $CD4^+$ lymphocyte, NK cell, and Treg cell counts were obtained using Flow-Count fluorospheres (Beckman Coulter, Catalogue No. 7547053), according to the manufacturer's instructions. B, $CD3^+CD4^+$T, $CD3^+CD8^+$T, and Treg cell counts were calculated by their percentages in $CD3^+$ or $CD4^+$ lymphocytes

multiplied by $CD3^+$ or $CD4^+$ lymphocyte counts.

The secondary outcomes of this study were plasma total cortisol and ACTH levels after ROSC. Venous blood samples were collected in heparin anticoagulant tubes, centrifuged 10 min at 3000 rpm, and then stored at -80 °C. Plasma total cortisol (IMMULITE 2000 Cortisol, L2KCO2, UK) and ACTH (IMMULITE 2000 ACTH, L2KAC2, UK) levels were assayed using a chemiluminescent immunoassay on a Siemens automated analyzer (IMMULITE 2000 XPi; Siemens Healthcare Diagnostics, Erlangen, Germany). The equipment and reagents were calibrated by engineers before use. The lower detection limit of total cortisol was 2.00 ng/mL, and that of ACTH was 5.00 pg/mL.

Statistical analyses

Data analysis was used in SPSS version 22.0 (IBM Corp., Armonk, NY, USA) and sample size calculation in PASS15.0 software (NCSS, LLC, Kaysville, UT, USA). For normally distributed data, continuous variables are expressed as means with standard deviations. Since the data for total cortisol and ACTH levels had a skewed distribution, we compared our results with the natural logarithmic conversion values after adding 1 (ln [total cortisol+ 1], ln [ACTH+ 1]). Measurement data with a skewed distribution are expressed as medians (25th and 75th percentiles). The Mann–Whitney U test was used to compare variables between groups. The qualitative parameters in the 2 × 2 contingency table were used for analysis. All statistical tests were two-tailed, and a P-value of <0.05 was considered statistically significant.

Follow-up

Patients were classified into survivor and non-survivor groups according to the 28-day survival endpoint. Those with all-cause mortality within the follow-up period were considered non-survivors. If data were lost, the corresponding candidate was excluded.

Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Results

Patient characteristics

40 healthy individuals and 85 patients who experienced CA were analyzed. The demographics and clinical characteristics of both groups are shown in Table 1. In this study, acute cardiac and brain events were the main causes of CA, with those in the latter category emanating from strokes. Other causes of CA included poisoning (including carbon monoxide poisoning) and hypokalemia. Sex and age were not significantly different between the CA and healthy control groups. The comparisons of clinical characteristics of the survivor and non-survivor groups based on 28-day survival are shown in Supplemental Table 2. The APACHE II and SOFA scores were significantly different between the CA and healthy control groups ($P<0.001$ for all) and survivor and non-survivor groups ($P<0.001$ and $P=0.011$, respectively).

Table 1. Patient Characteristics at Admission

Characteristics	Healthy Control Group (n=40)	Successful Resuscitation Group (n=85)
Age (years), median [IQR]	64.0 (54.3, 69.8)	65.0 (55.0, 74.0)
Male/Female (n)	23/17	58/27
Previous medical history, n (%)		
Hypertension	5 (12.5%)	38 (44.7%)
Diabetes	3 (7.5%)	27 (31.8%)
Coronary heart disease	2 (5.0%)	29 (34.1%)
Chronic lung disease	1 (2.5%)	9 (10.6%)
Chronic kidney disease	0	9 (10.6%)
Cardiac arrest cause (n, %)		
Cardiac		34 (40.0%)
Respiratory		20 (23.5%)
Cerebral		23 (27.1%)
Others		7 (8.2%)
Unknow		1 (1.2%)
Initial resuscitation		
Time to ROSC (min), median [IQR]		20.0 (10.0, 30.0)
Adrenaline (mg), median [IQR]		2.0 (0.0, 5.0)
Initial rhythm VF/VT, n (%)		30 (35.3%)
MAP (mmHg), median [IQR]	95.7 (86.0, 103.2)	74.3 (56.2, 97.2)
White cell count ($\times 10^9$/L), median [IQR]	5.81 (4.85, 6.53)	13.56 (10.84, 18.29)
APACHE Ⅱ score, mean±SD	0	32.9±6.5
SOFA score, median [IQR]	0	11.5 (8.5, 14.0)
28-day mortality, n (%)		65（76.5%）
28-day CPC 1–2, n (%)		14 (16.5%)

198 Abbreviations: IQR: interquartile range; ROSC: return of spontaneous circulation;
199 VF: ventricular fibrillation; VT: ventricular tachycardia; MAP: mean arterial pressure;
200 APACHE Ⅱ: acute physiology and chronic health evaluation; SOFA: sequential

organ failure assessment; SD: standard deviation; CPC: cerebral performance category.

Changes in circulatory T and B lymphocyte, NK cell, and Treg cell counts after ROSC

The T and B lymphocyte, NK cell, and Treg cell counts were significantly lower after ROSC in patients who experienced CA than in healthy controls ($P<0.001$ for all). Additionally, the $CD3^+CD4^+$/T lymphocyte, $CD3^+CD8^+$/T lymphocyte, and Treg cell/$CD4^+$ T lymphocyte ratios were significantly lower after ROSC in patients who experienced CA than in healthy controls ($P<0.001$ for all) (Fig. 1; Supplemental Table 3). However, there were no significant differences in these cell counts and ratios between survivors (n=20) and non-survivors (n=65) ($P>0.05$ for all) (Supplemental Table 4).

GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells after ROSC

The MFI and percentages of GR expression in B and T lymphocytes, NK cells, and $CD3^+CD8^+$ T lymphocytes were significantly lower after ROSC in patients who experienced CA than in healthy individuals ($P<0.01$ for all) (Fig. 2A–D, G, H, K, L). There were also significant reductions in the MFI in Treg cells and $CD3^+CD4^+$ T lymphocytes ($P<0.05$ for all) (Fig. 2E, I) but not in the percentages of GR expression ($P>0.05$ for all) (Fig. 2F, J; Supplemental Table 5). However, there were no significant differences in the MFI and percentages of GR expression in these cells between survivors and non-survivors ($P>0.05$ for all) (Supplemental Table 6).

Changes in plasma total cortisol and ACTH levels after ROSC

We measured the plasma total cortisol and ACTH levels of the 40 healthy individuals and 85 patients who experienced CA (two samples were excluded because their total cortisol levels were not measured). Plasma total cortisol levels were significantly higher in patients who experienced CA than in healthy controls ($P<0.001$), but ACTH levels were not (Fig. 3A, C). No significant differences in ln (total cortisol+1) and ln (ACTH+1) values were observed between survivors and non-survivors ($P>0.05$ for all) (Fig. 3B, D).

Discussion

In this study, we examined the levels of GR expression and plasma corticosteroids in patients with CA in the early period after ROSC. We found that GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells, cell counts and ratios in patients with CA was significantly lower compared to that in controls. Furthermore, plasma total cortisol levels in patients with CA were significantly higher compared to the controls.

The ischemia-reperfusion response initiates an acute inflammatory response that contributes to post-resuscitation shock after CA.[27] The immune response of patients who experience CA is impaired, and the systemic inflammatory response increases. [6, 28] The T and B lymphocyte, NK cell, and Treg cell counts and $CD3^+CD4^+$/T, $CD3^+CD8^+$/T, and Treg cell/$CD4^+$ T lymphocyte ratios were significantly reduced after ROSC. NK cells, which are special innate immune cells with cytotoxic functions

similar to $CD3^+CD8^+$ T lymphocytes, mainly distinguish infected and stressed cells from healthy cells and eliminate intracellular infection and dysfunctional cells. [29, 30] T lymphocytes are also crucial because they function as adaptive immune cells to control and eliminate the infection. [29] Moreover, B and T lymphocytes mediate humoral and cellular immunity, respectively. This study was performed earlier and involved a more comprehensive assessment of the immune system of patients who experienced CA. Our findings more substantially supported the rapid emergence of immune dysfunction in these patients after ROSC than in previous reports.

The binding of GCs to GR inside different peripheral blood mononuclear cells (PBMC) leads to changes in the ability of cells to regulate apoptosis, proliferation, and activity, and GC-GR complexes limit the transcription (trans-repression) of inflammatory genes, including those encoding for proinflammatory cytokines.[31, 32] This study is the first to explore GR expression in circulating immune cells in patients who experienced CA after ROSC. We observed that GR expression in B and T lymphocytes, NK cells, and $CD3^+CD8^+$ T lymphocytes decreased significantly in patients who experienced CA, whereas the percentage of GR^+ Treg cells and $CD3^+CD4^+$ T lymphocytes decreased slightly. Moreover, we observed a more significant decrease in the MFI of GR expression in Treg cells and $CD3^+CD4^+$ T lymphocytes but not in the percentage of GR expression. Previous studies have found decreased expression of GRs in peripheral polymorphonuclear cells in critically ill patients, [21] and antagonism to GRs aggravates viral and bacterial infections. [33] GCs induced upon infections help to maintain homeostasis and mitigate the life-

threatening impact of sepsis on the host.[31] Although studies have reported that the use of GCs during and after CPR seems to confer benefits concerning ROSC rates and long-term survival, the evidence is scant. [13,18,34,35] Since cortisol signaling is mediated by GRs, we hypothesized that the differential responses of CA patients to GC may be related to their levels of GR expression. This study suggests that the decrease in intracellular GR expression in patients who experienced CA is one of the causes of GC resistance due to insufficient binding of GRs and GCs, GC insensitivity, and the inability of GCs to exert anti-inflammatory and immunosuppressive effects effectively. These findings may also explain why different results regarding the clinical application of GCs have been reported previously. Furthermore, it is vital to measure GR levels as sufficient expression of GR is essential for mediating adequate GC effects during and after CPR.

We also found that the total plasma cortisol levels were significantly higher in patients who experienced CA, but ACTH levels were not. High levels of inflammatory cytokines inhibit ACTH release. [18] During critical illness, the body does not sufficiently metabolize cortisol. [36] In addition, the continuous increase in plasma cortisol levels may trigger the negative feedback pathway of the hypothalamic-pituitary-adrenal axis, inhibiting the release of ACTH and cortisol and eventually leading to adrenal insufficiency [37]. These factors may explain the opposite trends of plasma ACTH and cortisol levels in the patients included in this study and who experienced CA. Notably, this result suggests that low GR expression levels are not matched by high plasma total cortisol levels in patients who experienced CA. The

dissociation between low GR expression and high cortisol implies an abnormal stress response. [38] Although systemic cortisol levels may be high, its availability is low during cardiac arrest. Previous studies have found that GC use during resuscitation may benefit patients who experience CA. [13-16] Possible reasons for this response may be that large doses of GCs given to CA patients may stimulate the function of GRs, or that GR expression or GC sensitivity was better in some patients. The probability of systemic inflammatory response and immunosuppression may also have been reduced in some CA patients. This study did not provide data on plasma GC levels and GR expression in a group of patients who were administered GCs and successfully resuscitated; therefore, further studies are required.

Limitations

Our study has several limitations. First, to assess changes, we only enrolled patients who experienced CA and had signs of systemic ischemic hypoxia, such as GCS <8 after ROSC. The patients were not stratified by age, sex, the occurrence of comorbidities, or mild systemic ischemic hypoxia. Second, since this was a preliminary observational study, we observed only early changes. A more relevant control group and dynamic observations obtained over a longer duration would be helpful to understand the significance of GR expression in evolving immunity during the clinical course of CA after ROSC. Third, the samples used in this study were from clinical laboratories; thus, plasma total cortisol and ACTH in the samples were at risk of degradation before we collected the samples. Finally, we did not discuss the changes in and roles of GR

isoforms, free cortisol, and corticosteroid-binding globulin. Therefore, future studies on these aspects are warranted to better understand the immunosuppressive effects of ROSC among patients who experienced CA.

In conclusion, this study revealed that GR expression, cell counts and ratios rapidly decreased, whereas plasma total cortisol levels increased, in the early period after ROSC among CA patients. These findings may provide important information about GR expression levels and function, and immunosuppressive status in these patients. The assessment of GR expression in CA patients may help screening for those who are more sensitive to glucocorticoid therapy.

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Contributorship statement: CL designed the study and reviewed the manuscript. YNY searched the literature and contributed to the experimental studies, data analysis, and manuscript writing. ZRT, CCH, and LA collected and analyzed data. JBL and MRX helped with the statistical analyses. All authors have read and approved the final manuscript.

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Data sharing statement: All data relevant to the study are included in the article or uploaded as supplementary information. Due to privacy and ethical concerns, data can not be shared.

Ethics statements

Patient consent for publication: Not applicable.

Ethics approval: This study was approved by the Medical Ethics Committee of Beijing Chaoyang Hospital (2013-KE-1). After successful resuscitation, informed consent was obtained from the families of the patients to enroll them in the study.

