ASPECTS OF THE NEUROENDOCRINE STRESS RESPONSE IN PATIENTS WITH SEVERE SEPSIS OR TRAUMA

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, for the degree of Doctor of Philosophy

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DECLARATION

I declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy to the University of the Witwatersrand, Johannesburg.

This thesis is submitted in the integrated format, approved by the Faculty of Health Sciences, of an integrated narrative, which includes a synthesis and discussion of papers included in the thesis, a supporting introduction, the published works and a concluding chapter.

This work has not been submitted before for any degree or examination at any other University.

This thesis is presented for examination in fulfillment of the requirements for the degree of Doctor of Philosophy in Medicine.

Signed,

Gladness Dakalo Nethathe — 16 September 2025 (Date)

PREFACE

This thesis includes six journal manuscripts. Of these manuscripts, three (Chapter 6-8) have been published, Chapter 4 has been accepted for publication, and Chapters 3 and 5 are under review. The contents of these manuscripts remain unchanged from the accepted or published versions. The manuscripts are listed below, with a description of my contributions and the contributions of each author to the respective studies.

CHAPTER 3

Profiles of pro- and anti-inflammatory cytokines and total cortisol levels in severe sepsis and septic shock. Gladness Dakalo Nethathe, Hendrik Frederik Prinsloo Riekert, Ronald Anderson, Jeffrey Lipman, Helen C Steel, Jeremy Cohen, Tendesayi Kufa-Chakezha, Fathima Paruk, Nchafatso G. Obonyo, Charles Feldman.

This study was conducted on samples obtained from patients with septic shock at Steve Biko Academic Hospital in Pretoria, South Africa. The study concept and design was developed by Gladness Dakalo Nethathe, Associate Professor Jeremy Cohen, Professors Jeffrey Lipman, Ronald Anderson and Charles Feldman. Study coordination and data acquisition were conducted by Hendrik Frederik Prinsloo Riekert. Statistical analysis was performed by Tendesayi Kufa-Chakezha and Nchafatso G. Obonyo. Gladness Dakalo Nethathe drafted the original manuscript, which underwent critical revision by all co-authors for intellectual content. Gladness Dakalo Nethathe prepared the manuscript for submission for publication.

Gladness Dakalo Nethathe supervised the study alongside Jeffrey Lipman, Ronald Anderson, and Charles Feldman.

CHAPTER 4

The total and free cortisol response to ACTH and Glucocorticoid Sensitivity in Patients with Traumatic Brain Injury. Gladness Dakalo Nethathe, Jeremy Cohen, Jeffrey Lipman, Ronald Anderson, Karen Hay, Charles Feldman.

This study was conducted on samples obtained from patients with traumatic head injury at the Royal Brisbane and Women's Hospital and the Gold Coast Hospital. The control group was drawn from a concurrent study investigating glucocorticoid sensitivity in healthy adults (Chapter 5). The initial study concept was developed by Associate Professor. Jeremy Cohen, Professor Balasubramanian Venkatesh and Dr James Winearls. Gladness Dakalo Nethathe expanded and amended this design under the supervision of Associate Professor Jeremy Cohen, Professors Jeffrey Lipman, Ronald Anderson and Charles Feldman. Gladness Dakalo Nethathe was responsible for patient recruitment, clinical assessment, clinical data collection and analysis at the Royal Brisbane and Women's Hospital site. Gladness Dakalo Nethathe was the lead author, drafting and finalising the manuscript after consulting all co-authors. Melissa Lassig-Smith assisted with study recruitment, supported patient assessments, and clinical data recording. Anna Zournazi, Department of Chemical Pathology, Pathology Queensland, Queensland Health, Herston, Australia supervised the laboratory assessments. Karen Hay, Biostatistician at QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia. assisted with statistical analysis. Janine Stewart, Therese Starr and Amelia Livemore were also involved in patient assessment and recording of clinical data.

CHAPTER 5

Glucocorticoid sensitivity and plasma total and free cortisol responses to ACTH in healthy male and female volunteers. Gladness Dakalo Nethathe, Jeremy Cohen, Jeffrey Lipman, Ronald Anderson, Karen Hay, Charles Feldman.

The study investigated healthy volunteers at the Royal Brisbane and Women's Hospital. Gladness Dakalo Nethathe, Associate Professor Jeremy Cohen, Professors Jeffrey Lipman, Ronald Anderson and Charles Feldman developed the study concept and design. Gladness Dakalo Nethathe was responsible for recruitment, clinical assessment, clinical data collection, and analysis. Gladness Dakalo Nethathe was the lead author, drafting and finalising the manuscript after consulting all co-authors

Melissa Lassig-Smith, research nurse, intensive care services at the Royal Brisbane and Women's hospital assisted with study recruitment, data collection and entry. Anna Zournazi, Department of Chemical Pathology, Pathology Queensland, Queensland Health, Herston, Australia supervised the laboratory assessments.

Karen Hay, Biostatistician at QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia. assisted with data and statistical analysis.

The following were also involved in patient assessment and recording of clinical data:

Janine Stewart, Therese Starr and Amelia Livemore.

CHAPTER 6

Mineralocorticoid Dysfunction during Critical Illness: A Review of the Evidence. Anesthesiology. Nethathe GD, Cohen J, Lipman J, Anderson R, Feldman C.

This chapter is a narrative review that arose from the recognition that with regards to relative adrenal insufficiency, adrenal dysfunction syndromes in critical illness are unlikely to be localised to the zona glomerulosa of the adrenal cortex. This chapter critically examines the role of the mineralocorticoid axis in critical illness, emphasizing the broader implications of adrenal dysfunction. Gladness Dakalo Nethathe was the lead author and finalised the manuscript for submission after receiving input from all co-authors.

CHAPTER 7

CIRMI—a new term for a concept worthy of further exploration: a narrative review. Nethathe GD, Lipman J, Anderson R, Feldman C.

This paper introduces the concept of Critical Illness-Related Mineralocorticoid Insufficiency (CIRMI). This review explores the parallels between CIRMI and Critical Illness-Related Corticosteroid Insufficiency (CIRCI), highlighting gaps in the literature and providing novel insights into mineralocorticoid dysfunction in critical illness. Gladness Dakalo Nethathe led the manuscript preparation, consulted with all co-authors, and finalised the submission.

CHAPTER 8

Glucocorticoids with or without fludrocortisone in septic shock- a biochemical and molecular perspective. Nethathe GD, Fuller P, Anderson R, Lipman J, Feldman C.

This manuscript examines the potential role of fludrocortisone as an adjunct to hydrocortisone in septic shock. This narrative review discusses the molecular mechanisms underlying glucocorticoid and mineralocorticoid action and provides a rationale for adjunctive therapy in septic shock. Pending the publication of a well designed randomised controlled trial on the role of fludrocortisone in septic shock, a molecular perspective on the potential role of fludrocortisone, provides supportive rationale and guidance in informing clinical strategies. Gladness Dakalo Nethathe was the lead author and finalised the manuscript after incorporating feedback from all co-authors.

ABSTRACT

This thesis investigated the pathophysiology and clinical implications of adrenal insufficiency (AI) in the context of systemic inflammation with a particular focus on the interplay between the renin-angiotensin-aldosterone system (RAAS), cortisol metabolism, and glucocorticoid sensitivity. Through a series of observational studies and narrative reviews, a comprehensive analysis of hormonal alterations, their impact on critical care management, and the potential for targeted therapeutic strategies in critically ill patients is provided.

The first study examined the profiles of pro- and anti-inflammatory cytokines and total cortisol levels in patients with severe sepsis and septic shock. Elevated levels of interleukin (IL)-6 and tumour necrosis factor receptor 1 (TNFR1) were identified as significant predictors of mortality in this population, while the independent role of cortisol in predicting outcomes remains less definitive. IL-1 receptor antagonist (Ra) showed a significant median reduction from day 1 to day 7, with a significant association with an increased mortality ratio, suggesting its potential role as a marker of immune resolution and recovery in the current cohort. This study is the first to explore the trajectory of these biomarkers in a South African cohort with severe sepsis and septic shock. The findings highlight the importance of IL-6, TNFR1, ILRa and total cortisol in characterising disease severity, progression, and outcomes, emphasising their potential utility in risk stratification for critically ill patients.

A related investigation evaluated the free cortisol response to synacthen and glucocorticoid sensitivity in patients with traumatic brain injury (TBI). Findings from this study highlight altered glucocorticoid sensitivity and responsiveness in TBI. Significant variability in

measures of glucocorticoid sensitivity attributed to receptor-level changes and impaired hypothalamic-pituitary-adrenal (HPA) axis function was observed. These findings suggest that in traumatic brain injury, the sensitivity and responsiveness to glucocorticoids are altered with mechanistic and therapeutic implications.

To establish a baseline for understanding glucocorticoid receptor dynamics in critical illness, the thesis extended this investigation to glucocorticoid sensitivity in healthy volunteers to provide a baseline for interpreting cortisol metabolism and receptor activity in critically ill states. This comparative analysis established the extent to which critical illness modifies glucocorticoid receptor function.

Moreover, the concept of critical illness-related mineralocorticoid insufficiency is introduced, challenging the conventional focus on glucocorticoid insufficiency alone and advocating for a more comprehensive and nuanced approach to adrenal pathophysiology and support in critical illness. Finally, the thesis critically evaluated adjunctive corticosteroid therapy in septic shock. To integrate these findings into clinical practice, the thesis provides a narrative review of adjunctive corticosteroid therapy, particularly the use of adjunctive hydrocortisone with or without fludrocortisone in septic shock. The biochemical and molecular basis of corticosteroid action, the potential benefits of dual therapy targeting both glucocorticoid and mineralocorticoid pathways, and the limitations of current treatment protocols are discussed.

In summary, this thesis advances the understanding of adrenal insufficiency in critical illness by elucidating the complex interactions between cortisol production, RAAS dysregulation, and glucocorticoid and mineralocorticoid receptor dynamics. The integrated approach taken

here—spanning observational studies and comprehensive reviews—offers a holistic view of adrenal dysfunction and proposes actionable insights with the potential to refine current understanding, with the aim to improve outcomes for patients with septic shock and traumatic injury.

Key words: neuroendocrine response, hormone supplementation, critical illness, corticosteroids, mineralocorticoids, trauma, sepsis, septic shock, corticosteroid insufficiency, hypothalamic-pituitary-adrenal axis.

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I would like to extend my deepest gratitude to all the participants of these studies. Without their involvement, this research would not have been possible.

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I am profoundly thankful to my supervisors and collaborators—Associate Professor Jeremy Cohen, Professor Jeffrey Lipman, Professor Ronald Anderson, and Professor Charles Feldman. Their patience, motivation, unwavering support, and discussions have been invaluable throughout this journey.

A special thank you goes to Melissa Lassig-Smith and the research nursing team at Intensive Care Services, Royal Brisbane and Women's Hospital. Your dedication, patience, and encouragement, both within and beyond the hospital, have been pivotal in making this research a reality.

I am grateful to my colleagues and friends for their continued support and belief in my endeavors.

To my siblings, Azwinndini-Joy and Nelson, your faith in me has been a constant source of strength and motivation.

Finally, I would like to express my heartfelt thanks to Ulrich Kirchner for your unwavering encouragement and support, and to my mother, Constance, for fostering an environment that nurtured perseverance and fostered early independence.

DEDICATION

In memory of my father

Wilfred Tshisikhawe Nethathe

7th September 1952 – 6th May 1992

LIST OF RESEARCH OUTPUTS ARISING FROM THIS WORK

Publications

1. Nethathe GD, Cohen J, Lipman J, Anderson R, Feldman C. Mineralocorticoid Dysfunction during Critical Illness: A Review of the Evidence. Anesthesiology. 2020;133(2):439-457. Impact factor: 9.3

2. Nethathe GD, Lipman J, Anderson R, Feldman C. CIRMI- a new term for a concept worthy of further exploration: a narrative review. Annals of Translational Medicine. 2022;10(11):646. Impact factor: 3.9

3. Nethathe GD, Fuller P, Anderson R, Lipman J, Feldman C. Glucocorticoids with or without fludrocortisone in septic shock- a biochemical and molecular perspective. British Journal of Anaesthesia. 2024;132(1):53-65. Impact factor: 9.1

Submitted for publication

4. Profiles of pro- and anti-inflammatory cytokines and total cortisol levels in severe sepsis and septic shock. Gladness Dakalo Nethathe, Hendrik Frederik Prinsloo Riekert, Ronald Anderson, Jeffrey Lipman, Helen C Steel, Jeremy Cohen, Tendesayi Kufa-Chakezha, Fathima Paruk, Nchafatso G. Obonyo, Charles Feldman.

5. A comparison of the total and free cortisol response to a standard Synacthen test in patients with traumatic brain injury. Gladness Dakalo Nethathe, Jeremy Cohen, Jeffrey Lipman, Ronald Anderson, Charles Feldman.

6. Glucocorticoid sensitivity and plasma total and free cortisol responses to ACTH in healthy male and female volunteers. Gladness Dakalo Nethathe, Jeremy Cohen, Jeffrey Lipman, Ronald Anderson, Kate Hay, Charles Feldman.

Conference Presentations

Invited Speaker

Mineralocorticoid Dysfunction In Critical Illness. 2022 CCSSA National Congress, Critical Care Society of Southern Africa (CCSSA), 13-16 October 2022, Sandton Convention Centre, Johannesburg.

Mineralocorticoid Dysfunction In Critical Illness. 2024 CCSSA National Congress, Critical Care Society of Southern Africa (CCSSA), 22-25 August 2024, Sandton Convention Centre, Johannesburg.

Fludrocortisone in Septic Shock. 2024 All Africa Congress, 13th – 19th September 2024, Sandton Convention Centre, Johannesburg.

CIRCI/CIRMI Adrenal dysfunction in critical illness SASA Refresher Course Speaker, 13th – 19th September 2024, Sandton Convention Centre, Johannesburg.

Abstract. Adrenal dysfunction in critical illness. SASA Refresher course Abstract, All Africa Anaesthesia Congress, September 14, 2024

CHAPTER 1: Background and introduction

The stress response to critical illness involves intricate interactions involving the neuroendocrine axis, hypothalamic-pituitary-adrenal axis (HPA), the renin-angiotensin-aldosterone system (RAAS), as well as metabolic and immune system changes (1). These processes are generally proportional to the severity of the stress and reflect the body's attempt to restore homeostasis (1). Impairments in the HPA axis, including dysregulation of the hypothalamic-pituitary-axis, altered cortisol metabolism and tissue corticosteroid resistance, are hallmark features of critical illness-related corticosteroid insufficiency (CIRCI) (2). Such dysregulation has been associated with increased disease severity and mortality, yet gaps remain in understanding the mechanisms underlying these changes, with changes affecting various sub-populations being under-explored (2–5).

A number of studies have shown a positive correlation between serum total cortisol and severity of illness. However the free cortisol rather than the total cortisol is the active form and its levels might be a better reflection of the degree of stress. In addition tissue action is evidently increasingly relevant in the pathophysiology of CIRCI (2,6,7).

Furthermore, a growing body of evidence highlights the importance of glucocorticoid sensitivity—the responsiveness of tissues to circulating glucocorticoids—in the pathophysiology of CIRCI. Tissue-specific variability in glucocorticoid sensitivity influences inflammatory and metabolic outcomes, independent of circulating cortisol levels. This thesis explores the interplay between cortisol dynamics, glucocorticoid sensitivity, and

inflammatory responses in critically ill patients, focusing on severe sepsis, septic shock, and traumatic brain injury (TBI). By comparing findings in critically ill patients with healthy controls, this work sheds light on the complex and often independent mechanisms of adrenal dysfunction in severe sepsis and trauma.