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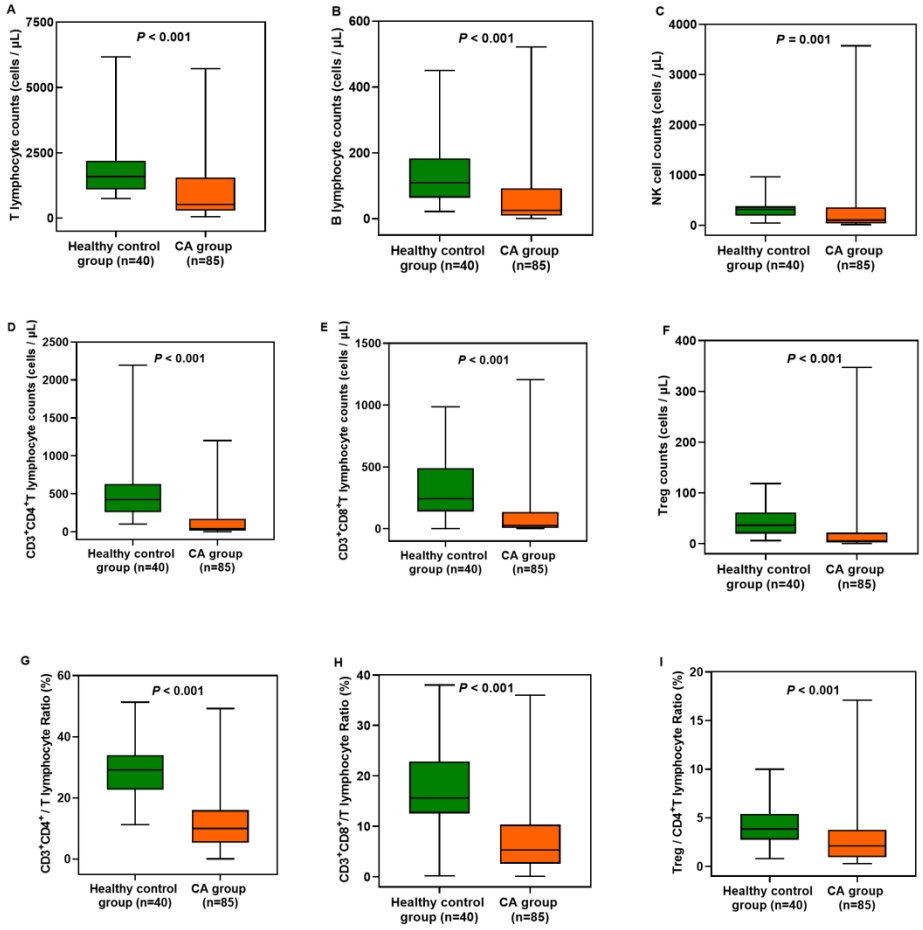
Figure legends

Fig. 1. Changes in circulatory T and B lymphocyte, NK cell, and Treg cell counts, $CD3^+CD4^+$/T, $CD3^+CD8^+$/T, and Treg/$CD4^+$T lymphocyte ratios between the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group ($P<0.001$). CA, cardiac arrest; CD, cluster-of-differentiation; NK, natural killer; Treg, regulatory T.

Fig. 2. Expression of GRs in circulatory T and B lymphocytes, NK cells, and Treg cells in the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group ($P<0.05$). CA, cardiac arrest; CD, cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; ROSC, return of spontaneous circulation; Treg, regulatory T.

Fig. 3. (A, B) Plasma total cortisol and ACTH levels (the natural logarithmic conversion values after adding 1) after ROSC in the healthy control group and CA group. (C, D) Plasma total cortisol and ACTH levels in survivors and non-survivors after ROSC. The CA group showed significant differences compared with the healthy control group ($P<0.05$). ACTH, adrenocorticotrophic hormone; CA, cardiac arrest; ROSC, return of spontaneous circulation.



Changes in circulatory T and B lymphocyte, NK cell, and Treg cell counts, and CD3+CD4+/T, CD3+CD8+/T, and Treg/CD4+T lymphocyte ratios between the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group (P<0.001). CA, cardiac arrest; CD, cluster-of-differentiation; NK, natural killer; Treg, regulatory T.

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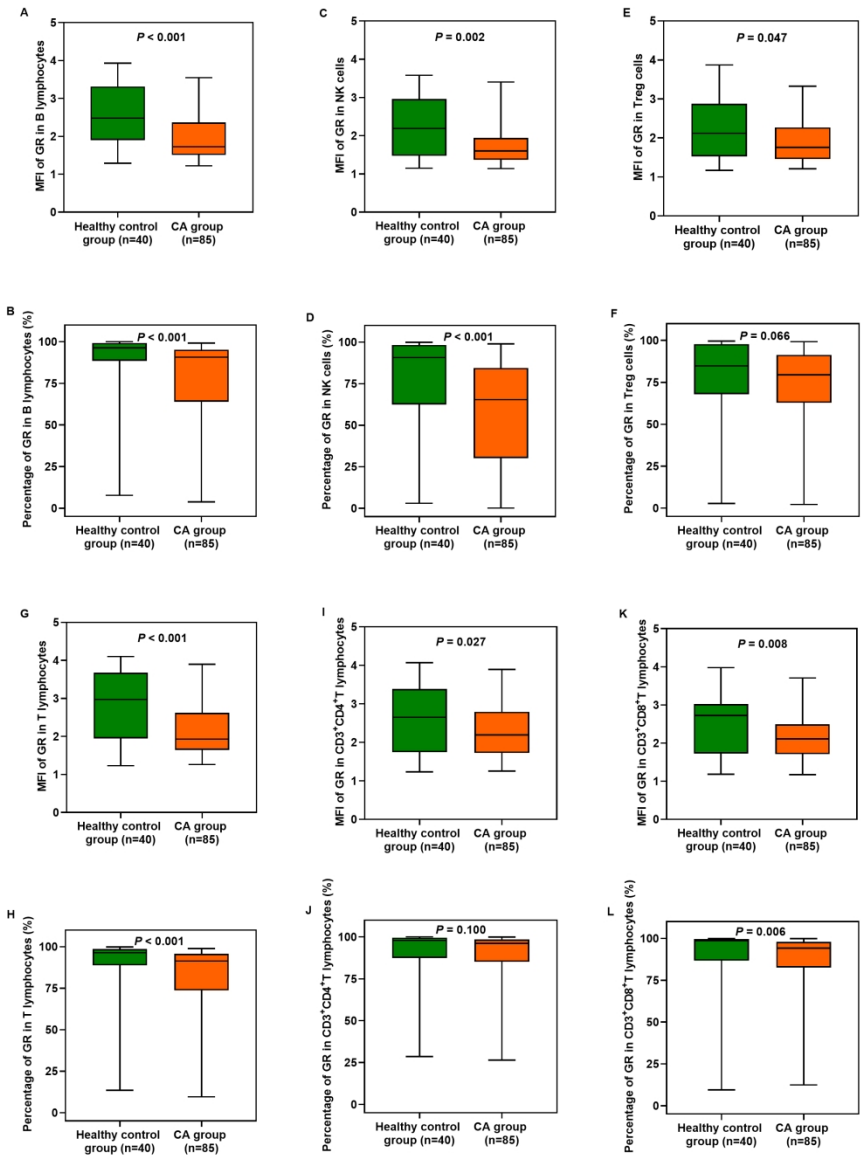

Expression of GRs in circulatory T and B lymphocytes, NK cells, and Treg cells in the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group (P<0.05). CA, cardiac arrest; CD, cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; ROSC, return of spontaneous circulation; Treg, regulatory T.

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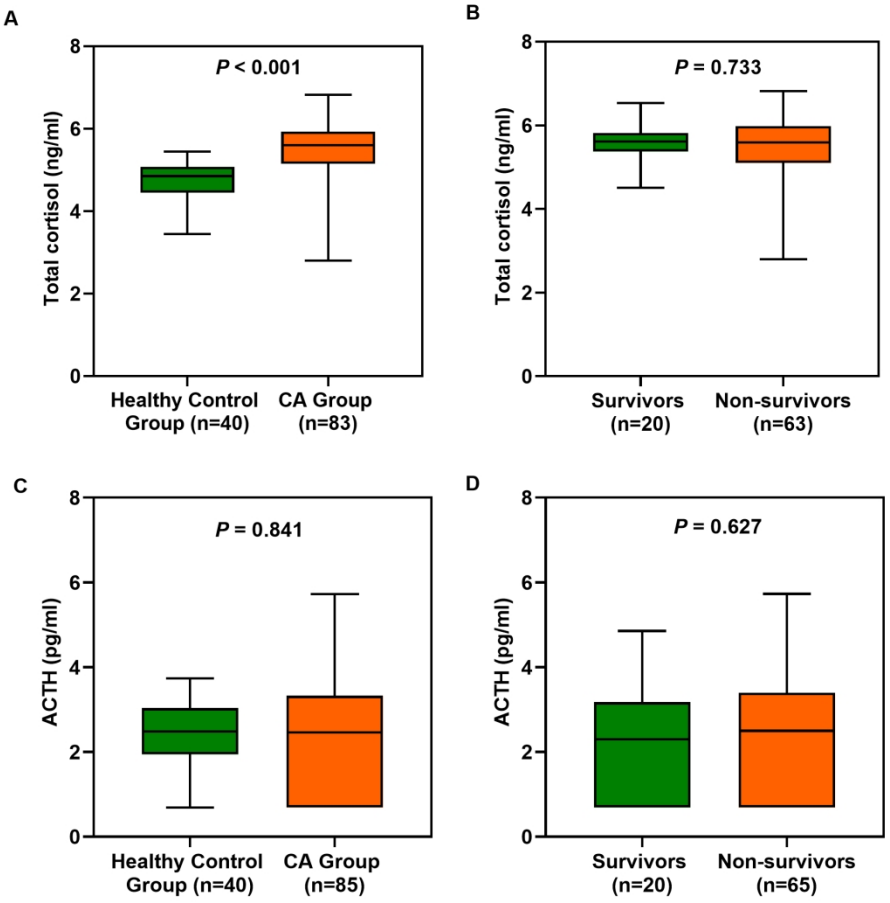

(A, B) Plasma total cortisol and ACTH levels (the natural logarithmic conversion values after adding 1) after ROSC in the healthy control group and CA group. (C, D) Plasma total cortisol and ACTH levels in survivors and non-survivors after ROSC. The CA group showed significant differences compared with the healthy control group (P<0.05). ACTH, adrenocorticotrophic hormone; CA, cardiac arrest; ROSC, return of spontaneous circulation.

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Electronic supplemental material

Glucocorticoid receptor expression in patients with cardiac arrest in the early period after the return of spontaneous circulation: A prospective observational study

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Contents

Supplemental Figure 1

Supplemental Figure 2

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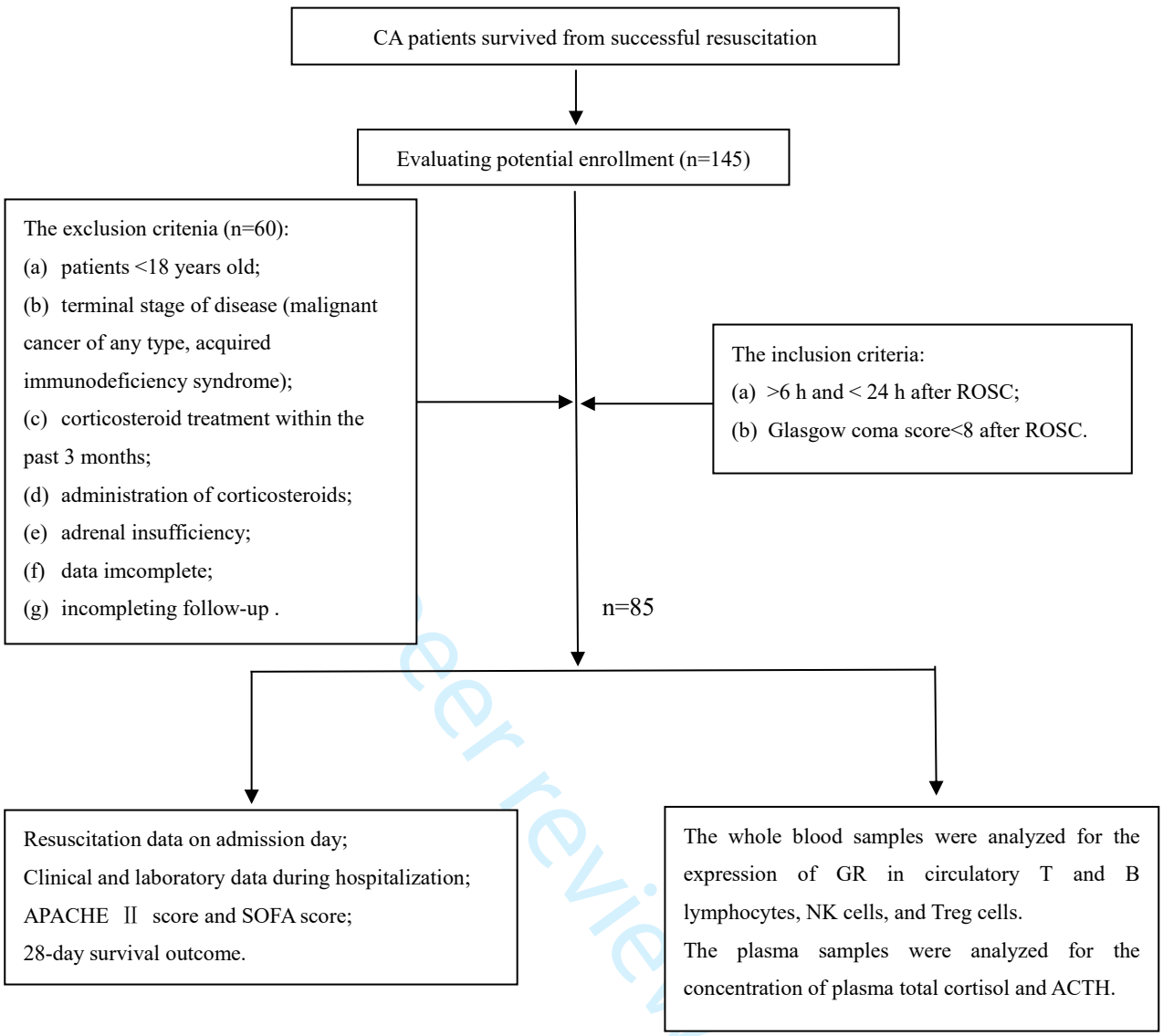
Supplemental Table 5

Supplemental Table 6



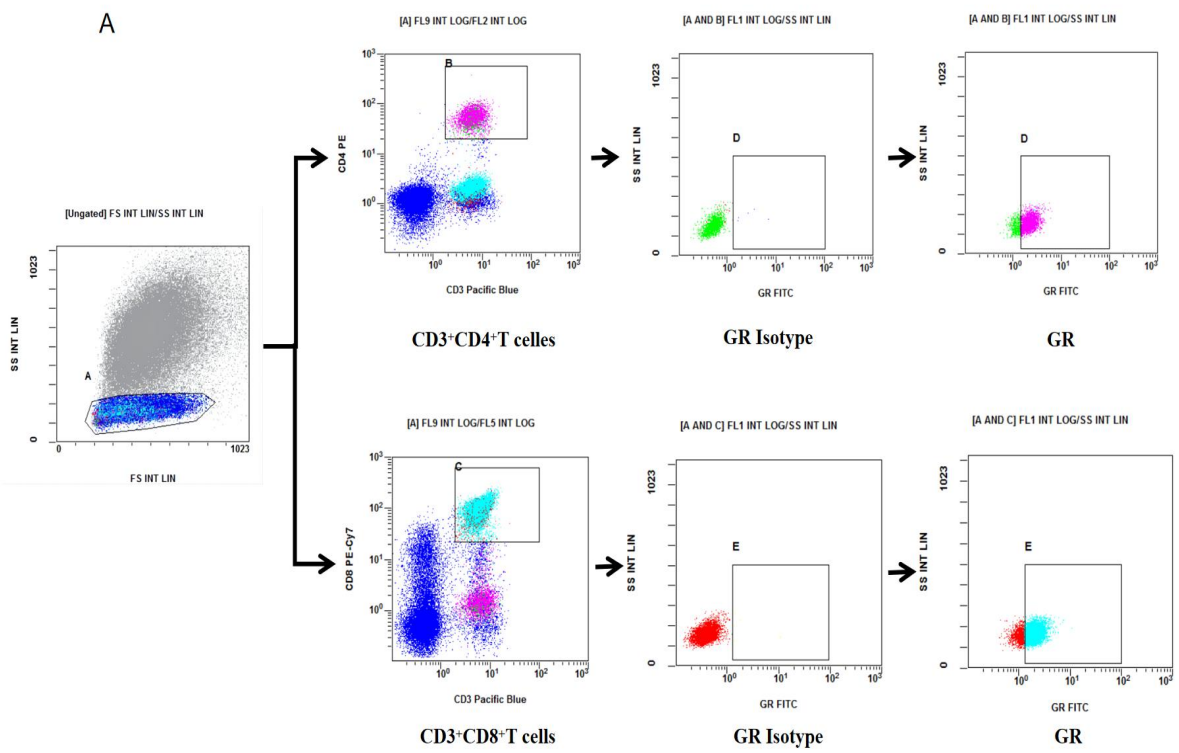
Supplemental Figure 1. The flow chart of the study.

Abbreviations: CA, cardiac arrest; ROSC, return of spontaneous circulation; APACHE II, acute physiology and chronic health evaluation II; SOFA, sequential organ failure assessment; GR, glucocorticoid receptor; Treg, regulatory T; ACTH, adrenocorticotrophic hormone.

Supplemental Figure 2. Representative plots and gating strategies for analyzing glucocorticoid receptor (GR) in the whole blood.

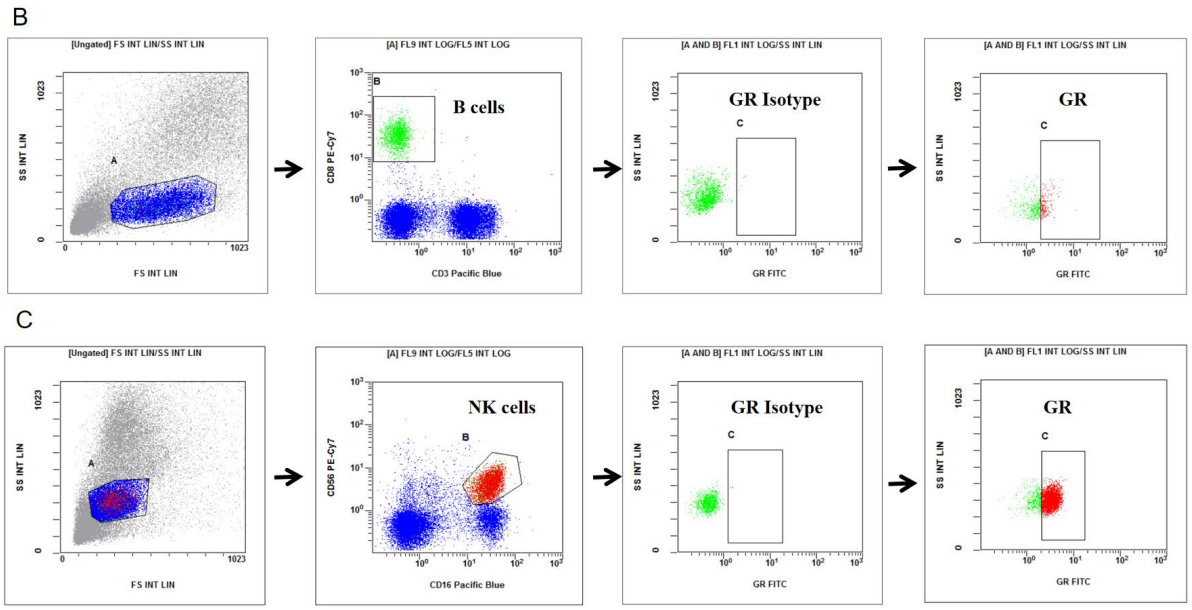
GR expression levels were determined on T cells, B cells, NK cells, and T regulatory (Treg) cells. Single cells were gated from all cellular events (FSC/SSC gate). B cells were identified as $CD3^{-}CD19^{+}$ cells. NK cells were identified as $CD16^{+}56^{+}$ cells. T cells were identified as $CD3^{+}CD4^{+}$ T cells and $CD3^{+}CD8^{+}$ T cells. Treg cells were identified as $CD4^{+}CD25^{high}CD127^{low}$.

A. Expression of GR on T cells

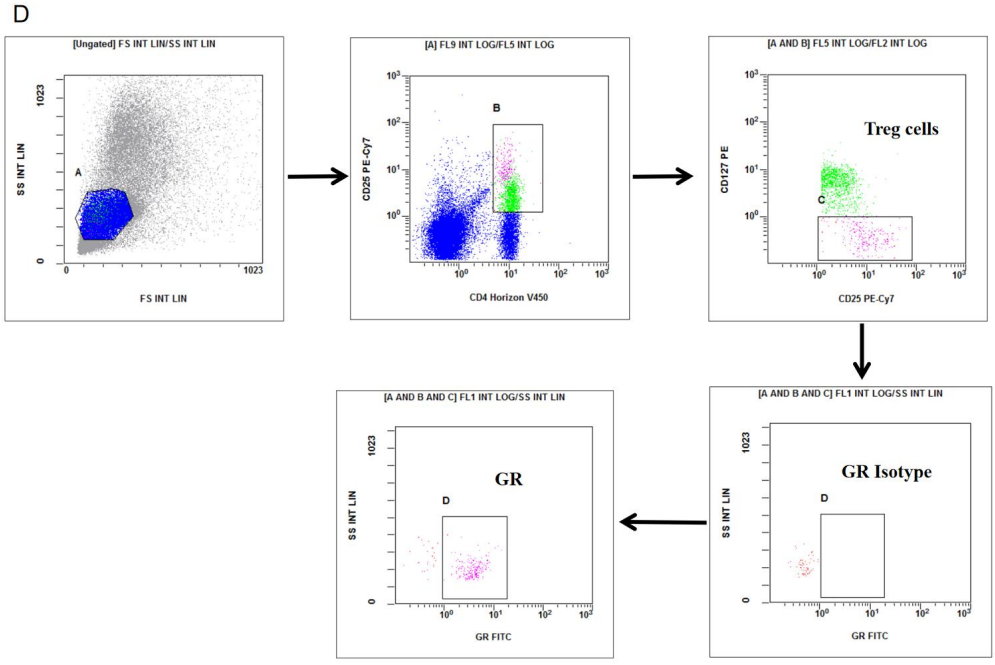


B. Expression of GR on B cells

C. Expression of GR on NK cells



D. Expression of GR on Treg cells



Supplemental Table 1. Details of antibodies for flow cytometry.

Antigen	Catalog Number	Fluorescein Conjugate	Source
CD3	558117	Pacific Blue	BD Pharmingen[a]
CD4	555347	PE	BD Pharmingen
CD4	560345	Horizon V450	BD Pharmingen
CD8	557746	PE-Cy7	BD Pharmingen
CD19	557835	PE-Cy7	BD Pharmingen
CD16	558122	Pacific Blue	BD Pharmingen
CD56	557747	PE-Cy7	BD Pharmingen
CD25	557741	PE-Cy7	BD Pharmingen
CD127	557938	PE	BD Pharmingen
GR	MCA2469F	FITC	Bio-Rad[b]
Mouse IgG1 Isotype	MCA928F	FITC	Bio-Rad
Mouse IgG1,κ Isotype	557872	PE-Cy7	BD Pharmingen
Mouse IgG1,κ Isotype	554680	PE	BD Pharmingen
Mouse IgG1,κ Isotype	558120	Pacific Blue	BD Pharmingen

[a] BD Pharmingen, San Diego, USA; [b] Bio-Rad AbD Serotec, Oxford, UK.

Abbreviations: CD, cluster-of-differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate; GR, glucocorticoid receptor; Ig: immunoglobulin.

Supplemental Table 2. Characteristics of CA survivors and non-survivors on admission.

	Survivors (n=20)	**Non-survivors (n=65)**
Age (years), median [IQR]	59.0 (53.3, 72.8)	66.0 (59.0, 75.5)
Male/Female (n)	12/8	46/19
Cardiac arrest cause (n, %)		
Cardiac	10 (50.0%)	24 (36.9%)
Non-Cardiac	10 (50.0%)	41 (63.1%)
Initial resuscitation		
Time to ROSC (min), median [IQR]	15.0 (7.3, 26.0)	20.0 (15.0, 30.0)
Adrenaline (mg), median [IQR]	1.0 (0.0, 3.0)	2.0 (0.0, 5.0)
Initial rhythm VF/VT, n (%)	11 (55.0%)	19 (29.2%)
MAP (mmHg), median [IQR]	89.9 (70.5, 104.9)	70.7 (50.0, 93.5)
White cell count ($\times 10^9$/L), median [IQR]	12.40 (6.98, 18.76)	13.80 (11.67, 18.20)
Lactate (mmol/L), median [IQR]	3.50 (1.33, 7.05)	7.50 (3.80, 11.20)
APACHE II score, mean±SD	27.8±6.6	34.4±5.6
SOFA score, median [IQR]	9.0 (7.3, 11.8)	12.0 (9.0, 15.0)

Data are presented as mean±SD or interquartile range (IQR) as appropriate. Abbreviations: ROSC: return of spontaneous circulation; VF: ventricular fibrillation; VT: ventricular tachycardia; MAP: mean arterial pressure; APACHE II: acute physiology and chronic health evaluation; SOFA: sequential organ failure assessment.