Despite its clinical significance, the role of glucocorticoid sensitivity in critical illness is poorly understood. Traditional measures of adrenal function, such as total cortisol levels and the short synacthen test, do not account for variability in tissue-level glucocorticoid action (2,6). By incorporating assessments of glucocorticoid sensitivity—using suppression assays of inflammatory cytokines like IL-6 and TNF-α—this thesis provides novel insights into how glucocorticoid action varies across critically ill populations. Findings from this work suggest that glucocorticoid sensitivity and cortisol dynamics operate through distinct mechanisms, with implications for individualized endocrine management in critical care settings.

Furthermore, mineralocorticoid dysfunction has emerged as a key, albeit under-explored component of adrenal insufficiency (3). A significant proportion of critically ill patients display dissociation of aldosterone and renin levels, a condition associated with poor outcomes, and potentially representative of adrenal gland adaptation to prioritise cortisol synthesis (8). This thesis introduces the concept of critical illness-related mineralocorticoid insufficiency (CIRMI), highlighting the comprehensive nature of adrenal dysfunction as a time-dependent condition involving both cortisol and aldosterone pathways, which is demonstrable by concurrent derangements in both cortisol and aldosterone handling.

The interplay between CIRCI, CIRMI, and glucocorticoid sensitivity offers a framework for

optimising adjunctive therapy in critically ill patients. Current evidence supports the use of adjunctive hydrocortisone in septic shock (9), yet the potential benefits of adjunctive fludrocortisone remain under-explored (10,11). This thesis investigates how combined glucocorticoid and mineralocorticoid therapies could address both cortisol and aldosterone dysfunction, thereby improving patient outcomes. A molecular perspective discussed in this thesis potentially offers valuable insights and guidance to help inform clinical strategies (12).

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CHAPTER 2: Literature review, incorporating the concept of the thesis

2.1 Stress response to trauma and sepsis

Stress may be defined as a state of threatened homeostasis. Critical illness represents a severe form of acute stress which evokes complex responses from the endocrine, autonomic and immune systems (1–3). These overlapping responses are collectively termed the stress response. During the stress response, the central nervous system (CNS) senses stress from a variety of hormonal and neurological inputs, which induce activation of both the sympathoadrenergic system and the hypothalamic-pituitary-adrenal axis (HPA) (2,4,5). The main mediators of this response include cytokines, catecholamines and corticosteroid hormones (3). The aim of this response is to maintain homeostasis through modulation of the neuro-endocrine axis, metabolic and immune systems (3). The stress response thus involves modulation of the autonomic nervous system, the neuro-endocrine axis, metabolic and immune systems. The magnitude of the stress response typically correlates with the severity of the stressor (2,5,6).

Trauma and sepsis are leading causes of critical illness associated with significant morbidity and mortality worldwide. Globally, traumatic injury remains a leading cause of mortality, hospitalisation, morbidity and disability (7–9). Traumatic injury is a devastating condition and a significant cause of mortality particularly among young individuals (9–11). Intensive care unit (ICU) admissions for multi-trauma cases accounted for 2.0% of all injury hospitalisations in Australia in 2021-22 (11). Trauma-induced tissue injury activates an inflammatory cascade, the immune system and the coagulation pathway (12). The subsequent systemic inflammatory response syndrome (SIRS), a result of immune system activation, is

correlated with adverse clinical outcomes (13).

Sepsis is a life-threatening condition characterised by a dysregulated host response to infection, leading to organ dysfunction and multi-organ failure. It is a major global health concern, with a substantial and increasing annual incidence in high-income countries (14–18). While mortality rates in sepsis have decreased over time, likely due to advances in pathophysiological insights and therapeutic strategies (19–21), it remains a leading cause of death (22). In sub-Saharan Africa, where data are scarce, the burden is anticipated to be higher due to prevalent infectious diseases (10).

The pathophysiology of sepsis is multifactorial, driven by the interactions between pathogens, host factors, neuroendocrine and immune responses (2). In septic shock, time-dependent derangements occur which result in increased risk of persistent shock, organ dysfunction and death. Inflammatory mediators lead to altered corticotropin synthesis, increased distribution volume for corticosteroids, modified corticosteroid delivery to tissues, prolonged corticosteroid metabolism and decreased tissue sensitivity to steroids (23–28). Termed critical illness-related corticosteroid insufficiency, these patients exhibit a dysregulated hypothalamic-pituitary-adrenal (HPA) axis, altered cortisol metabolism, and tissue resistance to glucocorticoids (26).

2.2 Adrenal dysfunction in critical illness

Adrenal insufficiency clinically manifests in the setting of inefficient production, or action of, glucocorticoids and is classified as primary, secondary, or relative (29,30). Primary disease

involves decreased production from the adrenal gland and stems from adrenal gland destruction from autoimmune disease, tuberculous adrenalitis and haemorrhage into the adrenals, leading to both glucocorticoid and mineralocorticoid deficiency (30). Secondary adrenal insufficiency arises from anterior pituitary dysfunction, most commonly following abrupt cessation of glucocorticoid therapy (30). Additional causes include hypopituitarism due to brain injury or post-pituitary surgery, or haemorrhage into the pituitary, as well as pituitary tumours (30).

There remains a concern that a subset of critically ill patients has an inadequate adrenal glucocorticoid response to their degree of illness, and that outcome could be improved by adjunctive glucocorticoid therapy in this group (31–33). Critical illness-related corticosteroid insufficiency (CIRCI) remains a topic of continued interest as a potential cause of increased mortality in critical illness (5). The diagnosis of CIRCI, however, poses a number of challenges. The standard method used to assess corticosteroid insufficiency (CI) in the non-critical care setting is a corticotrophin test, based on the administration of 250µg of synthetic ACTH followed by measurement of the adrenal response in the form of plasma total cortisol secretion. Furthermore, the currently accepted basal and stimulated cortisol levels of 15 ± 8 mg/dl (± 2SD) and 32 ± 12 mg/dl (± 2SD), respectively, were developed in healthy, non-stressed subjects (34). Application of this procedure in the setting of critical illness poses several difficulties, such as baseline variability of total plasma cortisol levels in critical illness. A false-positive result may occur when using the stimulated total cortisol level because of altered protein-binding. Conversely, the resultant drop in binding protein may result in a decrease in total cortisol (35). The alternative criteria of using the change from the basal to the stimulated level of 5–9 µg/dl is also problematic in critical illness as the delta

level is inversely related to the basal level, which is already high in the critically ill (36). In this context, studies have demonstrated a failure of as many as 30% of normal subjects to meet the delta criteria (37–39). Plasma cortisol levels are generally considerably raised in non-survivors of critical illness and less so in survivors, a finding considered inconsistent with the concept of relative adrenal insufficiency (31,33,36,40,41). Direct methods of free cortisol measurement such as ultra-high frequency tandem mass spectrometry may thus be more reliable in the assessment of cortisol levels in the critically ill (42).

2.3 Free cortisol in traumatic brain injury

Traumatic brain injury (TBI) is a recognised cause of secondary adrenal insufficiency; however, the biochemical diagnosis of adrenal insufficiency remains challenging in this context (43–45). Previous investigators have demonstrated that free cortisol levels provide better accuracy through demonstration of marked variation in free cortisol levels and nearly identical total plasma cortisol increments across healthy patients and those with septic shock (45,46). Significantly lower corticosteroid binding globulin (CBG) levels have been demonstrated in patients with TBI compared to controls (47). The reduction in CBG may contribute to the observed elevations in free cortisol, as well as highlighting the limitations of the use of indirect methods of free cortisol assessment (48).

2.4 Glucocorticoid sensitivity and tissue activity of glucocorticoids

Glucocorticoid sensitivity varies significantly across individuals and is influenced by genetic and disease-related factors (49). Understanding glucocorticoid action on target tissue may

allow for a more rational individualised approach to supplementation (36). Several factors, inclusive of glucocorticoid receptor density and function (50), as well as intracellular cortisol and cortisone metabolism as regulated by the functioning of the 11β-hydroxysteroid dehydrogenase enzyme (11β-HSD) system, play a role (51).

The glucocorticoid receptor is cytosolic (unbound form), expressed in almost every human cell and regulates genes that affect development, metabolism, and the immune system (50). Inflammation and cytokines impact significantly on glucocorticoid receptor number and function (52–54). A decrease in both binding activity and glucocorticoid receptor-mediated transcription, have been shown in acute respiratory distress syndrome (55). More recently glucocorticoid receptor expression and binding capacity have been shown to be increased in burn injury (54).

Suppression of 11β-HSD-2 activity, resulting in increased cellular cortisol availability has been shown in critical illness (35). Furthermore, the finding of reduced cortisol metabolism in sepsis, implying increased tissue cortisol exposure, suggests that lower doses of hydrocortisone than previously advocated may be sufficient if steroid supplementation is to be used (35,56–58). Interestingly, in critical illness, elevated serum cortisol levels are often observed, and the diminished or absence of a total cortisol response to ACTH may represent a physiological adaptation to a hypercortisolaemic state rather than a pathological deficiency (57).

Recently glucocorticoid sensitivity has been shown to be associated with disease severity (59). The association of *in vitro* glucocorticoid sensitivity with clinical outcome has not, however, yet been shown, regardless of whether glucocorticoid therapy has been employed.

Glucocorticoid sensitivity is influenced by genetic and disease-related factors and is thus highly variable in both healthy and critically ill individuals (59–61). Chronic conditions such as autoimmune diseases, obesity, and endocrine disorders have been shown to affect glucocorticoid sensitivity, with the diverse responses to glucocorticoids observed in conditions like rheumatoid arthritis highlighting the therapeutic relevance of considering individual variability in glucocorticoid sensitivity (61–64). The *in vitro* assessment of glucocorticoid sensitivity has previously been advocated in rheumatoid arthritis, a disease in which glucocorticoid therapy is known to be beneficial (61).

A number of methods have been employed in the assessment of *in vitro* glucocorticoid sensitivity. These commonly rely on glucocorticoid-induced suppression of peripheral blood mononuclear cell proliferation or glucocorticoid-induced suppression of cytokine release in stimulated or unstimulated human cell cultures derived from peripheral blood cells or alveolar macrophages, the stimulant being lipopolysaccharide (LPS) (63,65,65–67). Of note is that the suppression of cytokine release from LPS stimulated peripheral blood cells may be affected by the stimulation process itself, as well as by circulating glucocorticoids (63). The dexamethasone-induced suppression of cytokine production from LPS-stimulated monocytes, also called the dexamethasone suppression of cytokine production assay (DSCP), is one such assay with a reported greater ability to detect tissue glucocorticoid sensitivity than other methods (67). Although reduced glucocorticoid metabolism, has been observed in sepsis (35,57,68), tissue corticosteroid bioavailability has not been consistently shown to predict clinical outcome in critical illness.

2.5 Hyperreninemic hypoaldosteronism, a neglected entity

There is a novel and growing recognition that patients with CIRCI often exhibit endocrine dysfunction, encompassing both the hypothalamic-pituitary-adrenal axis and the RAAS (69). Angiotensin II was shown to effectively increase blood pressure in patients with vasodilatory shock non-responsive to conventional high doses of vasopressors bringing to light the role of the renin angiotensin system in outcomes of patients with critical illness (70). Furthermore, there has been demonstration of elevated plasma renin levels and inappropriately low aldosterone levels among a substantial cohort of critical illness patients (71). Hyperreninemic hypoaldosteronism, associated with a high mortality rate, has previously been described in a subset of critically ill patients (72). In this seminal study, a spectrum of aldosterone responsiveness in 18 patients (n=28) with persistent hypotension was described. Plasma renin activity was found to be increased in the total sample with aldosterone being inappropriately low in 18 of the 28 participants. The lack of an aldosterone response to angiotensin II or ACTH in most of the patients in this study suggested that the defect was at the level of the adrenal zona glomerulosa. Though the study was small and the methods used to measure both aldosterone and renin have since been improved, it nevertheless highlighted a neglected entity, namely selective hypoaldosteronism in haemodynamically unstable critically ill patients. Termed hyperreninaemic hypoaldosteronism, hyperreninaemia in septic shock is a clinically relevant marker of illness severity. This marker is associated with vasopressor-resistant vasodilatory shock, a form of shock characterised by increased or preserved cardiac output and peripheral vasodilation, as well as worsening renal failure and increased mortality (23,73,74).

Mineralocorticoid insufficiency, in trauma patients presenting in haemorrhagic shock, is

associated with increased disease severity, requirement for a more aggressive resuscitative effort, higher likelihood to experience hypotension, a higher risk of acute renal failure (AKI) and higher mortality rates (75). Critical illness–associated hyperreninemic hypoaldosteronism is identifiable when there is dissociation of plasma aldosterone and plasma renin as evidenced by a plasma aldosterone/plasma renin (PA/PR) ratio of less than 2. This definition is used in non-critically ill patients with mineralocorticoid deficiency and corresponds to the 98th percentile of the control population (76). Mineralocorticoid dysfunction may represent adrenal (mal)adaptation designed to optimise cortisol production as a result of precursor substrate diversion towards cortisol production in the critically ill (71).

Indeed, in sepsis, mineralocorticoid steroidogenesis has been shown to be more frequently impaired than glucocorticoid steroidogenesis (23)**.** Sepsis and trauma are both common aetiologies of adrenal insufficiency and vasodilatory shock in the critically ill.

Cortisol and aldosterone are the primary corticosteroids produced in critical illness and contribute to the stress response. Steroidogenesis, the process by which steroid hormones, including aldosterone and cortisol are synthesised, occurs in the adrenal gland and is dependent on the availability of steroid precursors (77). Cortisol precursors and their ratio to cortisol have been previously investigated to characterise steroidogenesis in the adrenal dysfunction of CIRCI in septic shock, leading to the recognition of substrate deficiency as a potential mechanism that contributes to CIRCI (77). Aldosterone is produced as a result of conversion of corticosterone and the synthesis of corticosterone is triggered exclusively by corticotrophin (23).

2.6 Therapeutic intervention

Both endogenous corticosteroids and exogenous corticosteroids play a pivotal role in modulating inflammatory and immune responses. Corticosteroids modulate immunomodulatory effects by inhibiting the transcription of pro-inflammatory genes, impede the migration of leukocytes to sites of inflammation and hinder the adherence of neutrophils to endothelial cells, thereby diminishing their subsequent release of humoral factors (78,79). Corticosteroids increase cardiac output, as well as regional and coronary blood flow through inhibition of nitric oxide synthesis (80). In septic shock corticosteroids increase arterial pressure through restoration of systemic vascular resistance and the vasopressor response to norepinephrine.