Supplemental Table 3. The flow cytometry results of cell counts and ratios of the healthy control group and successful resuscitation group

	Healthy Control Group （n=40）	**Successful Resuscitation Group (n=85)**	***Z*-value**	***P*-value**
T lymphocyte count (cells /μL)	1586.0 (1101.5, 2192.5)	514.0 (287.5, 1555.0)	-4.515	<0.001
NK cell count (/μL)	311.5 (191.0, 378.8)	101.0 (36.0, 351.5)	-3.332	0.001
B lymphocyte count (/μL)	109.3 (63.7, 183.3)	25.7 (9.4, 92.3)	-5.076	<0.001
Treg count (/μL)	0.259 (0.095, 0.516)	0.233 (0.135, 0.488)	-5.518	<0.001
Treg / $CD4^+$T lymphocyte Ratio	0.039 (0.028, 0.054)	0.021 (0.010, 0.038)	-4.418	<0.001
$CD3^+CD4^+$ T lymphocyte count (/μL)	421.7 (258.6, 627.4)	38.9 (17.6, 168.3)	-6.256	<0.001
$CD3^+CD4^+$ / T lymphocyte Ratio	0.292 (0.227, 0.340)	0.100 (0.054, 0.160)	-7.066	<0.001
$CD3^+CD8^+$ T lymphocyte count (/μL)	241.1 (139.5, 488.6)	26.3 (7.2, 135.9)	-5.287	<0.001
$CD3^+CD8^+$ / T lymphocyte Ratio	0.157 (0.126, 0.229)	0.053 (0.026, 0.104)	-5.719	<0.001

All the data in Supplemental table 3 are represented as the median [IQR]; IQR: Interquartile Range; CD: cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; Treg, regulatory T.

Supplemental Table 4. The flow cytometry results of cell counts and ratios of the CA patients on admission based on 28-day survival

	Survivors (n=20)	**Non-survivors (n=65)**	***Z*-value**	***P*-value**
T lymphocyte count (/μL)	502.0 (353.8, 1199.8)	514.0 (282.5, 1891.0)	-0.186	0.852
NK cell count (/μL)	167.0 (29.8, 309.3)	100.0 (36.0, 404.0)	-0.218	0.828
B lymphocyte count (/μL)	38.6 (15.7, 103.5)	19.2 (7.1, 65.7)	-0.632	0.527
Tregs count (/μL)	0.318 (0.145, 0.552)	0.212 (0.128, 0.479)	-0.611	0.396
Treg / $CD4^+$ T lymphocyte Ratio	0.025 (0.009, 0.043)	0.021 (0.010, 0.034)	-0.498	0.619
$CD3^+CD4^+$ T lymphocyte count (/μL)	55.1 (32.4, 228.0)	38.0 (16.0, 168.1)	-0.850	0.396
$CD3^+CD4^+$ / T lymphocyte Ratio	0.118 (0.070, 0.236)	0.097 (0.049, 0.142)	-1.565	0.118
$CD3^+CD8^+$ T lymphocyte count (/μL)	25.4 (12.5, 96.2)	26.3 (6.3, 138.8)	-0.021	0.983
$CD3^+CD8^+$ / T lymphocyte Ratio	0.054 (0.033, 0.104)	0.053 (0.025, 0.104)	-0.187	0.852

All the data in Supplemental table 4 are represented as the median [IQR]; IQR: Interquartile Range; CD: cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; Treg, regulatory T.

Supplemental Table 5. The flow cytometry results of GR expression in the CA group and successful resuscitation group.

	Healthy Control Group （n=40）	**Successful Resuscitation Group (n=85)**	***Z*-value**	***P*-value**
Percentage of GR on B lymphocytes	0.963 (0.885, 0.992)	0.896 (0.605, 0.949)	-3.742	<0.001
MFI of GR on B lymphocytes	2.48 (1.91, 3.31)	1.73 (1.50, 2.37)	-3.980	<0.001
Percentage of GR on T lymphocytes	0.964 (0.889, 0.986)	0.900 (0.703, 0.955)	-3.755	<0.001
MFI of GR on T lymphocytes	2.98(1.95, 3.68)	1.92 (1.36, 1.99)	-3.853	<0.001
Percentage of GR on NK cells	0.907 (0.624, 0.983)	0.611 (0.306, 0.840)	-3.792	<0.001
MFI of GR on NK cells	2.19 (1.48, 2.96)	1.60 (1.36, 1.99)	-3.171	0.002
Percentage of GR on Treg cells	0.848 (0.680, 0.978)	0.784 (0.589, 0.911)	-1.837	0.066
MFI of GR on Treg cells	2.12 (1.53, 2.88)	1.76 (1.44, 2.30)	-1.990	0.047
Percentage of GR on $CD3^+CD4^+$ T lymphocytes	0.980 (0.874, 0.996)	0.957 (0.824, 0.985)	-2.204	0.100
MFI of GR on $CD3^+CD4^+$ T lymphocytes	2.65 (1.75, 3.38)	2.17 (1.70, 2.92)	-1.646	0.027
Percentage of GR on $CD3^+CD8^+$T lymphocytes	0.986 (0.868, 0.996)	0.938 (0.823, 0.979)	-2.758	0.006
MFI of GR on $CD3^+CD8^+$ T lymphocytes	2.73 (1.73, 3.02)	2.10 (1.68, 2.54)	-2.668	0.008

All the data in Supplemental table 5 are represented as the median [IQR]. Abbreviations: IQR, interquartile range; CD, cluster-of-differentiation; NK, natural killer; Treg, regulatory T; GR, Glucocorticoid receptor; MFI, mean fluorescence intensity.

Supplemental Table 6. The flow cytometry results of GR expression in the survivors and non-survivors.

	Survivors (n=20)	**Non-survivors (n=65)**	***Z*-value**	***P*-value**
Percentage of GR on B lymphocytes	0.904 (0.595, 0.976)	0.906 (0.657, 0.946)	-0.787	0.431
MFI of GR on B lymphocytes	1.92 (1.52, 2.54)	1.72 (1.51, 2.31)	-0.881	0.378
Percentage of GR on T lymphocytes	0.899 (0.778, 0.969)	0.913 (0.692, 0.951)	-1.057	0.291
MFI of GR on T lymphocytes	2.05 (1.67, 2.83)	1.91 (1.64, 2.46)	-1.031	0.303
Percentage of GR on NK cells	0.717 (0.292, 0.886)	0.556 (0.302, 0.823)	-0.756	0.449
MFI of GR on NK cells	1.54 (1.37, 2.09)	1.61 (1.34, 1.87)	-0.565	0.572
Percentage of GR on Tregs	0.780 (0.667, 0.849)	0.799 (0.576, 0.923)	-0.440	0.660
MFI of GR on Tregs	1.61 (1.48, 2.30)	1.77 (1.45, 2.27)	-0.005	0.996
Percentage of GR on $CD3^+CD4^+$ T lymphocytes	0.975 (0.876, 0.985)	0.957 (0.845, 0.987)	-0.617	0.538
MFI of GR on $CD3^+CD4^+$ T lymphocytes	2.08 (1.72, 3.35)	2.22 (1.71, 2.69)	-0.865	0.387
Percentage of GR on $CD3^+CD8^+$ T lymphocytes	0.963 (0.816, 0.977)	0.938 (0.834, 0.980)	-0.254	0.800
MFI of GR on $CD3^+CD8^+$ T lymphocytes	2.08 (1.68, 3.10)	2.11(1.71, 2.46)	-0.653	0.514

All the data in Supplemental table 6 are represented as the median [IQR]. Abbreviations: IQR, Interquartile Range; CD, Cluster-of-differentiation; NK, natural killer; Treg, regulatory T; GR, glucocorticoid receptor; MFI, mean fluorescence intensity.

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3-5
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	Supplemental Figure 1
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5-9, Supplemental Figure 1
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5,6,8,9
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5, 6, 8, Supplemental Figure 1
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6, 8
Bias	9	Describe any efforts to address potential sources of bias	6-8
Study size	10	Explain how the study size was arrived at	Supplemental Figure 1
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8, 11

(*b*) Describe any methods used to examine subgroups and interactions	N/A
(*c*) Explain how missing data were addressed	8, 11
(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	11
(*e*) Describe any sensitivity analyses	

Continued on next page

Results			
Participants	13 *	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	9, Supplemental Figure 1
		(b) Give reasons for non-participation at each stage	9,12, Supplemental Figure 1
		(c) Consider use of a flow diagram	Supplemental Figure 1
Descriptive data	14 *	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9
		(b) Indicate number of participants with missing data for each variable of interest	Supplemental Figure 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	8
Outcome data	15 *	*Cohort study*—Report numbers of outcome events or summary measures over time	9-12
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-12, Electronic supplemental material
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N/A
Discussion			
Key results	18	Summarise key results with reference to study objectives	12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12-14
Generalisability	21	Discuss the generalisability (external validity) of the study results	15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	17

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely

available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Glucocorticoid receptor expression in patients with cardiac arrest in the early period after the return of spontaneous circulation: A prospective observational single-center study

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Glucocorticoid receptor expression in patients with cardiac arrest in the early period after the return of spontaneous circulation: A prospective observational single-center study

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Keywords: Cardiac arrest, glucocorticoid receptor, immunosuppression, cortisol

Word count of the main text: 3,437 words

Abstract

Objectives: Rapid changes in glucocorticoid (GC) levels and adrenal insufficiency are related to the development of post-cardiac arrest (CA) syndrome. However, GC receptor (GR) expression changes have not been studied. Hence, this study aimed to investigate the association of early changes in GR expression and prognosis and immune response in patients who experienced CA.

Design: Prospective observational study.

Setting: Emergency department.

Participants: Patients (85) in the early period of return of spontaneous circulation (ROSC) after CA were admitted between October 2018 and October 2019. After a physical examination, age- and sex-matched healthy individuals (40) were recruited for the control group.

Primary and secondary outcome measures: GR expression and cell counts of circulatory T and B lymphocytes, natural killer cells, and regulatory T (Treg) cells were assessed. Plasma total cortisol and adrenocorticotrophic hormone (ACTH) levels were also tested.

Results: All cell counts were lower, and plasma total cortisol levels were higher ($P<0.001$) in patients who experienced CA than in the healthy control group. GR expression in Treg cells and $CD3^+CD4^+$ T lymphocytes were not significantly different, but the mean fluorescence intensity and GR expression in other cells were lower in patients who experienced CA ($P<0.05$) than in the healthy control group. ACTH levels were not different. There were no significant differences between survivors and non-

survivors.

Conclusions: This study revealed that GR expression and cell counts rapidly decreased, whereas plasma total cortisol levels increased in the early period after ROSC among patients who experienced CA. Our findings provide important information about GR level and function, and immunosuppressive status in these patients. Assessing GR expression in CA patients may help screening for those who are more sensitive to glucocorticoid therapy.

Strengths and limitations of this study

1. The study was designed as single-center, prospective study.
2. This is the first study to evaluate the GR expression in the early period following ROSC among CA patients.
3. We only studied the GR expression of CA patients in the early period following ROSC; therefore, our results cannot be extrapolated to time points beyond 24 hours.
4. Decreased GR expression may affect the sensitivity of CA patients to GCs.
5. Decreased GR expression may affect potential immune consequences of CA patients.

Introduction

Cardiac arrest (CA) is a significant health problem globally; about 356,500 people experience medical emergencies due to CA in the United States, and over 544,000 people die from sudden CA in China annually. [1, 2] The systemic ischemia-reperfusion

response in patients who have experienced CA can present as post-cardiac arrest syndrome (PCAS) or systematic inflammatory response syndrome (SIRS), which increases the risk of multiple organ failure and infection and affects the inflammatory response and prognosis of patients after the return of spontaneous circulation (ROSC). [3-6]

CA is the most intense among acute stress events, which seriously affect the pituitary and adrenal axis function. [7] Studies have shown that abnormal cortisol levels and relative adrenocortical insufficiency after ROSC in patients who experienced CA are related to their prognosis. [8-11] However, the clinical application of glucocorticoids (GCs) is controversial. In the 2015 International Cardiopulmonary Resuscitation Guidelines, the routine use of GCs is not recommended for the resuscitation of patients with in-hospital or out-of-hospital CA. [12] Recent clinical studies have shown that early administration of corticosteroids after CA can improve the success rate of ROSC, nervous system functional outcome, and prognosis, which is speculated to be related to its influence on hemodynamics, and SIRS response, and other mechanisms. [12-17] Therefore, the role of GCs in the occurrence and development of PCAS needs to be studied further.

GCs combine with intracellular GC receptors (GRs) to exert anti-inflammatory and immunosuppressive effects and reduce the production and the release of inflammatory cytokines. [18, 19] The affinity of GRs to GCs in circulating monocytes is decreased in patients with acquired immunodeficiency syndrome. [20] The expression of GR alpha and beta in peripheral polymorphonuclear cells is decreased in patients with critical

illness, [21] pediatric septic shock, and high serum cortisol levels. [22] However, no study has reported the GR expression after ROSC in patients who experienced CA. Previous studies have found that the counts of circulating B and T lymphocytes, regulatory T (Treg) cells, and monocytes and expression of human leukocyte antigen DR (HLA-DR) on circulatory monocytes and B and T lymphocytes are reduced. [23, 24] Hence, this study aimed to investigate the relationship between GR expression and immune alteration in the early period after ROSC in patients who experienced CA by observing GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells, their cell counts, and total plasma cortisol and adrenocorticotrophic hormone (ACTH) levels.