A significant reduction in 90-day mortality has been demonstrated with adjunctive hydrocortisone administered with fludrocortisone (81,82). Potential underlying mechanisms for this mortality benefit have recently been described (83). Prior guidelines of the Society of Critical Care implicitly endorsed mineralocorticoid replacement by suggesting the preference of an oral daily dose of fludrocortisone 50 µg over dexamethasone given together with hydrocortisone as the latter lacks mineralocorticoid activity. Subsequent consensus statements by the Society of Critical Care Medicine (SCCM) and the European Society of Intensive Care Medicine (ESICM) 2017 did not address the role of mineralocorticoid replacement in patients with septic shock (84–86). Although evidence from a well-designed, randomised controlled trials is pending, recent insights suggest that a combination of fludrocortisone and hydrocortisone is more effective than hydrocortisone alone for septic shock (87,88)**.**

2.7 Conclusions and overall concept of the thesis

This introduction establishes a foundational understanding of the global burden of sepsis and

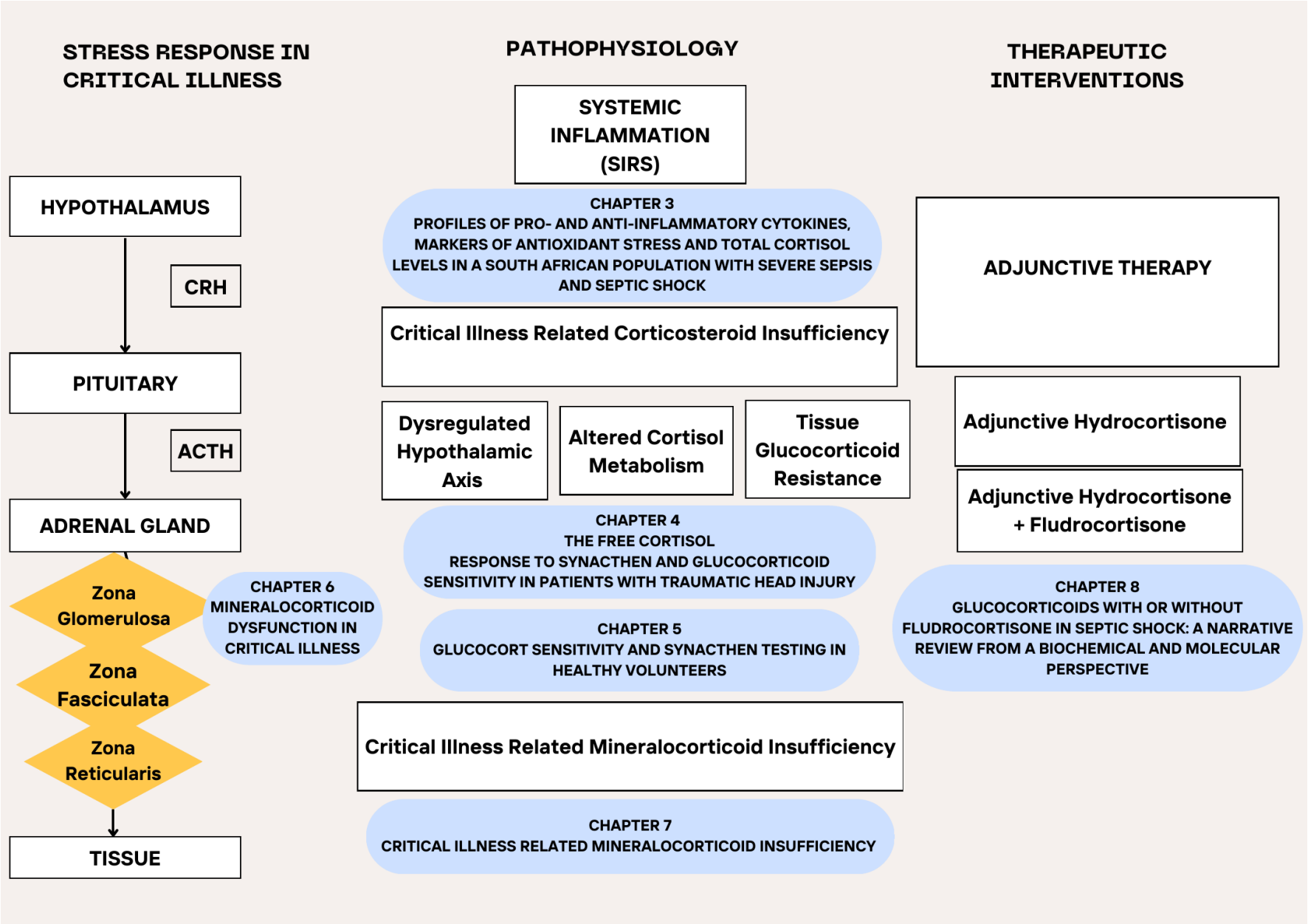
trauma, the complex interplay of the endocrine and immune responses in critical illness, and serves as the rationale for the inclusion of the various studies and reviews included in the subsequent chapters of the thesis. However, despite extensive work in this field, important knowledge gaps remain. There is a paucity of data describing the combined trajectory of pro- and anti-inflammatory cytokines and cortisol in critically ill patients with sepsis, in African cohorts, and it is unclear whether total cortisol provides independent prognostic information alongside inflammatory biomarkers.

While adrenal function has been studied in traumatic brain injury, prior limited research has almost exclusively measured total cortisol, leaving uncertainty about the role of free cortisol as a more reliable marker, and tissue glucocorticoid sensitivity has not previously been investigated in this population.

In healthy adults, although the ACTH stimulation test is widely used, reference data on free cortisol responses are limited, and the relationship between circulating cortisol responses and in vitro glucocorticoid sensitivity has not been characterised.

These knowledge gaps underpin the three original studies in this thesis. By addressing knowledge gaps in glucocorticoid sensitivity, cortisol metabolism, and mineralocorticoid dysfunction, this thesis seeks to advance the understanding of critical illness endocrinology and potentially inform targeted therapeutic strategies.

2.8 Research framework



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CHAPTER 3: Profiles of pro- and anti-inflammatory cytokines and total cortisol levels in severe sepsis and septic shock

TITLE

Profiles of pro- and anti-inflammatory cytokines, and total cortisol levels in severe sepsis and septic shock.

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KEYWORDS

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ABSTRACT

Background

Sepsis involves a complex interplay of immunoinflammatory interactions. We investigated pro- and anti-inflammatory cytokine and total cortisol levels (TC) in relation to disease severity and outcomes.

Methods

Forty-five patients with severe sepsis or septic shock were enrolled. Clinical data, including severity scores (APACHE II, SAPS II, SOFA) were recorded. Cytokines (interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-17, IL-1 receptor antagonist (Ra)), Tumour necrosis factor receptor 1 (TNFR1) and TC were evaluated on admission and on day 7. Regression analyses explored relationships between biomarkers, disease severity, and 28-day mortality.

Results

The median age was 49 years (IQR: 38–62). The most common infection site was abdominal (53%). Admission APACHE II, SAPS II, and SOFA scores were [median (IQR)] 22 (15–28), 40 (31–53), and 9 (6–12), respectively. Admission IL-6 [0.09 (0.03–0.32)] and TNFR1 [2050.4 (1292.1–4315.0)] levels (pg/mL) were elevated. IL-1Ra decreased significantly from day 1 [21.09 (12.88-60.19)] to day 7 [5.19 (3.38-26.84); $p = 0.032$] (pg/mL).

TC levels were at a median of 568 nmol/L (IQR: 335–1650). TNFR1 was a significant predictor of 28-day mortality [adjusted mortality ratio (aMR) = 1.0265, (95% CI: 1.0157–1.0373), $p < 0.001$]. IL-6 [MR = 1.0109, (95% CI 1.0060–1.0159), $p < 0.001$] and TC levels [MR = 1.0180, 95% CI: 1.005-1.031, $p = 0.007$] were significant in univariate analysis but not after adjustment.

Conclusions

TNFR1 was associated with mortality. The significant reduction in IL-1Ra suggests-a maladaptive anti-inflammatory response, with declining levels associated with increased mortality. Elevated TC levels were not independently associated with increased mortality.

INTRODUCTION

Sepsis remains a major global health concern contributing to substantial morbidity and mortality [1,2]. In low- and middle-income countries (LMICs) there is a paucity of data pertaining to the incidence and mortality of sepsis, and where available it depicts wide regional variability [1,3–8]. Sub-Saharan Africa remains a region with a high burden of sepsis, accounting for up to 28% of intensive care unit (ICU) admissions in South Africa [1,9,10]. As the understanding of sepsis pathophysiology evolves, accurately identifying the inflammatory and immune response and predicting outcomes remains a major challenge.

The clinical course of sepsis is dynamic and heterogeneous, influenced by pathogen virulence, patient comorbidities, treatment regimens and genetic predispositions, resulting in diverse inflammatory and immune trajectories, heterogenous treatment effects and treatment outcomes [3,4,6,11]. This forms the basis of exploring various sub-phenotypes of sepsis. In developing/LMICs,

delayed presentation and limited access to healthcare contribute disproportionately to higher mortality rates. Consequently, accurately identifying high-risk patient subgroups is crucial to personalising sepsis management and improving prognosis.

Pathogen-mediated activation of the innate immune system induces an upregulation of gene transcription and cytokine release. Pro-inflammatory cytokines, including tumour necrosis factor alpha (TNF-α), and its receptor tumour necrosis factor receptor 1 (TNFR1), interleukin (IL)-1 beta (IL-1β), IL-6, IL-8, and IL-17, trigger wide-spread inflammation, activate leukocytes, the complement system, and coagulation pathways. This pro-inflammatory state promotes acute-phase protein production, immune cell recruitment to infection sites and necrotic cell death [3,7,12]. TNF-α activates TNFR1 amplifying the inflammatory response [13]. In contrast, anti-inflammatory cytokines, including IL-1 receptor antagonist (IL-1Ra), IL-10, IL- 4, IL-11, and IL-13, suppress inflammation and promote recovery [14]. IL-1Ra antagonises IL-1 actions, while IL-10 inhibits pro-inflammatory mediators' synthesis. Imbalance between pro- and anti-inflammatory cytokines disrupts immune homeostasis in sepsis, potentially causing persistent inflammation, organ damage or immunosuppression.

Cytokines directly activate the hypothalamic pituitary axis axis further promoting cytokine production in the brain [12,15]. Cytokines activate nitric oxide synthase leading to nitric oxide release which diffuses into corticotrophin-secreting neurones, stimulating pituitary release of adrenocorticotropic hormone (ACTH) and cortisol synthesis [12,15–17]. Cortisol, a key regulator of metabolic, cardiovascular, and inflammatory responses, inhibits macrophage and neutrophil accumulation and suppresses inflammatory mediator synthesis [16,18]. In sepsis, cortisol dysregulation is associated with disease severity and outcomes [19].

The absence of reliable and discriminatory clinical parameters, coupled with the limitations in rapid current pathogen identification methods, has prompted the search for systemic biomarkers that can differentiate between systemic inflammatory response syndrome and sepsis, and in prognostication [20]. Numerous potential biomarkers have been investigated to address this need; however, gaps remains in understanding the dynamic changes in cytokine levels and their role in sepsis progression [20]. The aim of this study was thus to evaluate the trajectory and temporal profiles of pro- and anti-inflammatory cytokines, along with total cortisol levels (TC), in a South African population with severe sepsis and septic shock. Specifically, the study sought to explore the potential of these biomarkers to identify sepsis sub-phenotypes and to assess their relationship with disease severity and clinical outcomes in a sub-Saharan African population.

MATERIALS AND METHODS

This exploratory, prospective, observational cohort study included 45 consecutive patients (week-day enrolment) admitted with severe sepsis and septic shock to the surgical ICU at the Steve Biko Academic Hospital, Pretoria, South Africa, between June 2020 to September 2021. The sample size was determined pragmatically based on feasibility during the COVID-19 pandemic and resource limitations, which constrained ICU admissions and follow-up capacity. As an exploratory study, formal power calculations were not performed, and findings are intended to generate hypotheses for future, larger studies. The surgical intensivist-led unit is a university-affiliated tertiary academic hospital. The study was approved by the Human Research Ethics Committees of the University of the Witwatersrand (M190888) and the University of Pretoria (619/2019). Written informed consent was obtained from participants or their surrogate decision maker and the study was per-

formed in accordance with the Declaration of Helsinki. Patients were enrolled within 48 hours of ICU admission and were eligible if they were ≥18 years and met the consensus definitions of the American College of Chest Physicians/ Society of Critical Care Medicine for severe sepsis or septic shock [6]. Exclusion criteria comprised: patients below 18 years of age, known pregnancy, endocrine disease, current use of corticosteroids or within the past 3 months if previous chronic use (inhaled or oral), thyroid hormone replacement, etomidate or ketoconazole administration during admission, known human immunodeficiency virus (HIV) reactivity, and patients who fulfilled inclusion criteria after more than 48 hours before admission to ICU.

Data Collection and Assays

All patients in the ICU were monitored and managed as per best practice therapy by the treating intensivist. Study-specific samples were retrieved in appropriate blood collection tubes (through indwelling cannulas) within 48 hours of admission, timed in the morning before midday. Additional blood samples (10mL) were collected in tubes containing ethylene-diamine-tetra-acetic acid (EDTA) as an anticoagulant on the day of admission and again seven days following admission, provided the patient was still in ICU. These samples were processed, and the plasma fraction stored at -80 ^{0}C until analysis. Healthy control plasma samples obtained from laboratory volunteers served as assay controls. Routine pathology tests were performed by the National Health Laboratory Service (NHLS), Pretoria, South Africa. In addition, samples were analyzed for total plasma cortisol (nmol/L) at the NHLS.

Variables and outcomes

Clinical data collected included demographic characteristics (age, gender, weight and height, medical history, diagnosis or clinical condition requiring admission) and severity of illness and organ failure scores (Acute Physiology and Chronic Health Evaluation II [APACHE II], Simplified Acute Physiology II [SAPS II], Sequential Organ Failure Assessment [SOFA]) [21,22]. Various clinical data were collected to comprehensively characterise the patient population, including the source and site of sepsis, days on mechanical ventilation, length of ICU stay, and 28-day in-hospital mortality. Of these collected variables, days on mechanical ventilation, length of ICU stay, and 28-day in-hospital mortality were specifically analysed for this study.

Laboratory testing

Measurement of cytokine levels

Levels of circulating pro-inflammatory/anti-inflammatory cytokines (IL-1β, IL-1Ra, IL-6, IL-8, IL-10 and IL-17) were determined using a Luminex® Human Premixed Multi-Analyte kit (R&D Systems, Minneapolis, MN, USA) and the method was followed according to the manufacturers' instructions. Briefly, 50µL of standards of known concentration or sample, diluted two-fold, were added to appropriately designated wells of a microplate followed by the addition of 50 µL of microparticle cocktail. The microplate was sealed and incubated, protected from light, at room temperature for 2 hours with gentle agitation (800 rpm) on a horizontal orbital plate shaker (Thomas Scientific, Swedesboro, NJ, USA). Following the incubation step, the microplate was washed three times using an automated magnetic wash station (Bio-Rad Laboratories Inc., Hercules, CA, USA). Biotin-antibody (50 µL) was added to each well, the microplate was sealed and incubated for an additional 1 hour at room temperature with gentle agitation. Following the incubation period, the microplate

was washed three times as described above and streptavidin-phycoerythrin (50 µL) was added to each well. The microplate was again sealed and incubated for a further 30 minutes at room temperature as described above. The microplate was then washed a final three times using the automated magnetic wash station and the microparticles were resuspended in 100 µL of wash buffer followed by two minutes vigorous shaking using a Cooke AM69 microplate shaker (Dynatech AG, Bleichestrasse, ZUG, CH). The well contents were assayed using a Bio-Plex Suspension Array platform (BioRad Laboratories, Inc.). Bio-Plex Manager Software 6.0 was used for bead acquisition and analysis of median fluorescence intensity. Results are presented as picograms (pg)/mL.

Measurement of tumour necrosis factor receptor 1 levels

Concentrations of TNFR1 present in the plasma samples were determined using a Human TNFRSF1B (Tumour Necrosis Factor Receptor Superfamily, Member 1B) enzyme-linked immunosorbent assay (ELISA) kit (E-EL-H2436: Elabscience Biotechnology, Inc., Houston, TX, USA). The protocol was followed according to the manufacturer's instructions. Manufacturer-specified lower limits of detection (LOD) for each cytokine are provided in the product documentation. Levels of TNFR1 were determined as follows: the standards and appropriately diluted plasma samples (100 µ L) were added to the designated wells of a 96-well microplate. The microplate was sealed and incubated at 37 ^{o}C for 90 minutes. Following the incubation period, the plate contents were aspirated and 100 µL biotinylated detection antibody was immediately added to each well. The microplate was incubated at 37 ^{o}C for a further 60 minutes. The microplate was then washed three times using an automated plate washer (BioTek Instruments, Inc., Winooski, VT, USA) followed by the addition of 100 µL horseradish peroxidase conjugate to each well. The microplate was then sealed and incubated at 37 ^{o}C for 30 minutes followed by an additional five washes using the automated plate

washer as described above. Substrate reagent, 3,3 ′,5,5′-tetramethylbenzidine (100 µL), was then added to each well and the microplate was incubated, protected from light, at 37 ^{0}C for a further 15 minutes. The reaction was terminated by the addition of 50 µL stop solution and the optical density (OD) of each well was read using a PowerWaveX microplate spectrophotometer (BioTek Instruments, Inc.) set at a wavelength of 450 nm. The concentration of TNFR1 present in each sample was determined from the generated standard curve and the results are presented as pg/mL.