Materials and methods

Study participants

This was an observational study conducted in the Emergency Department (ED). According to the 2015 International Cardiopulmonary Resuscitation Guidelines, [25] we enrolled patients in the early ROSC period after CA (both in-hospital and out-of-hospital CA) and were admitted to the ED between October 2018 and October 2019. The inclusion criteria were patients with CA > 6 and < 24 hours after ROSC, with a Glasgow coma score < 8. The exclusion criteria were (a) <18 years of age, (b) terminal stage of disease (such as cancer of any type, acquired immunodeficiency syndrome), (c) corticosteroid treatment within the past three months, (d) administration of corticosteroids, and (e) adrenal insufficiency. All patients were treated according to the

2015 International Cardiopulmonary Resuscitation Consensus. [13] After a physical examination, age- and sex-matched healthy individuals were recruited for the control group.

Data collection

Data collection was performed according to the 2004 guidelines of the Utstein Style template. [26] We collected data on demographics, resuscitation (initial heart rhythm, ROSC time, and cumulative adrenaline [epinephrine] dose, and laboratory findings routine blood cell counts, blood gas analysis, and blood biochemical tests performed > 6 h and < 24 h after ROSC). Acute Physiology and Chronic Health Evaluation (APACHE) II and the Sequential Organ Failure Assessment (SOFA) were used to determine disease severity. Residual blood samples from routine clinical tests or physical health examinations in the morning were collected, maintained at 4 °C during transport and storage, and used to determine GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells and their cell counts. The plasma was maintained at -80 °C during storage and used to determine total cortisol and ACTH levels. During follow-up, 28-day survival data were also collected. Supplemental Figure 1 shows the workflow of this study.

Outcome measures

The primary outcomes of this study were GR expression and cell counts of T and B cells, NK cells, and Treg cells, measured by flow cytometry. Venous blood samples collected in ethylenediaminetetraacetic acid tubes, then used to measure GR expression in T and B lymphocytes, NK cells, and Treg cells. Briefly, a 100-μL peripheral blood

sample was stained for 20 min with surface antibodies (CD3, CD4, CD8, CD19, CD16, CD56, CD25, and CD127) in a dark place. Erythrocytes were lysed for 15 min, and the debris was washed away. Before staining of the intracellular GR antibody and its isotype control (Bio-Rad AbD Serotec, Oxford, UK), surface-stained cells were fixed and permeabilized using the BD Transcription Factor Buffer Set (BD Pharmingen, San Diego, USA, Catalogue No. 562574). Monoclonal antibodies and their isotype controls were all purchased from BD Biosciences (San Jose, CA, USA). Details of all antibodies are shown in Supplemental Table 1. According to the manufacturer's recommendations, all antibodies and their isotype controls were used at a concentration of 1 μL per 100 μL of whole blood. Samples were measured using the Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed using Gallios Software version 1.0 (Beckman Coulter). The flow cytometer was periodically calibrated by an engineer. Cells were stained for 20 min; thresholds were defined using the manufacturer's recommended isotype controls. Representative plots and gating strategy from a single sample are shown in Supplemental Figure 2. T cells were gated by $CD3^{+}CD4^{+}$ or $CD3^{+}CD8^{+}$, B cells were gated by $CD3^{-}CD19^{+}$, NK cells were gated by $CD16^{+}CD56^{+}$, and Tregs were gated by $CD4^{+}CD25^{high}CD127^{low}$. At least 10,000 events were collected in the lymphocyte cell gate for each sample. Results are expressed as percentages and mean fluorescence intensity (MFI) values.

Absolute $CD3^{+}$ and $CD4^{+}$ lymphocyte, NK cell, and Treg cell counts were obtained using Flow-Count fluorospheres (Beckman Coulter, Catalogue No. 7547053), according to the manufacturer's instructions. B, $CD3^{+}CD4^{+}$T, $CD3^{+}CD8^{+}$T, and Treg

cell counts were calculated by their percentages in $CD3^+$ or $CD4^+$ lymphocytes multiplied by $CD3^+$ or $CD4^+$ lymphocyte counts.

The secondary outcomes of this study were plasma total cortisol and ACTH levels after ROSC. Venous blood samples were collected in heparin anticoagulant tubes, centrifuged 10 min at 3000 rpm, and then stored at -80 °C. Plasma total cortisol (IMMULITE 2000 Cortisol, L2KCO2, UK) and ACTH (IMMULITE 2000 ACTH, L2KAC2, UK) levels were assayed using a chemiluminescent immunoassay on a Siemens automated analyzer (IMMULITE 2000 XPi; Siemens Healthcare Diagnostics, Erlangen, Germany). The equipment and reagents were calibrated by engineers before use. The lower detection limit of total cortisol was 2.00 ng/mL, and that of ACTH was 5.00 pg/mL.

Sample size calculation and statistical analysis

The sample size was calculated using the PASS15.0 software (NCSS, LLC, Kaysville, UT, USA) and the non-parametric test method. The median GR expression was 0.93 and 0.80 in the healthy and CA groups, respectively, and the interquartile spacing was 0.1 and 0.3. According to the ratio of 1:2 between the two groups, with a test level of 0.05 and a confidence interval of 0.90, a total of 105 samples were required, comprising at least 35 in the healthy group and 70 in the CA group. The number of people included in the two groups in this study was 40 and 85, respectively, which met our research requirements. Data analysis was used in SPSS version 22.0 (IBM Corp., Armonk, NY, USA). For normally distributed data, continuous variables are expressed as means with standard deviations. Since the data for total cortisol and ACTH levels

had a skewed distribution, we compared our results with the natural logarithmic conversion values after adding 1 (ln [total cortisol+ 1], ln [ACTH+ 1]). Measurement data with a skewed distribution are expressed as medians (25th and 75th percentiles). The Mann–Whitney U test was used to compare variables between groups. The qualitative parameters in the 2 × 2 contingency table were used for analysis. All statistical tests were two-tailed, and a P-value of <0.05 was considered statistically significant.

Follow-up

Patients were classified into survivor and non-survivor groups according to the 28-day survival endpoint. Those with all-cause mortality within the follow-up period were considered non-survivors. If data were lost, the corresponding candidate was excluded.

Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Results

Patient characteristics

40 healthy individuals and 85 patients who experienced CA were analyzed. The demographics and clinical characteristics of both groups are shown in Table 1. In this study, acute cardiac and brain events were the main causes of CA, with those in the latter category emanating from strokes. Other causes of CA included poisoning (including carbon monoxide poisoning) and hypokalemia. Sex and age were not

significantly different between the CA and healthy control groups. The comparisons of clinical characteristics of the survivor and non-survivor groups based on 28-day survival are shown in Supplemental Table 2. The APACHE II and SOFA scores were significantly different between the CA and healthy control groups (P<0.001 for all) and survivor and non-survivor groups (P<0.001 and P=0.011, respectively).

Table 1. Patient Characteristics at Admission

Characteristics	Healthy Control Group (n=40)	Successful Resuscitation Group (n=85)
Age (years), median [IQR]	64.0 (54.3, 69.8)	65.0 (55.0, 74.0)
Male/Female (n)	23/17	58/27
Previous medical history, n (%)		
Hypertension	5 (12.5%)	38 (44.7%)
Diabetes	3 (7.5%)	27 (31.8%)
Coronary heart disease	2 (5.0%)	29 (34.1%)
Chronic lung disease	1 (2.5%)	9 (10.6%)
Chronic kidney disease	0	9 (10.6%)
Cardiac arrest cause (n, %)		
Cardiac		34 (40.0%)
Respiratory		20 (23.5%)
Cerebral		23 (27.1%)
Others		7 (8.2%)
Unknow		1 (1.2%)
Initial resuscitation		
Time to ROSC (min), median [IQR]		20.0 (10.0, 30.0)
Adrenaline (mg), median [IQR]		2.0 (0.0, 5.0)
Initial rhythm VF/VT, n (%)		30 (35.3%)

MAP (mmHg), median [IQR]	95.7 (86.0, 103.2)	74.3 (56.2, 97.2)
White cell count ($\times 10^9$/L), median [IQR]	5.81 (4.85, 6.53)	13.56 (10.84, 18.29)
APACHE Ⅱ score, mean±SD	0	32.9±6.5
SOFA score, median [IQR]	0	11.5 (8.5, 14.0)
28-day mortality, n (%)		65（76.5%）
28-day CPC 1–2, n (%)		14 (16.5%)

Abbreviations: IQR: interquartile range; ROSC: return of spontaneous circulation; VF: ventricular fibrillation; VT: ventricular tachycardia; MAP: mean arterial pressure; APACHE Ⅱ: acute physiology and chronic health evaluation; SOFA: sequential organ failure assessment; SD: standard deviation; CPC: cerebral performance category.

Changes in circulatory T and B lymphocyte, NK cell, and Treg cell counts after ROSC

The T and B lymphocyte, NK cell, and Treg cell counts were significantly lower after ROSC in patients who experienced CA than in healthy controls (P<0.001 for all). Additionally, the $CD3^+CD4^+$/T lymphocyte, $CD3^+CD8^+$/T lymphocyte, and Treg cell/$CD4^+$ T lymphocyte ratios were significantly lower after ROSC in patients who experienced CA than in healthy controls (P<0.001 for all) (Fig. 1; Supplemental Table 3). However, there were no significant differences in these cell counts and ratios between survivors (n=20) and non-survivors (n=65) (P>0.05 for all) (Supplemental Table 4).

GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells after ROSC

The MFI and percentages of GR expression in B and T lymphocytes, NK cells, and

$CD3^+CD8^+$ T lymphocytes were significantly lower after ROSC in patients who experienced CA than in healthy individuals (P<0.01 for all) (Fig. 2A–D, G, H, K, L). There were also significant reductions in the MFI in Treg cells and $CD3^+CD4^+$ T lymphocytes (P<0.05 for all) (Fig. 2E, I) but not in the percentages of GR expression (P>0.05 for all) (Fig. 2F, J; Supplemental Table 5). However, there were no significant differences in the MFI and percentages of GR expression in these cells between survivors and non-survivors (P>0.05 for all) (Supplemental Table 6).

Changes in plasma total cortisol and ACTH levels after ROSC

We measured the plasma total cortisol and ACTH levels of the 40 healthy individuals and 85 patients who experienced CA (two samples were excluded because their total cortisol levels were not measured). Plasma total cortisol levels were significantly higher in patients who experienced CA than in healthy controls (P<0.001), but ACTH levels were not (Fig. 3A, C). No significant differences in ln (total cortisol+1) and ln (ACTH+1) values were observed between survivors and non-survivors (P>0.05 for all) (Fig. 3B, D).

Discussion

In this study, we examined the levels of GR expression and plasma corticosteroids in patients with CA in the early period after ROSC. We found that GR expression in circulatory T and B lymphocytes, NK cells, and Treg cells, cell counts and ratios in patients with CA was significantly lower compared to that in controls. Furthermore, plasma total cortisol levels in patients with CA were significantly higher compared to

the controls.

The ischemia-reperfusion response initiates an acute inflammatory response that contributes to post-resuscitation shock after CA.[27] The immune response of patients who experience CA is impaired, and the systemic inflammatory response increases. [6, 28] The T and B lymphocyte, NK cell, and Treg cell counts and $CD3^+CD4^+$/T, $CD3^+CD8^+$/T, and Treg cell/$CD4^+$ T lymphocyte ratios were significantly reduced after ROSC. NK cells, which are special innate immune cells with cytotoxic functions similar to $CD3^+CD8^+$ T lymphocytes, mainly distinguish infected and stressed cells from healthy cells and eliminate intracellular infection and dysfunctional cells. [29, 30] T lymphocytes are also crucial because they function as adaptive immune cells to control and eliminate the infection. [29] Moreover, B and T lymphocytes mediate humoral and cellular immunity, respectively. This study was performed earlier and involved a more comprehensive assessment of the immune system of patients who experienced CA. Our findings more substantially supported the rapid emergence of immune dysfunction in these patients after ROSC than in previous reports.

The binding of GCs to GR inside different peripheral blood mononuclear cells (PBMC) leads to changes in the ability of cells to regulate apoptosis, proliferation, and activity, and GC-GR complexes limit the transcription (trans-repression) of inflammatory genes, including those encoding for proinflammatory cytokines.[31, 32] This study is the first to explore GR expression in circulating immune cells in patients who experienced CA after ROSC. We observed that GR expression in B and T lymphocytes, NK cells, and $CD3^+CD8^+$ T lymphocytes decreased significantly in

patients who experienced CA, whereas the percentage of GR^+ Treg cells and $CD3^+CD4^+$ T lymphocytes decreased slightly. Moreover, we observed a more significant decrease in the MFI of GR expression in Treg cells and $CD3^+CD4^+$ T lymphocytes but not in the percentage of GR expression. Previous studies have found decreased expression of GRs in peripheral polymorphonuclear cells in critically ill patients, [21] and antagonism to GRs aggravates viral and bacterial infections. [33] GCs induced upon infections help to maintain homeostasis and mitigate the life-threatening impact of sepsis on the host.[31] Although studies have reported that the use of GCs during and after CPR seems to confer benefits concerning ROSC rates and long-term survival, the evidence is scant. [13,18,34,35] Since cortisol signaling is mediated by GRs, we hypothesized that the differential responses of CA patients to GC may be related to their levels of GR expression. This study suggests that the decrease in intracellular GR expression in patients who experienced CA is one of the causes of GC resistance due to insufficient binding of GRs and GCs, GC insensitivity, and the inability of GCs to exert anti-inflammatory and immunosuppressive effects effectively. These findings may also explain why different results regarding the clinical application of GCs have been reported previously. Furthermore, it is vital to measure GR levels as sufficient expression of GR is essential for mediating adequate GC effects during and after CPR.