DATA MANAGEMENT AND ANALYSIS

Data were captured into a study-specific REDCap database and exported to Stata® (version 18.5, Stata Corporation, College Station, TX, USA) for analysis [23]. Categorical variables were summarised as frequencies and percentages while continuous variables were tested for normality using the Shapiro-Wilk test and visual methods. Normally distributed continuous variables were summarised using mean and standard deviation (SD), and non-normally distributed variables with medians and interquartile ranges (IQR). Due to significant deviations from normality observed for many of the cytokine variables, power or log transformation was attempted for TNFR1, IL-1β, IL-6, IL-8, IL-10, IL-17 and IL-1Ra. However only IL-Ra showed approximate normality ($p = 0.0569$). Therefore, non-parametric methods were primarily used for further analyses of these variables. Comparisons between survivors and non-survivors were conducted using T-tests or Mann-Whitney tests for continuous variables and Chi-square (X^2) tests for categorical variables.

Univariable linear regression examined the relationships between admission total cortisol levels and circulating pro-/anti-inflammatory cytokines as well as 28-day mortality. The associations

between total cortisol on admission and clinical severity scores (APACHE II, SAPS II and SOFA scores), as well as the primary (28-day mortality) and secondary outcomes (ICU length of stay), was examined using linear regression analysis. Regression coefficients (β) with 95% confidence intervals (CIs) were reported, and a *p*-value of <0.05 was considered statistically significant.

Categorical variables were further analysed using frequency tables, and their association with total cortisol levels and other key variables were tested using the Chi-squared test. The Chi-squared test was also used to determine significant associations between total cortisol levels and various outcome measures, including (i) APACHE II, SOFA, and SAPS II scores; (ii) circulating pro-inflammatory/anti-inflammatory cytokines; (iii) ICU length of stay, ventilatory support duration, and (iv) 28-day all-cause mortality. Additionally, Chi-squared tests were used to examine associations between biomarkers of inflammation and clinical outcomes, such as ICU length of stay, ventilatory support duration, and 28-day all-cause mortality.

Univariable and multivariable Poisson regression with robust error variance assessed the strength of association between 28-day mortality (outcome) and predictors, including pro- and anti-inflammatory markers and severity scores (APACHE II, SAPS II, SOFA), adjusting for covariates and confounders such as age, sex, and BMI. Collinearity among pro- and anti-inflammatory markers was assessed prior to regression modelling using Pearson's correlation coefficient and linear regression to mitigate multicollinearity. Mortality ratios (MRs), representing the relative increase in 28-day mortality risk per scaled increment for each variable, were calculated and presented with 95% confidence intervals. Several continuous variables were transformed to enhance interpretability and avoid overly precise ratios (100 nmol/L for total cortisol, 100 pg/mL for TNFR1 and 0.1 pg/mL for IL-1Ra). A stepwise backward elimination approach refined the final multivariable model. Variables with a *p*-value <0.2 in the univariable analysis were included

initially, and the model was refined iteratively to retain one variable per 10 outcomes based on statistical significance and model fit. Multivariable models were constructed using baseline (Day 1) values of candidate predictors.

RESULTS

Patients

A total of 45 patients with severe sepsis or septic shock were included in the study. The median age of the cohort was 49 years (IQR 38–62), with a slight male preponderance (53%). The most common sources of sepsis were abdominal (53%), soft tissue (27%), respiratory (9%) and urological (4.4 %). The median BMI was 27.4 kg/m² (IQR 23.7–33.6). On admission, the median APACHE II, SAPS II, and SOFA scores were 22 (IQR 15–28), 40 (IQR 31–53), and 9 (IQR 6–12), respectively. Of the 45 patients included in the study, 26 (58%) had septic shock on admission, 17 (38%) were classified as having severe sepsis, and 2 (4%) had insufficient data for classification. The median number of days on mechanical ventilation was 6 (IQR 3- 9), the median ICU length of stay was 7 days (IQR 4–9), 19 patients had as ICU stay longer than 7 days (42.2%) and the 28-day in-hospital mortality rate was 42.2%.

Sites of infection in the study population are described in **Table 1**. Microbiology results showed growth of micro-organisms in 26 patients identifying *Acinetobacter baumannii* (n=6), *Enterobacter cloacae* (n=5), *Klebsiella pneumoniae* (n=4), *Escherichia coli* (n =4), *Pseudomonas aeruginosa* (n=2), *Candida species* (n=2), *Staphylococcus aureus* (n=2) and *Proteus mirabilis* (n=1). Median total cortisol levels were 568 nmol/L (IQR: 335–1650) **(Table 1).**

Table 1: Clinical and laboratory characteristics of patients with sepsis or septic shock.

Demographic characteristics	
Age in years (median, IQR)	49 (38-62)
Male (n, %)	24 (53.3)
BMI in kg/m^2 (median, IQR)	27.4 (23.7- 33.6)
Site of sepsis	**n (%)**
Abdominal	24 (53.3)
Respiratory	4 (8.9)
Soft tissues	12 (26.7)
Urological	2 (4.4)
Gynaecological	1 (2.2)
Joint	1 (2.2)
CNS	1 (2.2)
APACHE II score (median, IQR)	22 (15-28)
SAPS II score (median, IQR)	40 (31-53)
SOFA score (median, IQR)	9 (6-12)
Total cortisol nmol/L (median, IQR)	568 (335-1650)

Abbreviations: IQR, interquartile range; BMI, body mass index; CNS, central nervous system; APACHE II, Acute Physiology and Chronic Health Evaluation II; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment. Bold values indicate statistically significant differences ($p < 0.05$).

Cytokine Profiles

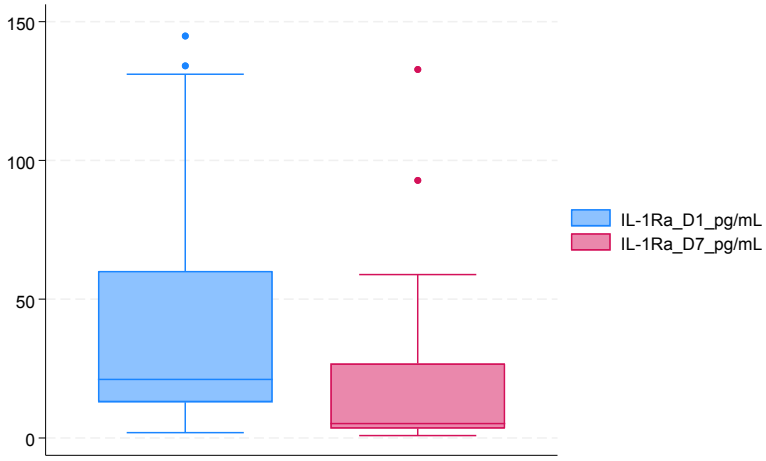
Paired data of circulating cytokines (day 1 and day 7) was available from 25 participants **(Table 2).** The reference cytokine levels, obtained from healthy controls, were as follows: TNFR1 were a median of 402.26 pg/mL (IQR: 331.64–411.37), and IL-1Ra a median of 0.565 pg/mL (IQR: 0.3675–0.725). Both IL-6 and IL-8 were at the lower detection limit, with medians of 0.01 pg/mL (IQR: 0.01–0.01) for each. IL-1β, IL-10, and IL-17, exhibited low detection rates, with fewer than 25% of samples having measurable levels above the assay's limit of detection (LOD). Specifically, IL-1β was detectable in 4.4% (2/45) of samples on day 1 and was undetectable in all samples on day 7. Similarly, IL-10 was detected in 22.2% (10/45) of samples on day 1 and 20.0% (5/25) on day 7, while IL-17 was detected in 11.1% (5/45) of samples on day 1 but undetectable in all day 7 samples. Given the limited number of detectable values for these cytokines, statistical analysis was not conducted. Interleukin-1Ra levels decreased significantly from a median of 21.09 pg/mL (IQR: 12.88-60.19) on day 1 to 5.19 pg/mL (IQR: 3.38-26.84) on day 7 ($p = 0.032$) **(Figure 1)**. Other

biomarkers, including, IL-6, IL-8 and TNFR1 did not exhibit statistically significant changes between day 1 and day 7 ($p > 0.05$ for all).

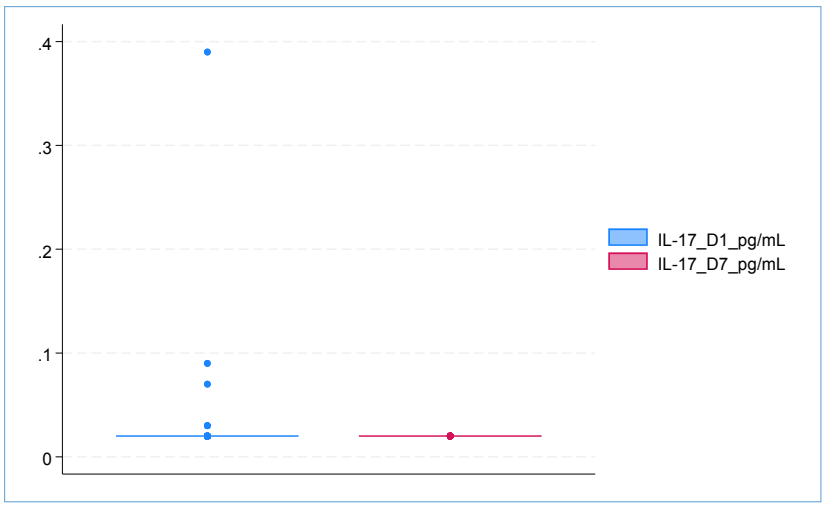
Table 2: Pro- and anti-inflammatory cytokine levels in patients with severe sepsis and septic shock at day 1 and day 7), N=25.

Cytokines*	**Day 1 levels (overall) (Median, IQR) N=45**	**Day 1 levels** (Median, IQR) N=25**	**Day 7 levels (Median, IQR) N=25**	**Wilcoxon Signed-rank p-value**
TNFR1 pg/mL	2050.4 (1292.1-4315)	2007 (1312.5- 3901.5)	1332.6 (951.8-3563.3)	0.149
IL-1Ra pg/mL	19.02(7.49-52.57)	21.09 (12.88 -60.19)	5.19 (3.38-26.84)	**0.032**
IL-6 pg/mL	0.09 (0.03-0.32)	0.14 (0.05-0.32)	0.03 (0.02-0.13)	0.112
IL-8 pg/mL	0.03 (0.01-0.09)	0.05 (0.03-0.09)	0.03 (0.01-0.11)	0.127

*all non-normally distributed; **among those with paired Day 7 results. Wilcoxon Signed-Rank Test: p-values reflect paired comparisons between Day 1 and Day 7 levels among patients with both measurements. Cytokine levels are reported in picograms per millilitre (pg/mL). Abbreviations: TNFR1 (tumour necrosis factor receptor 1), IL-6 (interleukin-6), IL-8 (interleukin-8), and IQR (interquartile range). Day 1 levels (overall) represent data from all available patients (N=45), while Day 1 levels for the subgroup (N=25) correspond to those with matched Day 7 data. Bold values indicate statistically significant differences ($p < 0.05$).



A) Interleukin-1Ra



B) Interleukin-17

Figure 1. Temporal changes in interleukin-1Ra (IL-1Ra) levels from day 1 (D1, (n = 45)) to day 7 (D7) of ICU admission in patients with severe sepsis and septic shock (n=25).

Association of cortisol levels with cytokines and clinical outcomes

Correlation analyses between cytokines and total cortisol

There was poor correlation between cortisol levels and cytokines.

Cortisol levels and their association with clinical outcomes

Total plasma cortisol levels on admission (day 1) were a median of 568 nmol/L (IQR: 335–1650). Total cortisol levels were significantly higher in non-survivors compared to survivors, with medians of 1207 nmol/L and 423.4 nmol/L, respectively (p=0.001) **(Table 3).** In univariate analysis, higher baseline cortisol levels were significantly associated with an increase in 28-day mortality ratio (**Table 4).**

Comparison of survivors and non-survivors

Table 3 presents a comparison of survivors and non-survivors in terms of demographics, sepsis sites, severity of illness scores, and admission total cortisol levels. A comparative analysis was conducted to evaluate differences between survivors and non-survivors across demographic and clinical variables at admission. No significant differences were found in age, sex, or BMI. However, non-survivors had significantly higher SAPS II scores ($p = 0.019$) and total cortisol levels ($p = 0.011$) compared to survivors. Non-survivors tended to have higher SOFA and APACHE II scores, although these did not reach statistical significance.

Table 3: Comparison of survivors and non-survivors among 45 patients with severe sepsis or septic shock.

Demographics	**Survivors (n=26)**	**Non-survivors (n=19)**	***p*-value**
Age in years (median, IQR)	46 (34-60)	55 (35-63)	0.499
Male (n,%)	13 (50)	11 (57.9)	0.600
BMI in kg/m^2 (median, IQR)	24.9 (23.5-31.6)	30.8 (24.4-37.6)	0.090
Site of sepsis (n, %)			
Abdominal	13 (50.0)	11 (57.9)	
Respiratory	3 (11.5)	1 (5.3)	
Soft tissues	6 (23.1)	6 (31.6)	
Urological	1 (3.9)	1 (5.3)	0.958
Gynaecological	1 (3.9)	0 (0)	
Joint	1 (3.9)	0 (0)	
CNS	1 (3.9)	0 (0)	
Severity of illness scores			
APACHE II score (Median, IQR)	20.5 (14-23)	27 (19-29)	0.137
SAPS II score (Median, IQR)	34 (28-49)	49 (36-55)	**0.019**
SOFA score (Median, IQR)	7 (4-11)	11 (7-14)	0.060
Total cortisol (Median, IQR)*	422.5 (328-1417)	1207 (396-3300)	**0.011**
Days on mechanical ventilation* (median, IQR)	5.5 (3-9)	6 (3-12)	0.851
Length of ICU stay in days* (median, IQR)	7 (5-9)	6 (3-12)	0.482

*non-normally distributed. Abbreviations: BMI, body mass index; CNS, central nervous system; APACHE II, Acute Physiology and Chronic Health Evaluation II; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment; IQR, interquartile range; ICU, intensive care unit. Total cortisol levels are reported in nanomoles per litre (nmol/L). Bold values indicate statistically significant differences ($p < 0.05$).

Multivariable regression analysis

A multivariable Poisson regression with robust error variance was conducted to assess baseline factors associated with 28-day mortality **(Table 4).** Unadjusted Poisson regression revealed that higher SAPSII and SOFA scores were significantly associated with increased 28-day mortality ratio. Elevated levels of total cortisol, day 1 TNFR1, IL-6, and IL-1Ra were also significantly associated with higher mortality ratios. The unadjusted MRs, scaled to clinically meaningful increments, indicated that higher total cortisol, Day 1 TNFR1, and IL-1Ra were significantly associated with increased 28-day mortality **(Table 4)**. These MRs reflect the relative increase in 28-day mortality risk per specified increment.

In the adjusted multivariable model, higher BMI and day 1 TNFR1, remained significant predictors of 28-day mortality. Other variables, including SOFA and SAPSII scores, IL-6 and IL-8, did not retain statistical significance after adjustment. The final model identified BMI and Day 1 TNFR1 levels as associated with a relative increase in mortality ratio **(Table 4)**.

Table 4: Baseline factors associated with 28-day mortality ratio (n = 45).

Variable	**Mortality ratio (95% CI)**	***p*-value**	**Adjusted mortality ratio (95% CI)**	***p*-value**
Age (per year)	1.0080 (0.9843-1.0322)	0.511		
Sex (Male vs Female‡)	0.8312 (0.4106-1.68)	0.607		
BMI (per kg/m^2)	1.0376 (0.9857 -1.0921)	0.158	1.0538 (1.0066-1.1032)$^{\gamma}$	**0.025**
APACHE II score (per score unit)	1.0240 (0.9861-1.0633)	0.218		
SAPS II score (per score unit)	1.0192 (1.0021-1.0364)	**0.027**		
SOFA score (per score unit)	1.0828 (1.0077-1.1635)	**0.030**		
Total cortisol (per 100 nmol/L increment)	1.0180 (1.005-1.031)	**0.007**		
Day 1- TNFR1 (per 100 pg/mL increment)	1.0231 (1.0141-1.0321)	**<0.001**	1.0265 (1.0157-1.0373)$^{\gamma}$	**<0.001**
D1 IL-6 (pg/mL)	1.0109 (1.0060 -1.0159)	**<0.001**		
D1 IL-1Ra (per 0.1 pg/mL increment)	1.0654 (1.0078 -1.1262)	**0.025**		
D1 IL-8 (pg/mL)	1.0187 (1.0113 -1.0261)	**<0.001**		

Adjusted mortality ratios (aMR) were calculated for BMI and Day 1 TNFR1 based on their significant associations in univariable analysis ($p < 0.05$). Variables without adjusted values were not included in multivariable models due to lack of statistical or clinical relevance.[γ] the last two variables in the final model. ‡Females serve as the reference group (MR = 1.00); the MR for males represents the relative mortality rate compared to females. Mortality ratios (MRs) for continuous variables represent the change in mortality risk per unit increase unless specified otherwise. Continuous variables are power-transformed to clinically relevant increments: 100 nmol/L for cortisol, 100 pg/mL for TNFR1 and 0.1 pg/mL for IL-1Ra.

Change in pro- and anti-inflammatory markers and association with 28-day mortality

Table 5 presents the percent change in pro- and anti-inflammatory markers from day 1 to day 7 and their association with 28-day mortality ratios (MRs). Median percent changes, crude MRs (cMR), 95% confidence intervals (CIs) and *p*-values are reported. IL-1Ra demonstrated a significant median reduction of 59.3% from day 7 to day 1 and was significantly associated with an increased mortality ratio. In contrast, TNFR1, and IL-8, were not significantly associated with increased mortality.

Table 5: Change in pro- and anti-inflammatory markers and association with 28-day mortality (n=25)

Marker	% change (D7- D1) Median (IQR)	Crude Mortality ratio (95% CI)	*p*-value
TNFR1 pg/mL	-10.6 (- 38.8- 18.6)	1.01 (0.99- 1.02)	0.244
IL-6 pg/mL	-75.5 (-89.3 – 14.3)	1.00 (1.00- 1.00)	0.690
IL-1Ra pg/mL	-59.3 (- 90.3- 7.9)	1.005 (1.003- 1.008)	**<0.001**
IL-8 pg/mL	-20 (-70.2- 0)	1.000 (0.999- 1.001)	0.915

Abbreviations: TNFR1, tumour necrosis factor receptor 1; IL-6, interleukin-6; IL-1Ra, interleukin-1 receptor antagonist; IL-8, interleukin-8; IQR, interquartile range; CI, confidence interval. Percent change (% change) from day 1 (D1) to day 7 (D7) is presented as the median with interquartile ranges. Bold p-values indicate statistically significant differences ($p < 0.05$). Cytokine levels are reported in picograms per millilitre (pg/mL).

DISCUSSION

The current study investigated the profiles of pro- and anti-inflammatory cytokines, TNFR1 and total cortisol levels in patients with severe sepsis and septic shock in a South African ICU setting whose characteristics (e.g infection sites) aligned with regional sepsis populations [24,25]. Intra-

abdominal sepsis was the most common source of infection [24,25]. The overall mortality rate of 42.2% whilst high is not surprising considering the admission severity of illness scores and is in keeping with the diagnosis of septic shock that was observed in the majority of the patients [26]. In Africa reported mortality rates vary for sepsis and septic shock and are estimated to be in the region of 30-47% taking into consideration the Sepsis-3 definition. The overall mortality rate in the ICU was 22%. Mortality in septic shock is reported to be 30% to 40% in high-income countries [27–29], whereas in low- and middle-income countries, rates often surpass 40% in sepsis and 50% in septic shock. In LMICs, mortality rates are often much higher, frequently exceeding 40% in sepsis and 50% in septic shock [29–31]. Higher mortality rates in LMICs may stem from patient demographics, comorbidities, delayed presentation, limited advanced care, and resource constraints. In this study, the COVID-19 pandemic likely impacted mortality by straining healthcare resources, reducing ICU capacity, and delaying patient presentation, and subsequently treatment, due to lockdowns and travel restrictions. Operational changes, including the redeployment of ICU staff to a neighbouring COVID-19 unit, contributed to bed shortages and fewer admissions during the study period.

Baseline TNFR1 emerged as an independent predictor of mortality. BMI was independently associated with mortality in our cohort. Prior studies in sepsis and critical illness have variably reported an ‘obesity paradox,’ in which moderately obese patients experience lower mortality. Meta-analyses support a U- or J-shaped association between BMI and mortality, although mechanisms remain uncertain and may relate to metabolic reserves, immune and inflammatory modulation, or pharmacokinetics of therapy. Our finding of increased mortality with higher BMI should therefore be interpreted with caution and may reflect population-specific factors, sample size limitations, or residual confounding. Conversely, IL-6 was significantly associated with increased mortality in the unadjusted analysis but did not retain statistical significance in the

adjusted model. IL-1Ra levels declined significantly between day 1 and day 7. While IL-1Ra was associated with increased 28-day mortality ratio in unadjusted analyses, this relationship also did not persist after adjustment. IL-1β, IL-10, and IL-17 were largely below the assay's detection limit, limiting their utility as prognostic markers in this cohort. In the univariate analysis, higher baseline cortisol levels were associated with an increase in 28-day mortality ratio but were not independently associated with an increased mortality ratio after adjustment. No correlations were observed between cortisol and cytokine levels.

As mentioned above, plasma levels of IL-17 and IL-10 were predominantly below the detection threshold of the assay at both day 1 and day 7, with only a few samples showing detectable levels. Similarly, IL-1β was detectable in only two samples at day 1 and was undetectable in all samples by day 7. IL-1β, which is produced predominantly by activated macrophages and monocytes, is a key mediator of the inflammatory response, initiating pro-inflammatory pathways and promoting the release of cytokines such as IL-6 and TNF-α [14,32]. Previous investigators have associated persistently high levels of IL-1β with ongoing inflammation and worse prognosis in patients with sepsis [33]. Leng and colleagues identified IL-1β as an independent risk factor for both 28-day and 90-day mortality in septic patients, with multivariable Cox regression analysis showing that elevated IL-1β significantly increased the hazard of mortality [33]. In contrast, IL-1β levels in the current cohort were predominantly below the detection threshold of the assay at both day 1 and day 7, limiting our ability to directly assess its role in this study. This discrepancy may reflect the cohort's immune response, sepsis severity or sensitivity limits of the assay.

IL-1Ra showed a significant median reduction from day 1 to day 7, with a significant association with an increased mortality ratio, suggesting that declining IL-1Ra may reflect a maladaptive anti-

inflammatory response. IL-1Ra inhibits the actions of IL-1α and IL-1β. In sepsis, IL-1Ra levels rise and elevated levels are associated with poor outcomes in critically ill patients [34,35]. In neutropenic patients, IL-1Ra has been proposed as a biomarker for predicting severe sepsis and septic shock. Intke and colleagues, found that IL-1Ra was able to predict severe sepsis earlier than CRP and PCT, showing a significant increase on day 1 post-onset of febrile neutropenia [34]. Fischer and colleagues observed that while IL-1Ra levels were markedly elevated in sepsis, its diagnostic performance was lower compared to IL-8 and granulocyte colony-stimulating factor (G-CSF) in distinguishing sepsis from other inflammatory conditions [35]. In the current study cohort, IL-1Ra showed a median reduction of 59.3% from day 1 to day 7 in survivors, suggesting a resolution of inflammation. Persistently elevated levels in non-survivors may indicate an ongoing inflammatory response emphasising the potential role of IL-1Ra in identifying high-risk patients [34,35]. The role of IL-1Ra as a distinguishing marker between sepsis and sterile inflammation has been previously demonstrated [36]. Our findings add prognostic relevance to IL-1Ra dynamics, suggesting that a sustained or insufficient decrease in IL-1Ra may reflect ongoing dysregulation in non-survivors.

While IL-6 and IL-8 are known to drive inflammatory cascades and multi-organ dysfunction, and despite observable fluctuations between day 1 and day 7, neither was significantly associated with an increased 28-day mortality ratio after adjustment. Elevated IL-6 and IL-8 levels have been consistently linked with increased mortality due to their roles in promoting multi-organ dysfunction [33].

A median reduction of 10.6% in TNFR1 levels was observed from day 1 to day 7. Despite this reduction, TNFR1 levels remained significantly associated with an increased 28-day mortality ratio in adjusted models, identifying TNFR1 as an independent predictor of increased mortality ratio.

Admission cortisol levels reflected the physiological stress response to sepsis. Cortisol, a key regulator of stress and inflammation, has been extensively studied for its role in modulating the immune response in sepsis [19,37–39]. Elevated cortisol levels have been associated with both adaptive and maladaptive responses in sepsis. Leng and colleagues identified distinct cortisol trajectories that were associated with significantly different outcomes in patients with sepsis, suggesting the need for individualised management strategies based on cortisol dynamics [33]. The current study supports this notion. In the current study, higher baseline cortisol levels were not significantly associated with an increased 28-day mortality ratio or ICU length of stay in the multivariable analysis. However, trends suggest that elevated cortisol levels may contribute to worse outcomes in some patients, supporting its role as a potential biomarker for risk stratification [33].

Despite these findings, this study has several limitations. The relatively small sample size may limit generalisability. Only patients who remained in the ICU for seven days had repeat biomarker measurements, potentially introducing selection bias. Excluding HIV-reactive patients further narrows the applicability of these findings to broader populations in sub-Saharan Africa, where HIV co-infection is prevalent. Low levels of IL-1β, IL-10, and IL-17 on both day 1 and day 7 in the majority of patients suggests limited expression or relevance in this cohort's sepsis response, as well as potential limitations due to assay detection sensitivity. While IL-1β is a well-established mediator of sepsis pathophysiology and has been identified as a predictor of poor prognosis in prior studies, its role could not be meaningfully characterized in this study due to limited detectability.

The short half-life of many cytokines, including IL-1β, may have influenced detectability. Cytokine levels can fluctuate rapidly, and the timing of sample collection relative to sepsis onset or therapy

may have influenced detectability. This limitation highlights the challenge of capturing the dynamic inflammatory response in sepsis using single time-point measurements.

An additional limitation is that treatment regimens were not standardised and could not be fully accounted for in the analysis. This reflects the pragmatic reality of ICU sepsis care, where therapies such as antibiotics, vasopressors, fluids, corticosteroids, and renal replacement therapy are individualised and change dynamically in response to patient physiology. Given the small sample size, it was not feasible to adjust for these treatment variables in regression analyses without risking overfitting. The high cost of cytokine assays, including multiplex assays, represents a significant constraint in resource-limited settings and may limit their widespread use, restricting the feasibility of routine cytokine profiling in clinical practice.

CONCLUSION

In this cohort, elevated baseline IL-6 and IL-1Ra levels were associated with mortality in univariable analyses, whereas BMI and Day 1 TNFR1 emerged as the only independent predictors in multivariable modelling. While cortisol may play a role in the stress response, its independent association with mortality was less clear. This is the first study to evaluate the trajectory of pro- and anti-inflammatory cytokines, together with baseline total cortisol levels, in a South African population with severe sepsis and septic shock. The role of biomarkers IL-6, TNFR1, and total cortisol in characterising disease severity, progression and outcomes highlights their potential as biomarkers for risk stratification in critically ill patients with severe sepsis and septic shock.

DECLARATIONS

Ethics approval and consent to participate: The study was approved by the Human Research Ethics Committees of the University of the Witwatersrand (M190888) and the University of

Pretoria (619/2019). Written informed consent was obtained from participants or their surrogate decision maker and the study was performed in accordance with the Declaration of Helsinki

Clinical trial number: Not applicable

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Availability of data and material: Some or all datasets generated during and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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CHAPTER 4: The total and free cortisol response to ACTH and Glucocorticoid Sensitivity in Patients with Traumatic Brain Injury

The Total and Free Cortisol Response to ACTH and Glucocorticoid Sensitivity in Patients with Traumatic Brain Injury

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Abstract:	OBJECTIVES To determine plasma free (PFC) and total cortisol (TC) levels in patients with traumatic brain injury (TBI) to ACTH stimulation and assess the relationship with glucocorticoid sensitivity (GS). DESIGN Prospective, multi-centre observational. SETTING Two level 1 trauma centers in Queensland, Australia. PATIENTS Twenty-nine patients with TBI and 48 healthy controls (24 males and 24 females). INTERVENTIONS ACTH stimulation diagnostic test. MEASUREMENTS AND MAIN RESULTS Basal and ACTH-stimulated TC, PFC (measured using measured using liquid chromatography-tandem mass spectrometry), and baseline corticosteroid binding globulin (CBG) levels were determined. GS was assessed using the dexamethasone suppression of cytokine production (DSCP) assay. PFC levels were significantly higher in TBI patients

	compared to controls, at baseline, 30 and 60 minutes (P<0.001). TC levels were elevated at all time points in patients with TBI; however, only significantly at 30 and 60 minutes (P<0.001). CBG levels were significantly lower in patients with TBI (median CBG 25 [IQR: 21–26] vs. 27 [IQR: 26–31], p = 0.001). GS, assessed via IL-6 and TNF-α suppression ratios, was lower in patients with TBI (IL-6: P=0.038; TNF-α: P=0.048). No association was observed between GS and cortisol response to ACTH. CONCLUSION PFC may be a more reliable marker of adrenal response in TBI while TC potentially underestimates early responses. Elevated GS in TBI and the lack of its association with the cortisol response suggests that these mechanisms may function independently in TBI. Despite limitations, these findings offer mechanistic insights and highlight the need for further research into GS testing in patients with TBI.

TITLE PAGE

TITLE

The total and free cortisol response to ACTH and Glucocorticoid Sensitivity in Patients with Traumatic Brain Injury

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N/A

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KEYWORDS

Adrenal insufficiency, Traumatic brain injury, Critical illness, Glucocorticoids, Corticosteroid insufficiency, Stress response.

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ABSTRACT

OBJECTIVES

To determine plasma free (PFC) and total cortisol (TC) levels in patients with traumatic brain injury (TBI) to ACTH stimulation and assess the relationship with glucocorticoid sensitivity (GS).

DESIGN

Prospective, multi-centre observational.

SETTING

Two level 1 trauma centres in Queensland, Australia.

PATIENTS

Twenty-nine patients with TBI and 48 healthy controls (24 males and 24 females).

INTERVENTIONS

ACTH stimulation diagnostic test.

MEASUREMENTS AND MAIN RESULTS

Basal and ACTH-stimulated TC, PFC (measured using measured using liquid chromatography-tandem mass spectrometry), and baseline corticosteroid binding globulin (CBG) levels were determined. GS was assessed using the dexamethasone suppression of cytokine production (DSCP) assay. PFC levels were significantly higher in TBI patients compared to controls, at baseline, 30 and 60 minutes (P<0.001). TC levels were elevated at all time points in patients with TBI; however, only significantly at 30 and 60 minutes (P<0.001). CBG levels were significantly lower in patients with TBI (median CBG 25 [IQR: 21–26] vs. 27 [IQR: 26–31], p = 0.001). GS, assessed via IL-6 and TNF-α suppression ratios, was lower in patients with TBI (IL-6: P=0.038; TNF-α: P=0.048). No association was observed between GS and cortisol response to ACTH.