We also found that the total plasma cortisol levels were significantly higher in patients who experienced CA, but ACTH levels were not. High levels of inflammatory cytokines inhibit ACTH release. [18] During critical illness, the body does not

sufficiently metabolize cortisol. [36] In addition, the continuous increase in plasma cortisol levels may trigger the negative feedback pathway of the hypothalamic-pituitary-adrenal axis, inhibiting the release of ACTH and cortisol and eventually leading to adrenal insufficiency [37]. These factors may explain the opposite trends of plasma ACTH and cortisol levels in the patients included in this study and who experienced CA. Notably, this result suggests that low GR expression levels are not matched by high plasma total cortisol levels in patients who experienced CA. The dissociation between low GR expression and high cortisol implies an abnormal stress response. [38] Previous studies have reported that GR-action was clearly suppressed throughout critical illness; GR resistance could not be overcome by further increasing glucocorticoid availability.[21,39,40] Adequate GR levels and function are also required for normal GC function, which may explain differences in the responsiveness of cardiac arrest patients to exogenous steroid administration or endogenous cortisol secretion. Thus, actual GR levels cannot be reflected by measuring total cortisol levels alone. Therefore, the GR level should be considered when applying personalized GC therapy. The determination of GR expression might help to screen those who might respond better to glucocorticoid prescription.

Limitations

Our study has several limitations. First, to assess changes, we only enrolled patients who experienced CA and had signs of systemic ischemic hypoxia, such as GCS <8 after ROSC. The patients were not stratified by age, sex, the occurrence of comorbidities, or

mild systemic ischemic hypoxia. Second, since this was a preliminary observational study, we observed only early changes. A more relevant control group and dynamic observations obtained over a longer duration would be helpful to understand the significance of GR expression in evolving immunity during the clinical course of CA after ROSC. Third, the samples used in this study were from clinical laboratories; thus, plasma total cortisol and ACTH in the samples were at risk of degradation before we collected the samples. Finally, we did not discuss the changes in and roles of GR isoforms, free cortisol, and corticosteroid-binding globulin. Therefore, future studies on these aspects are warranted to better understand the immunosuppressive effects of ROSC among patients who experienced CA.

In conclusion, this study revealed that GR expression, cell counts and ratios rapidly decreased, whereas plasma total cortisol levels increased, in the early period after ROSC among CA patients. These findings may provide important information about GR expression levels and function, and immunosuppressive status in these patients.

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Contributorship statement: CL designed the study and reviewed the manuscript. YNY searched the literature and contributed to the experimental studies, data analysis, and manuscript writing. ZRT, CCH, and LA collected and analyzed data. JBL and MRX

helped with the statistical analyses. All authors have read and approved the final manuscript.

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Data sharing statement: All data relevant to the study are included in the article or uploaded as supplementary information. Due to privacy and ethical concerns, data can not be shared.

Ethics statements

Patient consent for publication: Not applicable.

Ethics approval: This study was approved by the Medical Ethics Committee of Beijing Chaoyang Hospital (2013-KE-1). Because CA is sudden and life-threatening, the consent was usually obtained orally from relatives or bystanders and in writing with some delay from relatives or bystanders after successful resuscitation.

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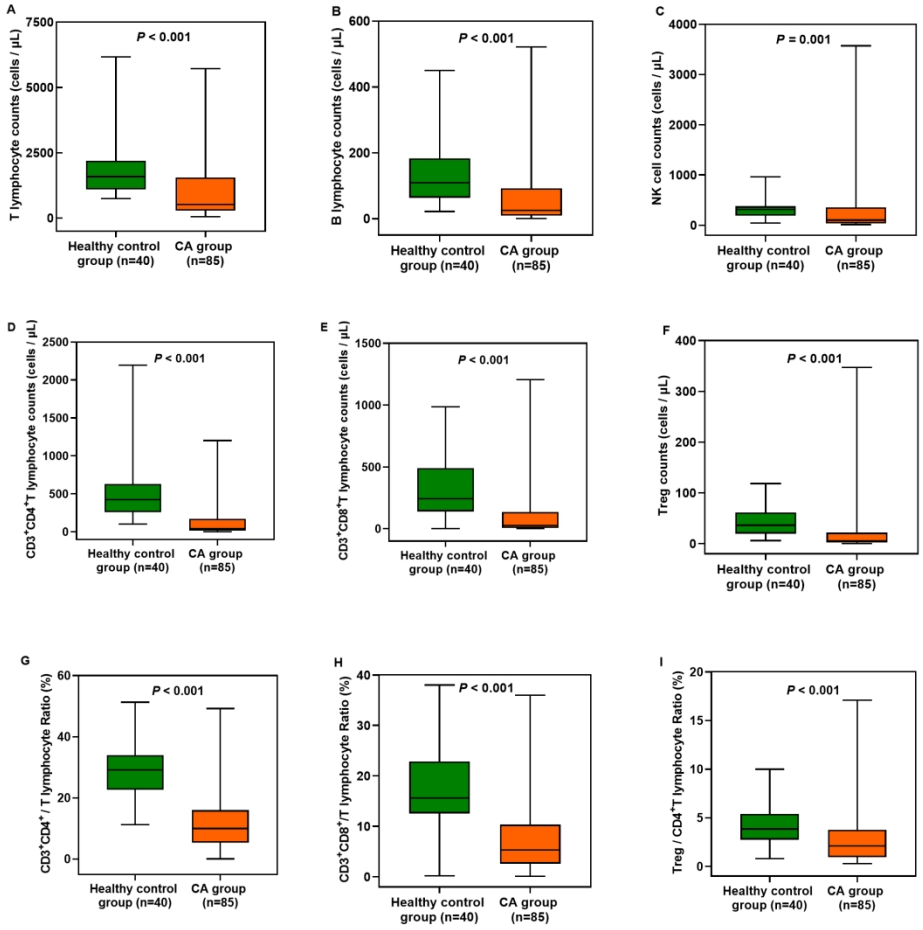
Figure legends

Fig. 1. Changes in circulatory T and B lymphocyte, NK cell, and Treg cell counts, $CD3^+CD4^+$/T, $CD3^+CD8^+$/T, and Treg/$CD4^+$T lymphocyte ratios between the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group ($P<0.001$). CA, cardiac arrest; CD, cluster-of-differentiation; NK, natural killer; Treg, regulatory T.

Fig. 2. Expression of GRs in circulatory T and B lymphocytes, NK cells, and Treg cells in the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group ($P<0.05$). CA, cardiac arrest; CD, cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; ROSC, return of spontaneous circulation; Treg, regulatory T.

Fig. 3. (A, B) Plasma total cortisol and ACTH levels (the natural logarithmic conversion values after adding 1) after ROSC in the healthy control group and CA group. (C, D) Plasma total cortisol and ACTH levels in survivors and non-survivors after ROSC. The CA group showed significant differences compared with the healthy control group ($P<0.05$). ACTH, adrenocorticotrophic hormone; CA, cardiac arrest; ROSC, return of spontaneous circulation.



Changes in circulatory T and B lymphocyte, NK cell, and Treg cell counts, and CD3+CD4+/T, CD3+CD8+/T, and Treg/CD4+T lymphocyte ratios between the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group (P<0.001). CA, cardiac arrest; CD, cluster-of-differentiation; NK, natural killer; Treg, regulatory T.

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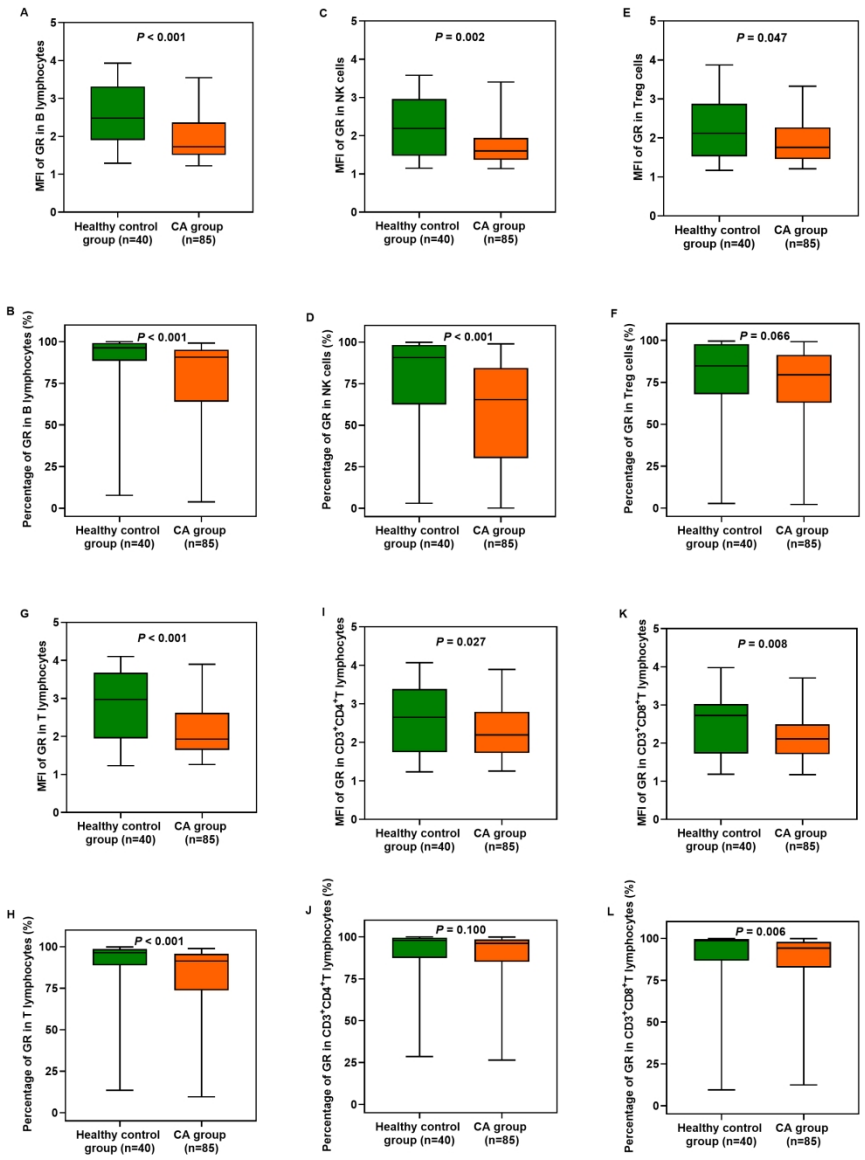

Expression of GRs in circulatory T and B lymphocytes, NK cells, and Treg cells in the healthy control group and CA group. The CA group showed significant differences compared with the healthy control group (P<0.05). CA, cardiac arrest; CD, cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; ROSC, return of spontaneous circulation; Treg, regulatory T.

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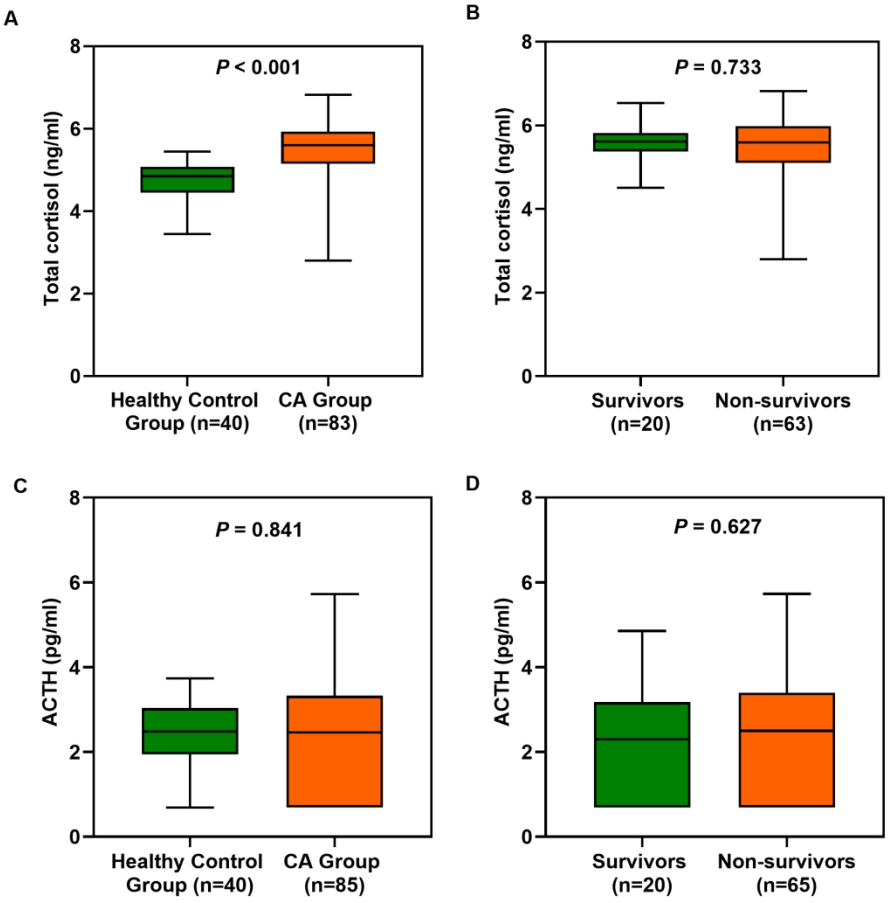

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Electronic supplemental material

Glucocorticoid receptor expression in patients with cardiac arrest in the early period after the return of spontaneous circulation: A prospective observational single-center study

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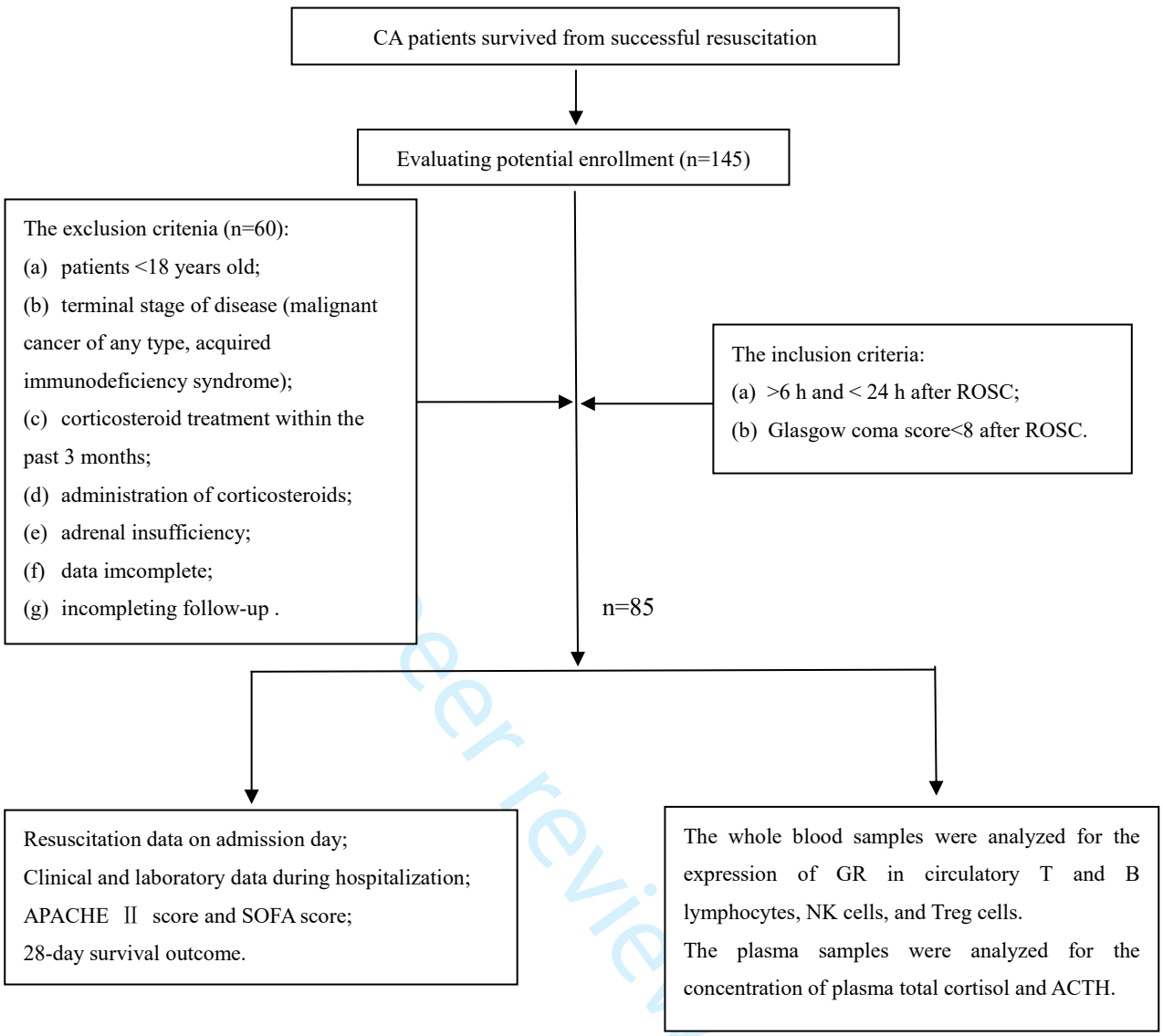


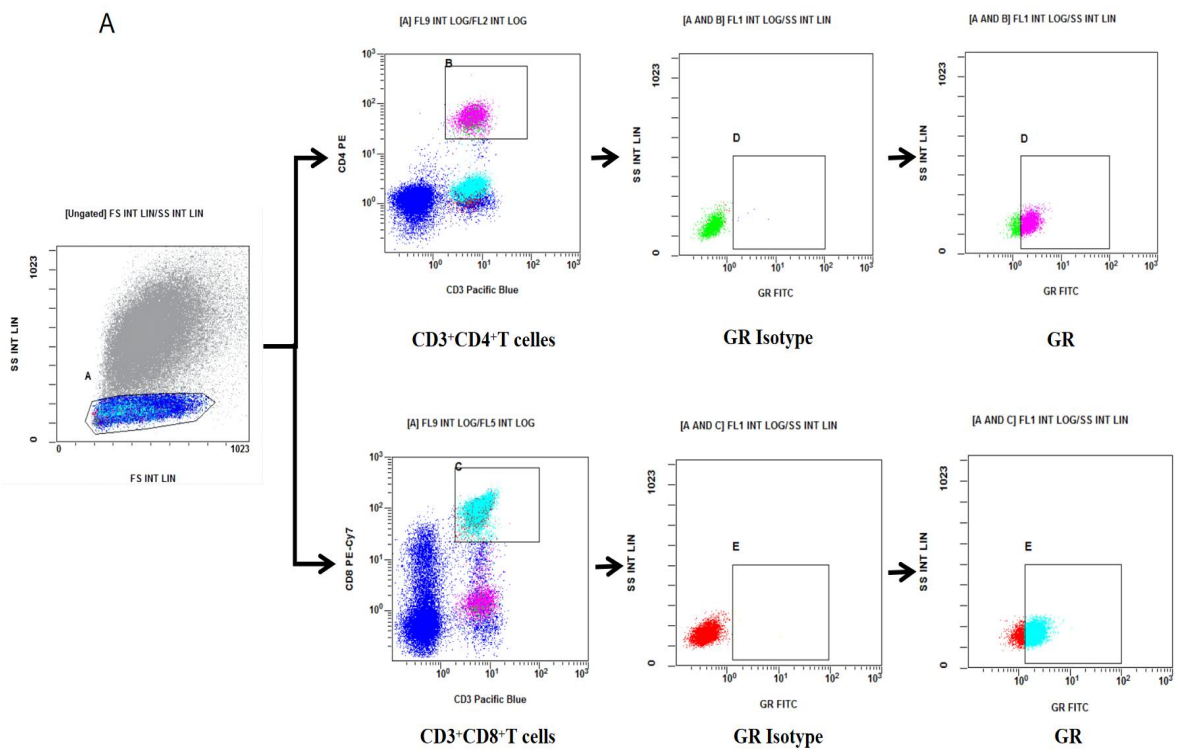
Supplemental Figure 1. The flow chart of the study.

Abbreviations: CA, cardiac arrest; ROSC, return of spontaneous circulation; APACHE II, acute physiology and chronic health evaluation II; SOFA, sequential organ failure assessment; GR, glucocorticoid receptor; Treg, regulatory T; ACTH, adrenocorticotrophic hormone.

Supplemental Figure 2. Representative plots and gating strategies for analyzing glucocorticoid receptor (GR) in the whole blood.

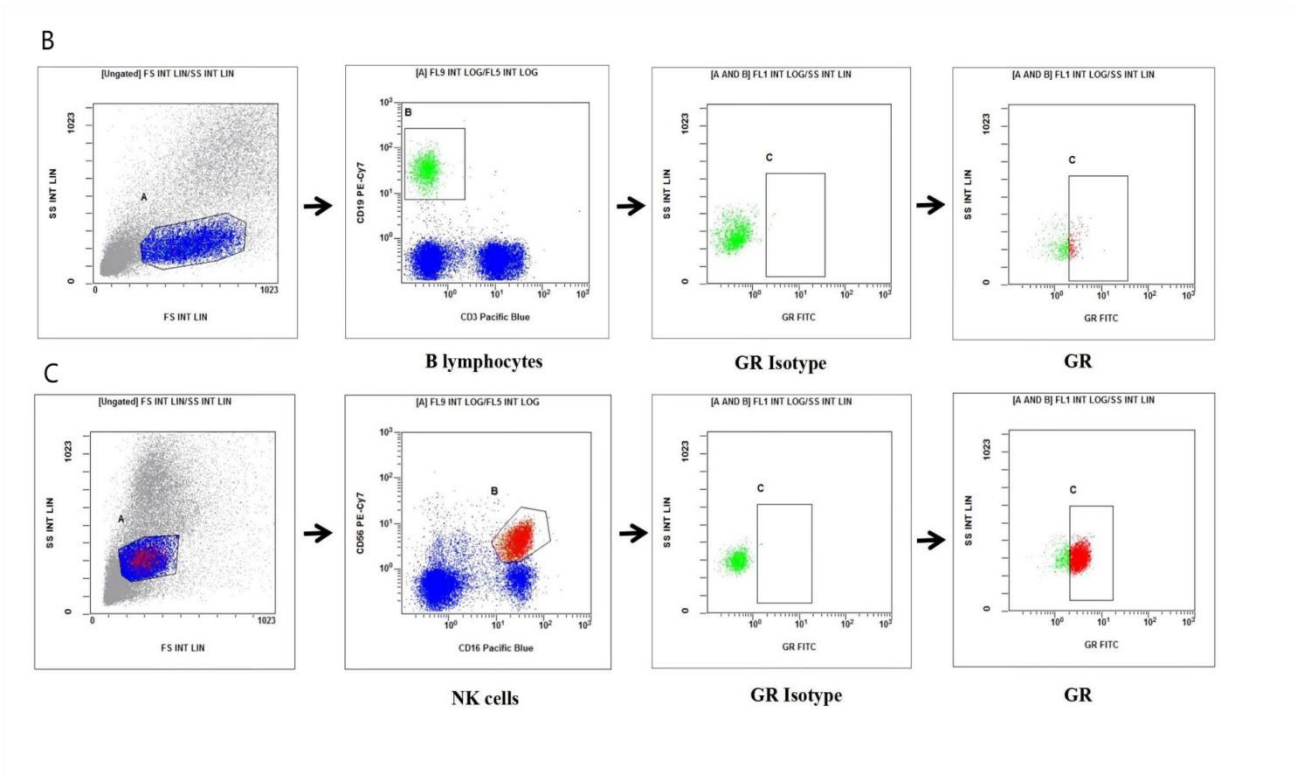
GR expression levels were determined on T cells, B cells, NK cells, and T regulatory (Treg) cells. Single cells were gated from all cellular events (FSC/SSC gate). B cells were identified as $CD3^{-}CD19^{+}$ cells. NK cells were identified as $CD16^{+}56^{+}$ cells. T cells were identified as $CD3^{+}CD4^{+}$ T cells and $CD3^{+}CD8^{+}$ T cells. Treg cells were identified as $CD4^{+}CD25^{high}CD127^{low}$.