CONCLUSION

PFC may be a more reliable marker of adrenal response in TBI while TC potentially underestimates early responses. Elevated GS in TBI and the lack of its association with the cortisol response suggests that these mechanisms may function independently in TBI. Despite limitations, these findings offer mechanistic insights and highlight the need for further research into glucocorticoid sensitivity testing in patients with TBI.

Keywords: Cortisol, Adrenal insufficiency, Traumatic brain injury, Glucocorticoid resistance

INTRODUCTION

Traumatic brain injury (TBI) carries a significant global socioeconomic impact and is a leading cause of mortality and prolonged disability, among young adults (1). Neuroendocrine dysfunction is a well-documented complication following TBI (2–4). The incidence of adrenal insufficiency in the

acute phase of TBI ranges widely from 25% to 100% (4). In critically ill trauma patients with an abnormal cortisol response to the short synacthen test, treatment with intravenous hydrocortisone has been demonstrated to reduce the incidence of ventilator-associated pneumonia significantly, with pronounced effects observed in patients with TBI (5). However, diagnosing adrenal dysfunction in TBI is challenging due to factors such as testing methodology and varying definitions of adrenal insufficiency (4). In particular, the use of total plasma cortisol concentrations in the short synacthen test may be unreliable in this population due to factors such as acute changes in binding protein levels (6–8). Previous investigators have shown a dissociation between total and free plasma cortisol concentrations in septic shock, with a greater relative increase in free cortisol levels in response to a short synacthen test. This difference leads to significant variability in the diagnosis of adrenal insufficiency depending on whether total or free cortisol criteria are used (8, 9).

While measurement of free cortisol maybe more reliable, there is limited data on the free cortisol response to corticotrophin in patients with TBI (4, 10). Available evidence suggests that corticosteroid binding globulin (CBG) levels are reduced in this population and hence dissociation between total and free plasma cortisol values is anticipated (8, 10–12). However, the measurement of free cortisol is not readily available outside of specialised centres. The alternative, the estimation of the calculated free fraction by means of the Coolens' equation, has been shown to be unreliable in the critically ill (13). To date, however, the only published prospective data examining free cortisol in TBI patients comes from two investigations, using the Coolens' equation (10, 11).

Glucocorticoid sensitivity varies significantly among critically ill patients and is closely associated with disease severity (14). Such variability is driven by factors such as alterations in glucocorticoid receptor function and the influence of inflammatory cytokines (14, 15).

While these dynamics have been studied in septic shock, there is a notable lack of comparative data on glucocorticoid sensitivity in patients with TBI. A recent study by Lotsios and colleagues provided insight into this area by examining glucocorticoid receptor alpha (GRα) expression and its downstream target glucocorticoid-inducible leucine zipper (GILZ) in ICU patients with various brain injuries, including TBI, revealing differential temporal expression profiles when compared to healthy controls (16). Nevertheless, functional assessments such as the dexamethasone suppression of cytokine production (DSCP) test, which evaluate glucocorticoid receptor activity more directly, remain under-explored in this population. The dexamethasone suppression of cytokine production (DSCP) test, though promising for evaluating glucocorticoid receptor function, has limited validation in critically ill populations beyond specific genetic contexts, such as mutations in the NR3C1 gene (17). The current study, therefore, aimed to evaluate the dynamics of total and free cortisol in patients with TBI during the acute phase of injury (initial 72 hours) using the ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method of free cortisol assessment, the currently accepted gold standard of free cortisol measurement, and to explore the relationship between the total and free cortisol response to ACTH and glucocorticoid sensitivity as assessed by the DSCP test (17, 18).

MATERIALS AND METHODS

Study design and participants

This prospective, multi-centre observational study was conducted from 2017 to 2020 at The Royal Brisbane and Women's (RBWH) and Gold Coast University Hospitals (Queensland Health) and was approved by the Metro-North Human Research Ethics Committee (HREC/14/QRBW/7), with written informed consent obtained from all participants prior to inclusion. Demographic data (age, sex, body mass index (BMI)), and clinical scores (Injury Severity Score (ISS), Acute Physiology and Chronic Health Evaluation II (APACHE II), Simplified Acute Physiology II (SAPS II) and Sequential Organ Failure Assessment (SOFA) score) and clinical details (date and time of injury, presence or absence of a skull base fracture on imaging, documented pre-hospitalisation hypotension (systolic blood pressure <90 mmHg), and hypoxia (SpO_2 <90%), the Glasgow Coma Scale (GCS) score at the scene of injury) were recorded. On the day of sampling, clinical and laboratory data collected included blood biochemistry results, including serum sodium level, presence or absence of an intracranial pressure (ICP) monitor, initial ICP reading, mean arterial pressure (MAP), cerebral perfusion pressure (CPP), and length of stay (LOS) in the intensive care unit (ICU). Follow-up assessments included 28-day mortality, 6-month mortality and Glasgow Outcome Score (GOS) at 6 months, which was obtained via phone call where necessary.

Study procedures

Participants were eligible for inclusion if they were aged 18 years or older, had sustained a severe TBI (defined as a Glasgow Coma Scale [GCS] score of 8 or less), and had evidence of brain injury confirmed by a computed tomographic (CT) scan within the previous 72 hours. Exclusion criteria included being under 18 years of age, the presence of bilateral fixed dilated pupils, recent treatment with long-term glucocorticoids within the past 6 months or short-term glucocorticoids within the last 4 weeks, documented hypoadrenalism, or a prognosis of imminent or inevitable

death with no commitment to active treatment. Patients expected to die from underlying disease within 90 days, or those meeting all inclusion criteria more than 72 hours prior were excluded. All participants had an indwelling arterial line as part of routine care, and samples were collected by qualified staff. The control group consisted of 48 healthy volunteers aged 18 years or older. Control participants were recruited via newsletters from RBWH and the University of Queensland, and through social media outreach. Exclusion criteria included age under 18 years, a documented diagnosis of hypoadrenalism, or recent glucocorticoid use (defined as oral long-term use within the preceding six months or short-term use within the last four weeks). No participants were on corticosteroids, individuals with stable chronic conditions (eg asthma, depression, essential hypertension), were eligible. Upon study entry, participants underwent a medical assessment, which included collection of demographic data (age, sex, body mass index (BMI)), medical history and a physical examination.

Total and free cortisol measurements

The standard short synacthen test (250 µg Synacthen, Novartis, NSW, Australia) was conducted in the ICU between 7:30 and 10:00 am. Blood samples were drawn at baseline, and at 30- and 60-minutes following adrenocorticotrophic hormone (Synacthen, ACTH) administration, processed for serum and ultra-filtrate and stored at -20°C until analysis. Assays included measurements of plasma total cortisol and plasma free cortisol at all time points (baseline, 30- and 60-minutes, following ACTH administration), and baseline cortisol-binding globulin levels. Plasma total cortisol and plasma free cortisol concentrations were measured using UHPLC-MS/MS. Where ACTH testing was declined by the surrogate decision maker, patients were invited to contribute baseline samples.

Glucocorticoid sensitivity assessment

Glucocorticoid sensitivity was assessed in a subset of patients using the dexamethasone suppression of cytokine production (DSCP) test (17). Due to logistical constraints, including site-specific availability, specialised laboratory staff availability and the test's time-sensitivity (requiring processing between 8:00 and 10:00 AM), DSCP testing was feasible for participants recruited at the site where the laboratory was situated (RBWH). Glucocorticoid sensitivity analysis was thus conducted on all healthy participants and 13 patients with TBI. This assay evaluates the ability of exogenous dexamethasone to suppress lipopolysaccharide (LPS)-stimulated monocyte production of interleukin (IL)-6 and tumour necrosis factor-α (TNF-α), by measuring the *in vitro* suppression of production of these pro-inflammatory cytokines (IL-6 and TNF-α) by lipopolysaccharide (LPS)-stimulated leukocytes following dexamethasone administration.

Lithium heparinised whole blood (1 mL) was separated into three aliquots and incubated in wells containing buffer (baseline), LPS with dexamethasone (LPS+Dex) or LPS without dexamethasone (LPS) to a final dexamethasone concentrations of 1000 nmol/L. After a 3-hour incubation, at 37°C the samples were centrifuged, to separate the plasma and the plasma was stored at –20°C until analysis on a Luminex analyser for IL-6 and TNF-α (17). TNF-α and IL-6 production by monocytes was measured in the supernatants (ng/L). The buffer portion served as the baseline, LPS represented maximum cytokine stimulation, and LPS + Dex indicated glucocorticoid suppression. Results are expressed as a ratio.

Glucocorticoid sensitivity was calculated as the ratio of cytokine concentrations (IL-6 and TNF-α) in the LPS + Dex condition relative to the LPS-only condition (i.e., LPS+Dex / LPS). A lower ratio reflects increased sensitivity (greater cytokine suppression by dexamethasone), while a higher

ratio reflects reduced sensitivity (17). Although baseline cytokine production (buffer only) is measured, it is not included in the ratio calculation; and serves to confirm minimal spontaneous cytokine release and validate the assay conditions. Based on previously established reference ranges, the 95% reference intervals for normal glucocorticoid sensitivity are 0.244–0.472 for IL-6 and 0.372–0.693 for TNF-α. Values below these intervals indicate increased glucocorticoid sensitivity, whereas values above indicate reduced sensitivity (14, 17). The DSCP assay is an *in vitro* test, distinct from the standard dexamethasone suppression test used for Cushing's syndrome (17). and demonstrates superior sensitivity in detecting tissue glucocorticoid responsiveness compared to the thymidine assay (17)**.** The test's sensitivity and specificity have been validated in contexts involving genetic variations in glucocorticoid receptors, and it may be influenced by cytokine variability, sample timing, and delayed processing (17). The DSCP test has limited validation in critical illness and its applicability in TBI has not been previously explored.

DATA ANALYSIS

Statistical Methods

Based on an anticipated incidence of adrenal insufficiency (AI) of 76% in patients with TBI (5), a sample size of 50 patients was estimated to provide 99% power to detect a 30% diagnostic discrepancy between total and free cortisol criteria. This estimate was informed by a prior study of 43 patients with stable severe liver disease, which observed such a discrepancy (9). However, only 29 patients with TBI were ultimately recruited due to enrolment challenges and logistical constraints. Outcome measures of interest were total cortisol, plasma free cortisol and IL-6 and TNF-α, ratios. Because ratio measures were only available for 13 patients with TBI, differences in

patient characteristics for those with/without ratio measures were tested to ensure they were not systematically different. Patient characteristics and measures of interest were compared between groups and cohorts. Continuous variables were summarised as median (IQR) and differences between groups were tested using Wilcoxon's rank sum test. Categorical variables were summarised as frequency (percentage) and compared between groups using Pearson's chi-square test or Fisher's exact test where more than 20% of cells had fewer than five observations. Generalised linear mixed effects models with a log-link and patient-level random intercepts were fitted to assess the total cortisol and plasma free cortisol responses to ACTH stimulation. Age was centred, and time (baseline, 30- and 60-minutes) was fitted as a categorical variable. Model-based predictions were obtained for each time-point. The effects of cohort (TBI vs normal) and patient characteristics (age, sex, BMI) were tested in these models. Peak total and plasma-free cortisol values and changes from baseline were derived with missing values replaced by model-based predictions. Statistical analyses were conducted using Stata software, version 18 (StataCorp, College Station, TX, USA) (19).

RESULTS

Participant characteristics

The TBI cohort comprised 29 patients (76% male) with a median (IQR) age of 45 (28-62) years **(Table 1)**. There was a large percentage of missing/incomplete data for some variables. Although IL-6 and TNF-α dexamethasone ratio measures were only available for 13 patients with TBI **(Figure 1)**, baseline patient characteristics and serum total cortisol and plasma free cortisol measures did not differ significantly irrespective of whether or not these ratio measures were available.

The age and sex distributions differed significantly by cohort **(Table 2)**. The control cohort was on average younger (median (IQR) age 33 (27-41) compared to 45 (28-62) years (P=0.042)) with a higher percentage of females (50% vs 24%; P=0.025) compared to the TBI cohort. Mechanism of injury was available for 20 of the 29 patients with TBI. These included motor vehicle accidents (n=7), falls (n=5), recreational incidents (n=4), and pedestrian injuries (n=4). Mechanism of injury could not be determined for 9 patients due to insufficient injury documentation. At each of the three measured time points, plasma free cortisol (FC) was significantly higher in the TBI group than in controls ($p < 0.05$). For total cortisol, levels were significantly higher in the TBI group at 30- and 60-minutes compared to the control cohort. For the subset with glucocorticoid sensitivity test results (n=13 TBI and 45 healthy control), ratio measures were significantly lower in the TBI cohort compared to the control cohort. Based on the published reference intervals, 9/13 (69%) TBI patients were classified as having low IL-6 ratios and 11/13 (85%) had low TNF-α ratios. However, of 45 control patients, 36% (IL-6) (P=0.11) and 67% (TNF-α) (P=0.47) had low ratios. There was considerable variation in glucocorticoid sensitivity ratio measures in both groups, with estimated coefficients of variation of 55% (control) and 79% (TBI) for IL-6 and 47% (control) and 51% (TBI) for TNF-α. Box plots comparing cortisol responses between the control and TBI cohort are shown in **Figure 2** and glucocorticoid sensitivity ratio measures are shown in **Figure 3**.

Results of regression modelling are presented in **Table 3** and **Figure 4**. Age was associated with both total and plasma free cortisol levels in the TBI cohort but, not in the control cohort. There was a significant difference in plasma free cortisol levels between the TBI and the control group and a significant interaction between cohort and time was observed, which persisted after adjusting for age. For a patient of average age (40 years), baseline free cortisol levels were an

estimated average 5.2 (95%CI: 3.0-9.0; P<0.001) times higher in TBI patients compared to control patients. After adjusting for age, no significant association was observed between cohort and total cortisol levels.

DISCUSSION

This study assessed the total and free cortisol response to ACTH administration, and glucocorticoid sensitivity in patients with TBI within 72 hours of injury. Plasma free cortisol levels were significantly elevated in patients with TBI compared to controls at all time points while total cortisol levels were higher at all time points in patients with TBI; however, only significantly elevated at 30- and 60-minutes post-stimulation. After age adjustment, no significant difference in total cortisol levels remained between patients with TBI and controls (Table 3, and Figure 3). Glucocorticoid sensitivity, demonstrated by significantly lower ratio measures of IL-6 and TNF-α, was increased in patients with TBI. However, substantial variability in values was observed in both groups. Our findings align with prior research in septic shock, where similar median IL-6 and TNF-α suppression ratios were reported (14), suggesting common sensitivity profiles, though further validation is necessary given the variability in values and the limited sample size.

No association was observed between glucocorticoid sensitivity and cortisol response to ACTH administration. Data on glucocorticoid sensitivity in patients with TBI is lacking and data on free cortisol levels in patients with TBI is extremely limited. This study's is the first to use UHPLC-MS/MS for free cortisol measurement in this context.

TBI is a recognised cause of secondary adrenal insufficiency; however, its biochemical diagnosis remains challenging (4, 20, 21). This study is one of three prospective investigations assessing and comparing free and total cortisol measurements in patients with TBI (10, 11). Consistent with previous investigations, free cortisol levels showed marked variation while total plasma cortisol increments remained nearly identical across healthy and critically-ill patients, suggesting that free cortisol may provide greater accuracy (6, 21).

Kusmenkov and colleagues, reported lower total and free cortisol levels (and increased CBG) in patients with TBI without significant increase within the first 24 hours post-injury (10). In contrast. our study demonstrated significantly elevated free cortisol levels, and decreased CBG, in patients with TBI at all time points following ACTH stimulation, with total cortisol elevated only at 30- and 60- minutes. This disparity reflects methodological differences, as UHPLC-MS/MS, used in this study, provides greater sensitivity for free cortisol detection (22).

Our CBG findings align with other critically ill populations, indicating significantly lower levels in patients with TBI compared to controls ($p = 0.001$) (12). This supports concerns about Coolen's equation in the assessment of free cortisol, which assumes normal albumin levels and does not account for altered binding affinities or abnormal binding proteins commonly observed in critical illness (23).

Savaridas and colleagues similarly observed elevated free cortisol and normal total cortisol due to reduced CBG in acute brain injury, suggesting higher cortisol bioavailability despite normal total cortisol levels, and that total cortisol may underestimate adrenal function in this population (11).

Altered CBG likely explains the observed dissociation between total and free cortisol in TBI, reflecting stress-induced cortisol elevation (12). Our findings support previous work showing elevated free cortisol in critical illness and variability in total cortisol responses (8, 20, 21).

A novel and key finding was enhanced glucocorticoid sensitivity in patients with TBI, contrasting with glucocorticoid resistance typically seen in critical illness (24, 25). This suggests pathophysiological differences between TBI and conditions such as sepsis. Enhanced glucocorticoid sensitivity in patients with TBI is likely influenced by receptor dynamics, inflammatory cytokines, and patient-specific factors and might reflect a compensatory mechanism aimed at mitigating inflammation following brain injury. (15, 26). This finding highlights the complexity of the neuroendocrine stress response in TBI and that glucocorticoid sensitivity can vary depending on disease pathophysiology. While TBI triggers a prolonged focal neuro-inflammatory response characterised by blood-brain barrier disruption and glial cell activation (27–29), sepsis triggers a systemic cytokine storm (27, 30, 31).

Variability in glucocorticoid sensitivity is influenced by complex genetic and environmental factors (20). In this study, patients with TBI demonstrated increased glucocorticoid sensitivity with less inter-individual variability compared to controls, in contrast to the substantial variability and resistance reported in septic shock populations (14, 25). This lower variability supports the notion that uniform corticosteroid strategies may be inappropriate in critical illness (15, 32).

Notably, the CRASH trial demonstrated increased mortality in TBI patients who received high-dose methylprednisolone within 8 hours of injury, leading to broad caution regarding corticosteroid use in this population (33, 34), However, our finding of elevated free cortisol and altered glucocorticoid sensitivity may offer mechanistic insights into these adverse outcomes, suggesting that endogenous cortisol dynamics play a significant role.

In contrast, a multi-centre randomised controlled trial by Roquilly and colleagues demonstrated that physiological doses of intravenous hydrocortisone significantly reduced the incidence of

hospital-acquired pneumonia in critically ill trauma patients with an abnormal cortisol response to ACTH (Δ cortisol <250 nmol/L) (5). The reduction in pneumonia was pronounced in the TBI subgroup suggesting that modulating the glucocorticoid response in TBI patients may have ~~important~~ clinical implications, including reduced infection risk and improved ICU outcomes (35). Of interest, the CRASH trial demonstrated increased mortality in TBI patients who received high-dose methylprednisolone within 8 hours of injury (33). These divergent outcomes highlight that both dose and patient selection are critical in determining whether glucocorticoid therapy is harmful or beneficial following TBI.

This study's strengths, include the novel use of UHPLC-MS/MS and the use of a healthy control group. The study did not reach its intended sample size of 50 patients, limiting power to detect some differences and increasing the potential for variability in observed outcomes. Nevertheless, this remains larger than prior prospective studies of free cortisol in patients with TBI (10, 11), Variability in IL-6 and TNF-α suppression ratios limits generalisability, though the data remain hypothesis-generating.

Sex differences in TBI are an important consideration in this study (36). We acknowledge the 76% male representation, reflecting the epidemiology of TBI. Although this limits generalisability, it mirrors real-world trauma patterns (36). Sex and gender differences represent an important consideration in interpreting these findings (36–38). Oestrogen and testosterone have been shown to modulate the hypothalamic–pituitary–adrenal (HPA) axis and cytokine responses, potentially influencing both cortisol levels and glucocorticoid sensitivity. Oestrogen has been demonstrated to dampen cortisol's suppressive effect on inflammatory cytokines, and sex-based variation in glucocorticoid receptor expression has been observed (39).

Nonetheless, prior studies in critically ill populations have shown that sex does not significantly affect cortisol response to ACTH or baseline free cortisol levels, reducing the likelihood of systematic bias from sex imbalance in this study. Roelfsema and colleagues demonstrated that although ACTH secretion is higher in males than females, cortisol secretion rates did not significantly differ between sexes, indicating equivalent adrenal output despite varying ACTH drive (40, 41). Similarly, Waltman and colleagues found no significant differences in basal or stimulated ACTH and cortisol secretion, or in glucocorticoid feedback sensitivity, between young and older adults (42). These findings suggest a physiological compensation in the HPA axis that maintains stable cortisol levels across sex and age groups.

Glucocorticoid sensitivity testing was limited to one site (RBWH) due to its time-sensitive nature and laboratory constraints (requiring collection and analysis between 8:00 and 10:00 AM), resulting in subset analysis. Although no differences were found between those with glucocorticoid sensitivity measures and those without, representativeness may be affected. Missing data and logistical constraints further highlight the need for validation in larger studies. Patients with TBI had higher cortisol concentrations and altered glucocorticoid sensitivity compared with controls. We also noted considerable variance in glucocorticoid sensitivity ratios within both the control and TBI groups. Such variability has been described previously and is thought to reflect inter-individual differences in receptor expression and signalling as well as the influence of the cytokine milieu (14).

This study did not systematically evaluate hypothalamic or pituitary injury. While basal skull fractures, identified in four patients (**Table 1)**, may suggest potential damage to the hypothalamic–pituitary axis, the low frequency of this finding precludes meaningful subgroup analysis.

CONCLUSION

This study contributes to growing evidence that plasma free cortisol may be a more reliable marker of adrenal function in patients with TBI, while total cortisol may fail to capture early adrenal responses. Enhanced glucocorticoid sensitivity, contrary to typical critical illness patterns, and its independence from ACTH response suggest unique endocrine dynamics in TBI. Despite limitations, these findings offer mechanistic insights and highlight the need for further research into glucocorticoid sensitivity testing in this population.

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Table 1: Summary statistics for the cohort with traumatic brain injury. Continuous variables are presented as median (IQR); categorical variables as n (%).

Variable	N*		Summary measure (median, IQR)
Age	29		45 (28-62)
Sex (n(%))		Female	7 (24%)
		Male	22 (76%)
BMI	20		26 (23-29)
ISS on admission	19		25 (20-29)
Base of skull fracture (n(%))	19	No	15 (79%)
		Yes	4 (21%)
APACHE II	22		20 (19-24)
SAPS II	18		38 (31-45)
SOFA Score	20		4 (2-6)
Sodium level at enrolment	26		140 (138-145)
ICP Monitor in situ (n(%))	23	No	8 (35%)
		Yes	15 (65%)
CPP (mmHg)	14		68 (61-83)
ICP (mmHg)	15		9 (6-10)
Pre-hospital hypotension (n(%))	21	No	17 (81%)
		Yes	4 (19%)
Pre-hospital hypoxia (n(%))	21	No	18 (86%)
		Yes	3 (14%)
Glasgow coma scale			
GCS Initial Motor score	18		1 (1-5)
GCS Initial Total score	22		5 (3-8)
GCS Admission Motor score	19		1 (1-4)
GCS Admission Total score	19		3 (3-7)
GCS Enrolment Motor score	22		1 (1-5)
GCS Enrolment Total score	22		6 (3-8)
Days on mechanical ventilation	21		8.0 (3.0-12.0)
Glasgow outcome scale	29		4 (3-4)
28 day mortality (n(%))	29		5 (17%)
6 months mortality[c]	29	No	24 (83%)
		Yes	5 (17%)

*Number of available (non-missing) observations.
ISS, injury severity score; APACHE, acute physiology age and chronic health evaluation; SAPS, simplified acute physiology s*core;* SOFA, sequential organ failure assessment; GCS, Glasgow coma scale; ICP, intracranial pressure; CPP, cerebral perfusion pressure.

Table 2: Comparison of summary statistics for patient characteristics and cortisol measures by cohort

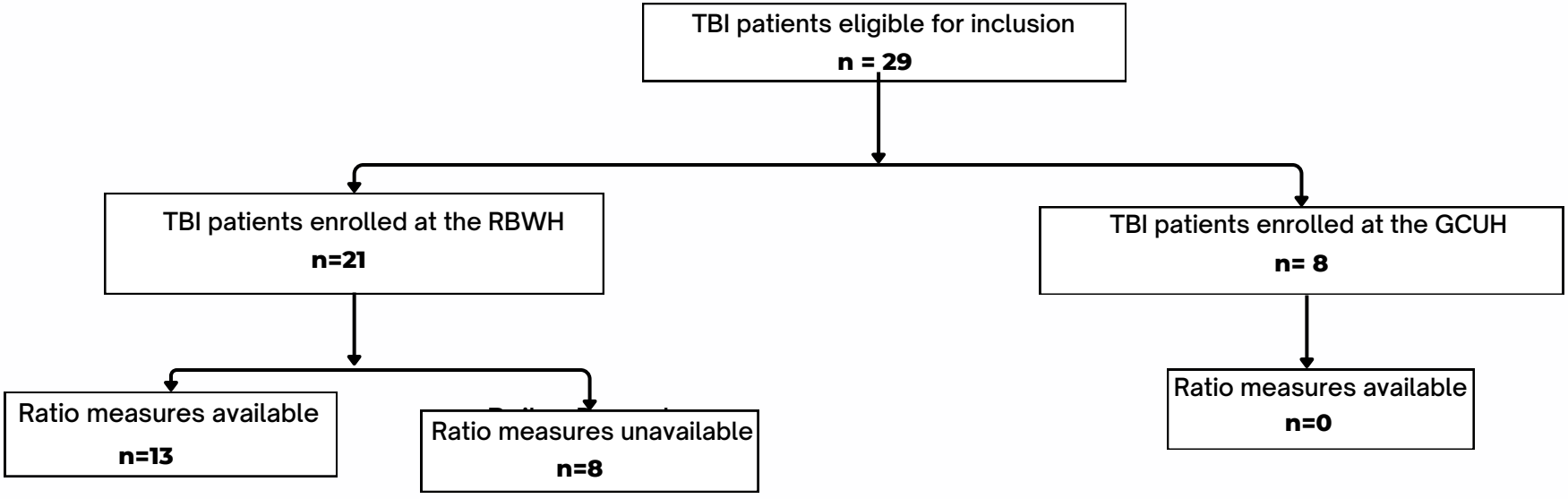
Variable	N*		Control N=48	TBI N=29	Total N=77	*p*-value
Age[a] (years)	77		33 (27-41)	45 (28-62)	33 (27-50)	0.042
Sex[b]	77	Female	24 (50%)	7 (24%)	31 (40%)	0.025
		Male	24 (50%)	22 (76%)	46 (60%)	
Body mass index[a] kg/m²	68		24 (22-26)	26 (23-29)	25 (22-27)	0.095
Plasma free cortisol[a] (nmol/L)						
Baseline	75		10 (6-17)	44 (10-74)	12 (7-30)	<0.001
30 minutes	66		46 (37-52)	149 (99-180)	53 (40-111)	<0.001
60 minutes	69		62 (53-72)	179 (146-218)	71 (55-147)	<0.001
Peak[c]	77		62 (54-70)	175 (116-211)	71 (59-133)	<0.001
Change from baseline[c]	77		49 (42-62)	107 (89-126)	59 (45-102)	<0.001
Total cortisol[a] (nmol/L**)**						
Baseline	77		306 (226-385)	437 (224-565)	352 (225-455)	0.064
30 minutes	73		541 (493-578)	722 (621-855)	565 (497-727)	<0.001
60 minutes	74		622 (550-747)	820 (667-972)	652 (568-842)	0.003
Peak[c]	**77**		622 (557-747)	789 (667-935)	659 (568-842)	0.007
Change from baseline[c]	**77**		306 (233-398)	372 (286-440)	327 (246-406)	0.20
CBG[a] (mg/L)	**61**		27 (26-31)	25 (21-26)	27 (25-30)	0.001
Ratio_Dex_IL-6av[a]	58		0.31 (0.18-0.40)	0.18 (0.14-0.26)	0.28 (0.18-0.38)	0.038
Ratio_Dex_TNF-α av[a]	58		0.33 (0.26-0.43)	0.25 (0.15-0.33)	0.32 (0.23-0.42)	0.048

[a] Median (IQR) with *p*-value derived from Wilcoxon's rank rum test; (N (%)) with *p*-value from [b]Pearson's chi-square test; [c]Missing values replaced with model-based predicted values**;** [*]Number of non-missing observations. Peak: highest value among baseline, 30 min, and 60 min. Change from baseline: peak value minus baseline value.

Table 3: Results of regression modelling of cortisol response to Synacthen stimulation testing

Outcome measure	Variable	Category	RR[a] (95% CI)	*p*-value
Plasma free cortisol				
	Time (age age=40 & cohort=normal)			<0.001
		Baseline	Reference	
		30 minutes	4.0 (2.4-6.9)	<0.001
		60 minutes	5.6 (3.3—9.4)	<0.001
	Cohort (at baseline & age=40)			
		Normal	Reference	
		TBI	5.2 (3.0-9.0)	<0.001
	Age	(per 10 years)	1.15 (1.08-1.21	<0.001
	Interaction	(Cohort*time)		0.002
		30 mins*TBI	0.48 (0.27-0.82)	0.008
		60 mins*TBI	0.41 (0.24-0.70)	0.001
Total cortisol				
	Time	Baseline	Reference	
		30 minutes	1.6 (1.5-1.7)	<0.001
		60 minutes	1.8 (1.6-1.9)	<0.001
	Cohort	Normal	Reference	
		TBI	1.1 (1.0-1.3)	0.15
	Age	(per 10 years)	1.08 (1.03-1.14	0.001

[a] Estimates derived from generalised linear mixed effects models fitted with a log link and patient-level random intercept. Time and cohort represent fixed effects. Interaction terms (Cohort*time) assess whether the cortisol response over time differs between TBI and control groups. 30 mins*TBI and 60 mins*TBI represent the estimated difference in cortisol levels at those time points for patients with TBI, compared to baseline and to controls. *Denotes interaction term; RR, relative risk; TBI, traumatic brain injury.


TBI patients eligible for inclusion
n = 29
TBI patients enrolled at the RBWH
n=21
TBI patients enrolled at the GCUH
n= 8
Ratio measures available
n=13
Ratio measures unavailable
n=8
Ratio measures available
n=0

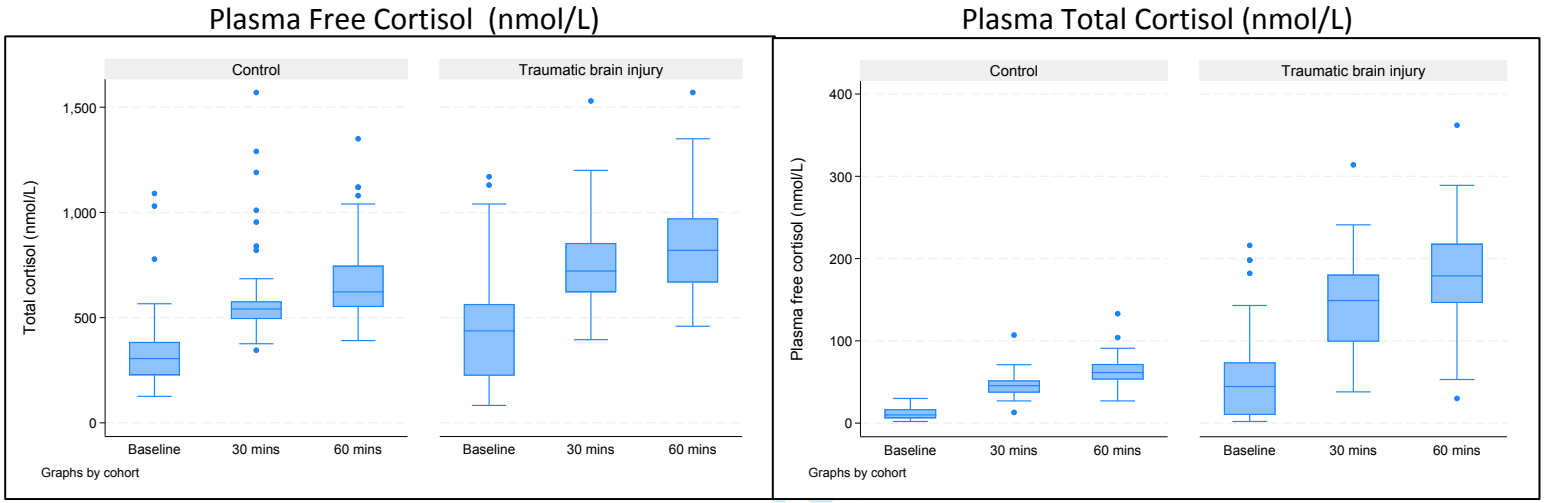


Figure 2: Box plots of plasma free cortisol and total cortisol levels at baseline, 30 minutes, and 60 minutes post-ACTH stimulation in patients with traumatic brain injury (TBI) and healthy controls. Plasma free cortisol levels were significantly higher in TBI patients than controls at all timepoints (baseline, 30 minutes, and 60 minutes post-ACTH) (all $p < 0.001$). Total cortisol levels were significantly elevated in TBI patients at 30 and 60 minutes ($p < 0.001$ and $p = 0.003$), with no significant difference at baseline ($p = 0.064$).