A. Expression of GR on T cells

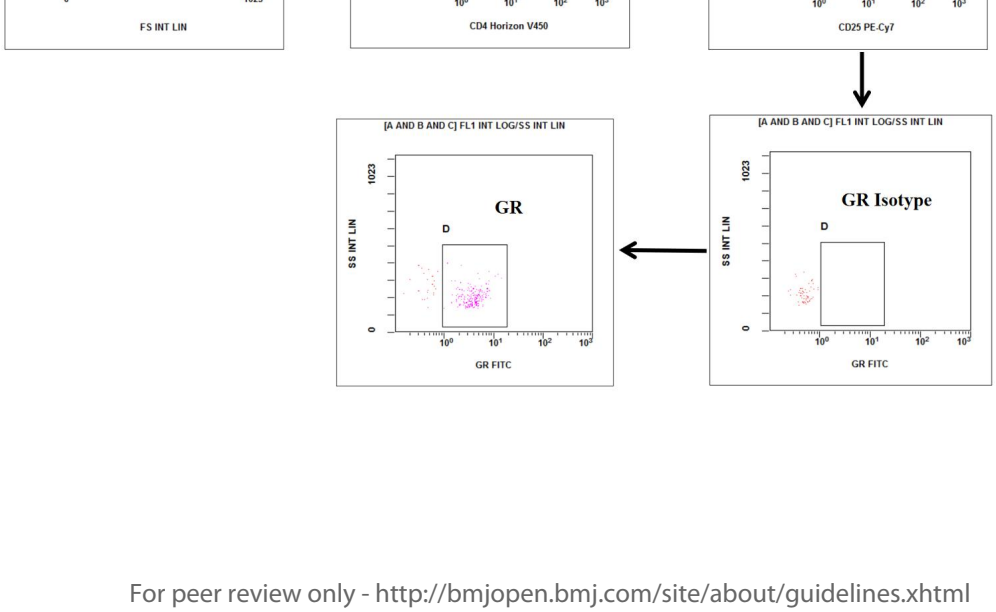


B. Expression of GR on B cells

C. Expression of GR on NK cells



D. Expression of GR on Treg cells



Supplemental Table 1. Details of antibodies for flow cytometry.

Antigen	Catalog Number	Fluorescein Conjugate	Source
CD3	558117	Pacific Blue	BD Pharmingen[a]
CD4	555347	PE	BD Pharmingen
CD4	560345	Horizon V450	BD Pharmingen
CD8	557746	PE-Cy7	BD Pharmingen
CD19	557835	PE-Cy7	BD Pharmingen
CD16	558122	Pacific Blue	BD Pharmingen
CD56	557747	PE-Cy7	BD Pharmingen
CD25	557741	PE-Cy7	BD Pharmingen
CD127	557938	PE	BD Pharmingen
GR	MCA2469F	FITC	Bio-Rad[b]
Mouse IgG1 Isotype	MCA928F	FITC	Bio-Rad
Mouse IgG1,κ Isotype	557872	PE-Cy7	BD Pharmingen
Mouse IgG1,κ Isotype	554680	PE	BD Pharmingen
Mouse IgG1,κ Isotype	558120	Pacific Blue	BD Pharmingen

[a] BD Pharmingen, San Diego, USA; [b] Bio-Rad AbD Serotec, Oxford, UK.

Abbreviations: CD, cluster-of-differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate; GR, glucocorticoid receptor; Ig: immunoglobulin.

Supplemental Table 2. Characteristics of CA survivors and non-survivors on admission.

	Survivors (n=20)	**Non-survivors (n=65)**
Age (years), median [IQR]	59.0 (53.3, 72.8)	66.0 (59.0, 75.5)
Male/Female (n)	12/8	46/19
Cardiac arrest cause (n, %)		
Cardiac	10 (50.0%)	24 (36.9%)
Non-Cardiac	10 (50.0%)	41 (63.1%)
Initial resuscitation		
Time to ROSC (min), median [IQR]	15.0 (7.3, 26.0)	20.0 (15.0, 30.0)
Adrenaline (mg), median [IQR]	1.0 (0.0, 3.0)	2.0 (0.0, 5.0)
Initial rhythm VF/VT, n (%)	11 (55.0%)	19 (29.2%)
MAP (mmHg), median [IQR]	89.9 (70.5, 104.9)	70.7 (50.0, 93.5)
White cell count ($\times 10^9$/L), median [IQR]	12.40 (6.98, 18.76)	13.80 (11.67, 18.20)
Lactate (mmol/L), median [IQR]	3.50 (1.33, 7.05)	7.50 (3.80, 11.20)
APACHE II score, mean±SD	27.8±6.6	34.4±5.6
SOFA score, median [IQR]	9.0 (7.3, 11.8)	12.0 (9.0, 15.0)

Data are presented as mean±SD or interquartile range (IQR) as appropriate. Abbreviations: ROSC: return of spontaneous circulation; VF: ventricular fibrillation; VT: ventricular tachycardia; MAP: mean arterial pressure; APACHE II: acute physiology and chronic health evaluation; SOFA: sequential organ failure assessment.

Supplemental Table 3. The flow cytometry results of cell counts and ratios of the healthy control group and successful resuscitation group

	Healthy Control Group （n=40）	**Successful Resuscitation Group (n=85)**	***Z*-value**	***P*-value**
T lymphocyte count (cells /μL)	1586.0 (1101.5, 2192.5)	514.0 (287.5, 1555.0)	-4.515	<0.001
NK cell count (/μL)	311.5 (191.0, 378.8)	101.0 (36.0, 351.5)	-3.332	0.001
B lymphocyte count (/μL)	109.3 (63.7, 183.3)	25.7 (9.4, 92.3)	-5.076	<0.001
Treg count (/μL)	0.259 (0.095, 0.516)	0.233 (0.135, 0.488)	-5.518	<0.001
Treg / $CD4^+$T lymphocyte Ratio	0.039 (0.028, 0.054)	0.021 (0.010, 0.038)	-4.418	<0.001
$CD3^+CD4^+$ T lymphocyte count (/μL)	421.7 (258.6, 627.4)	38.9 (17.6, 168.3)	-6.256	<0.001
$CD3^+CD4^+$ / T lymphocyte Ratio	0.292 (0.227, 0.340)	0.100 (0.054, 0.160)	-7.066	<0.001
$CD3^+CD8^+$ T lymphocyte count (/μL)	241.1 (139.5, 488.6)	26.3 (7.2, 135.9)	-5.287	<0.001
$CD3^+CD8^+$ / T lymphocyte Ratio	0.157 (0.126, 0.229)	0.053 (0.026, 0.104)	-5.719	<0.001

All the data in Supplemental table 3 are represented as the median [IQR]; IQR: Interquartile Range; CD: cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; Treg, regulatory T.

Supplemental Table 4. The flow cytometry results of cell counts and ratios of the CA patients on admission based on 28-day survival

	Survivors (n=20)	**Non-survivors (n=65)**	***Z*-value**	***P*-value**
T lymphocyte count (/μL)	502.0 (353.8, 1199.8)	514.0 (282.5, 1891.0)	-0.186	0.852
NK cell count (/μL)	167.0 (29.8, 309.3)	100.0 (36.0, 404.0)	-0.218	0.828
B lymphocyte count (/μL)	38.6 (15.7, 103.5)	19.2 (7.1, 65.7)	-0.632	0.527
Tregs count (/μL)	0.318 (0.145, 0.552)	0.212 (0.128, 0.479)	-0.611	0.396
Treg / $CD4^+$ T lymphocyte Ratio	0.025 (0.009, 0.043)	0.021 (0.010, 0.034)	-0.498	0.619
$CD3^+CD4^+$ T lymphocyte count (/μL)	55.1 (32.4, 228.0)	38.0 (16.0, 168.1)	-0.850	0.396
$CD3^+CD4^+$ / T lymphocyte Ratio	0.118 (0.070, 0.236)	0.097 (0.049, 0.142)	-1.565	0.118
$CD3^+CD8^+$ T lymphocyte count (/μL)	25.4 (12.5, 96.2)	26.3 (6.3, 138.8)	-0.021	0.983
$CD3^+CD8^+$ / T lymphocyte Ratio	0.054 (0.033, 0.104)	0.053 (0.025, 0.104)	-0.187	0.852

All the data in Supplemental table 4 are represented as the median [IQR]; IQR: Interquartile Range; CD: cluster-of-differentiation; GR, glucocorticoid receptor; NK, natural killer; Treg, regulatory T.

Supplemental Table 5. The flow cytometry results of GR expression in the CA group and successful resuscitation group.

	Healthy Control Group （n=40）	**Successful Resuscitation Group (n=85)**	***Z*-value**	***P*-value**
Percentage of GR on B lymphocytes	0.963 (0.885, 0.992)	0.896 (0.605, 0.949)	-3.742	<0.001
MFI of GR on B lymphocytes	2.48 (1.91, 3.31)	1.73 (1.50, 2.37)	-3.980	<0.001
Percentage of GR on T lymphocytes	0.964 (0.889, 0.986)	0.900 (0.703, 0.955)	-3.755	<0.001
MFI of GR on T lymphocytes	2.98(1.95, 3.68)	1.92 (1.36, 1.99)	-3.853	<0.001
Percentage of GR on NK cells	0.907 (0.624, 0.983)	0.611 (0.306, 0.840)	-3.792	<0.001
MFI of GR on NK cells	2.19 (1.48, 2.96)	1.60 (1.36, 1.99)	-3.171	0.002
Percentage of GR on Treg cells	0.848 (0.680, 0.978)	0.784 (0.589, 0.911)	-1.837	0.066
MFI of GR on Treg cells	2.12 (1.53, 2.88)	1.76 (1.44, 2.30)	-1.990	0.047
Percentage of GR on $CD3^+CD4^+$ T lymphocytes	0.980 (0.874, 0.996)	0.957 (0.824, 0.985)	-2.204	0.100
MFI of GR on $CD3^+CD4^+$ T lymphocytes	2.65 (1.75, 3.38)	2.17 (1.70, 2.92)	-1.646	0.027
Percentage of GR on $CD3^+CD8^+$T lymphocytes	0.986 (0.868, 0.996)	0.938 (0.823, 0.979)	-2.758	0.006
MFI of GR on $CD3^+CD8^+$ T lymphocytes	2.73 (1.73, 3.02)	2.10 (1.68, 2.54)	-2.668	0.008

All the data in Supplemental table 5 are represented as the median [IQR]. Abbreviations: IQR, interquartile range; CD, cluster-of-differentiation; NK, natural killer; Treg, regulatory T; GR, Glucocorticoid receptor; MFI, mean fluorescence intensity.

Supplemental Table 6. The flow cytometry results of GR expression in the survivors and non-survivors.

	Survivors (n=20)	**Non-survivors (n=65)**	***Z*-value**	***P*-value**
Percentage of GR on B lymphocytes	0.904 (0.595, 0.976)	0.906 (0.657, 0.946)	-0.787	0.431
MFI of GR on B lymphocytes	1.92 (1.52, 2.54)	1.72 (1.51, 2.31)	-0.881	0.378
Percentage of GR on T lymphocytes	0.899 (0.778, 0.969)	0.913 (0.692, 0.951)	-1.057	0.291
MFI of GR on T lymphocytes	2.05 (1.67, 2.83)	1.91 (1.64, 2.46)	-1.031	0.303
Percentage of GR on NK cells	0.717 (0.292, 0.886)	0.556 (0.302, 0.823)	-0.756	0.449
MFI of GR on NK cells	1.54 (1.37, 2.09)	1.61 (1.34, 1.87)	-0.565	0.572
Percentage of GR on Tregs	0.780 (0.667, 0.849)	0.799 (0.576, 0.923)	-0.440	0.660
MFI of GR on Tregs	1.61 (1.48, 2.30)	1.77 (1.45, 2.27)	-0.005	0.996
Percentage of GR on $CD3^+CD4^+$ T lymphocytes	0.975 (0.876, 0.985)	0.957 (0.845, 0.987)	-0.617	0.538
MFI of GR on $CD3^+CD4^+$ T lymphocytes	2.08 (1.72, 3.35)	2.22 (1.71, 2.69)	-0.865	0.387
Percentage of GR on $CD3^+CD8^+$ T lymphocytes	0.963 (0.816, 0.977)	0.938 (0.834, 0.980)	-0.254	0.800
MFI of GR on $CD3^+CD8^+$ T lymphocytes	2.08 (1.68, 3.10)	2.11(1.71, 2.46)	-0.653	0.514

All the data in Supplemental table 6 are represented as the median [IQR]. Abbreviations: IQR, Interquartile Range; CD, Cluster-of-differentiation; NK, natural killer; Treg, regulatory T; GR, glucocorticoid receptor; MFI, mean fluorescence intensity.

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3-5
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	Supplemental Figure 1
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5-9, Supplemental Figure 1
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5,6,8,9
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5, 6, 8, Supplemental Figure 1
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6, 8
Bias	9	Describe any efforts to address potential sources of bias	6-8
Study size	10	Explain how the study size was arrived at	Supplemental Figure 1
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8, 11

(*b*) Describe any methods used to examine subgroups and interactions	N/A
(*c*) Explain how missing data were addressed	8, 11
(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	11
(*e*) Describe any sensitivity analyses	

Continued on next page

Results			
Participants	13 *	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	9, Supplemental Figure 1
		(b) Give reasons for non-participation at each stage	9,12, Supplemental Figure 1
		(c) Consider use of a flow diagram	Supplemental Figure 1
Descriptive data	14 *	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9
		(b) Indicate number of participants with missing data for each variable of interest	Supplemental Figure 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	8
Outcome data	15 *	*Cohort study*—Report numbers of outcome events or summary measures over time	9-12
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-12, Electronic supplemental material
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N/A
Discussion			
Key results	18	Summarise key results with reference to study objectives	12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12-14
Generalisability	21	Discuss the generalisability (external validity) of the study results	15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	17

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely

available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.