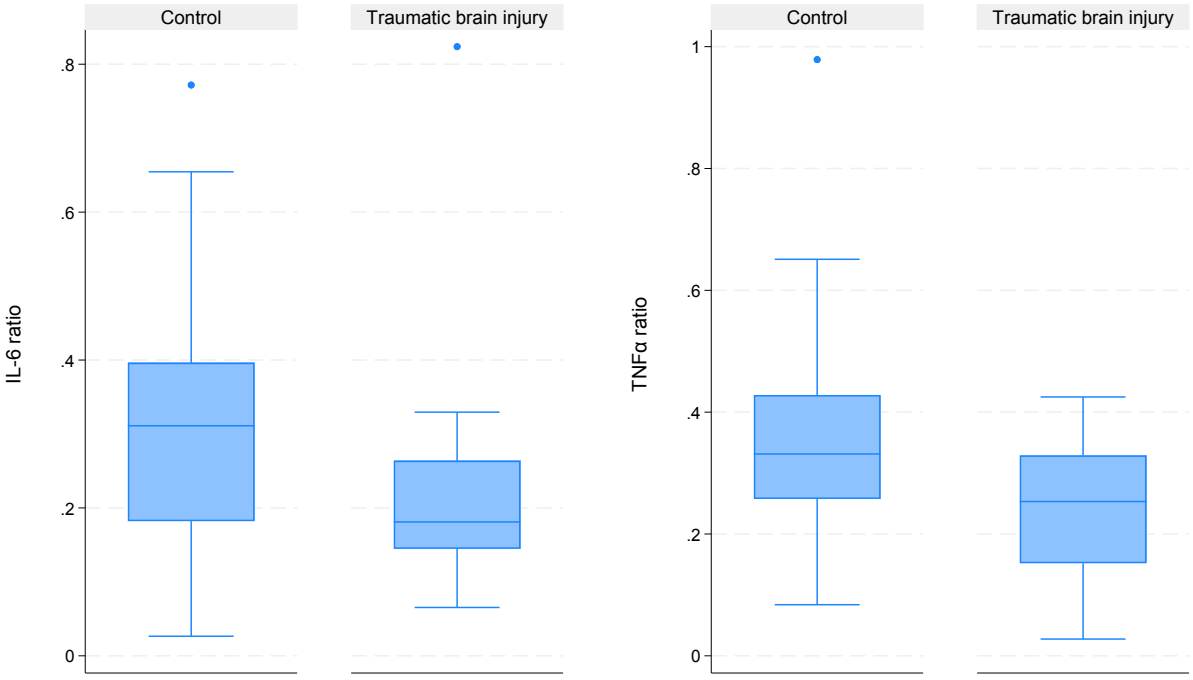
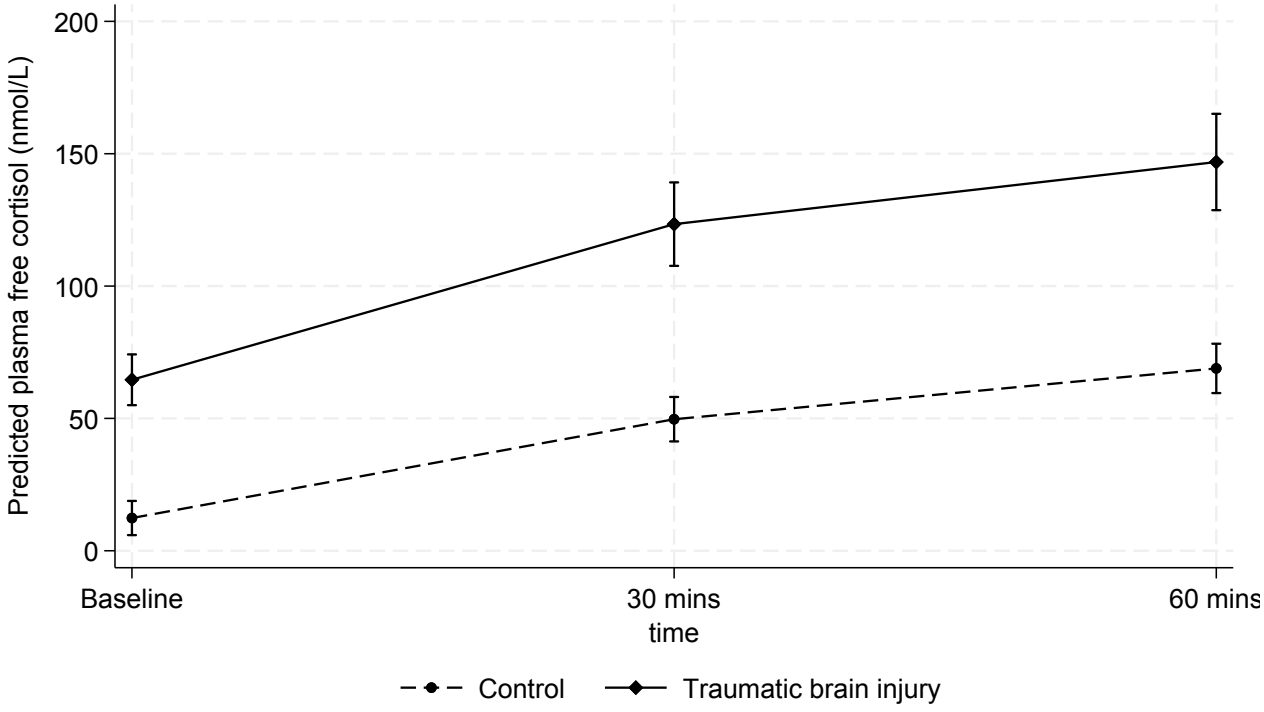


Figure 3: Box plots showing distribution of glucocorticoid sensitivity ratio measures by cohort. IL-6 and TNF-α suppression ratios were significantly lower in TBI patients compared to controls (IL-6: p = 0.038; TNF-α: p = 0.048), indicating differences in glucocorticoid sensitivity between groups.



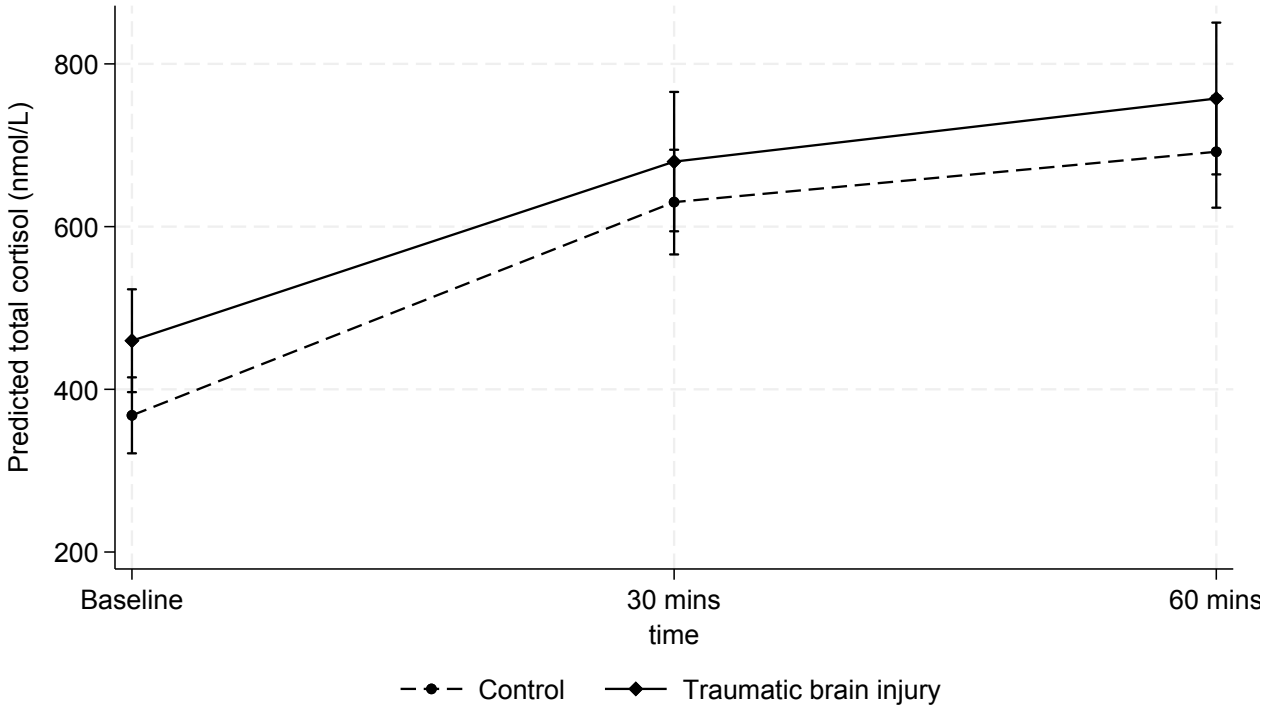


Figure 4: Comparison of total cortisol and free cortisol responses between traumatic brain injury and control cohorts.

CHAPTER 5: In Vitro Dexamethasone Suppression of Cytokine Production Assay and plasma total and free cortisol responses to ACTH (1-24) in healthy volunteers

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***In Vitro* Dexamethasone Suppression of Cytokine Production Assay and plasma total and free cortisol responses to ACTH (1-24) in healthy volunteers**

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ABSTRACT

Introduction

Glucocorticoid sensitivity varies between individuals, influencing stress and inflammatory responses, however its relationship with the synthetic ACTH (1–24) stimulation test, is not well evaluated.

Objective

To evaluate glucocorticoid sensitivity and plasma total cortisol (TC) and plasma free cortisol (PFC) responses to stimulation with 250 µg of synthetic ACTH (1-24) in healthy adults.

Design

Prospective, single-centre observational study.

Methods

Forty-eight healthy adults (24 females, 24 males) underwent basal and stimulated TC and PFC level measurements using liquid chromatography-tandem mass spectrometry. Peak cortisol values were defined as the highest TC or PFC at either 30- or 60-minutes. Glucocorticoid sensitivity was measured using the dexamethasone suppression of cytokine production (DSCP) assay, based on interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) production from lipopolysaccharide-stimulated (LPS) monocytes. A whole blood sample was incubated with buffer, LPS, or LPS with dexamethasone (Dex). TNF-α and IL-6 concentrations were measured in the supernatants (ng/L). The ratio of cytokine levels between LPS + Dex and LPS indicated glucocorticoid sensitivity.

Results

Data from 45 participants were analysed. Peak TC levels ranged from 391 to 1570 nmol/L (median: 618 nmol/L, IQR: 555–729), while PFC levels ranged from 27 to 133 nmol/L (median: 62 nmol/L,

IQR: 53–73). No significant associations were found between baseline or peak TC and PFC levels and glucocorticoid sensitivity ratios. Synthetic ACTH (1-24) test results did not predict glucocorticoid sensitivity. Males exhibited higher BMI (median: 26 vs. 22; p = 0.031), however no significant differences were observed in age, baseline PFC and TC levels between sexes.

Conclusion

No significant relationship was found between glucocorticoid sensitivity, and cortisol post-synthetic ACTH (1-24). Standard measures of adrenal function may not reflect individual variability in glucocorticoid sensitivity.

Introduction

Glucocorticoid sensitivity (GS), which refers to the extent to which tissues or cells already capable of responding to glucocorticoids, exhibit changes in response to these hormones, plays a crucial role in regulating stress and inflammatory responses [1]. It is increasingly recognised that GS is a function of factors including circulating cortisol concentrations, tissue-specific factors such as glucocorticoid receptor (GR) expression, isoforms, polymorphisms, and local cortisol activation through 11β-HSD1 and 11β-HSD2 [2,3]. These factors influence the genomic and non-genomic signalling pathways of GCs, contributing to variability in glucocorticoid effects across individuals and tissues, even when circulating levels are similar. Polymorphisms associated with increased or decreased GS have been linked to differences in metabolic risk, adiposity, and insulin resistance [2].

Variability in GS has been observed among individuals, influencing clinical outcomes in conditions where the hypothalamic-pituitary-adrenal (HPA) axis is involved [1]. This variation appears to be at

least partially ultradian in nature, aligning with the known pulsatile secretion of cortisol and the circadian acetylation of GRs, which modulate receptor activity throughout the day [2].

Despite its clinical importance, the relationship between GS and glucocorticoid responsiveness, as measured by the standard short Synacthen (synthetic ACTH (1–24), also known as tetracosactide) test, remains under-explored in healthy populations.

The synthetic ACTH (1-24) stimulation test is widely used to assess adrenal function by measuring cortisol production following an exogenous stimulus (synthetic ACTH (1-24)) [4]. While traditionally focused on total cortisol (TC) levels, measurements of these levels obtained using immunoassays demonstrate significant variability [5]. Furthermore, TC responses to synthetic ACTH (1-24) are influenced by factors such as testing methodology and patient characteristics [6]. Immunoassays are more prone to cross-reactivity with other corticosteroids, which may lead to falsely elevated TC levels and liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered more accurate due to its high specificity and sensitivity [7]. With regards to patient factors, younger adults tend to have a more robust cortisol response to synthetic ACTH (1-24) stimulation compared to older adults [8]. Total cortisol levels further depend on corticosteroid binding globulin (CBG), the primary carrier protein for cortisol in the blood. Thus factors that alter CBG levels, such as oestrogen therapy, pregnancy, or liver disease, can affect TC measurements without necessarily reflecting changes in plasma free cortisol levels (PFC) [9,10]. However, PFC is also influenced by several factors, including the binding affinity of corticosteroid binding globulin. Thus, interpreting PFC variability in healthy individuals provides an opportunity to examine its relationship to tissue-level glucocorticoid sensitivity, which may underlie inter-individual differences in HPA axis dynamics. Although our study does not directly assess these mechanisms, we acknowledge their potential to modulate PFC levels. Notably reference data for PFC in the healthy population are not

well established and examining inter-individual variation in PFC responses may nonetheless provide insight into functional glucocorticoid exposure and sensitivity [11].

Sex is increasingly recognised as a factor influencing TC and PFC [12,13]. Differences in steroid-binding proteins, the effect of interfering steroids on cortisol immunoassays and hormonal effects of oestrogen and progesterone, may dampen the cortisol response [13]. However, findings are conflicting, with some studies demonstrating higher stimulated cortisol responses in females, depending on the methodology [13–15]. Previous investigators have demonstrated higher peak TC and PFC levels in males following synthetic ACTH (1-24) stimulation, suggesting inherent sex-specific differences in adrenal function [12,16]. These findings are supported by Sofer and colleagues, who reported elevated PFC levels in men, and Roelfsema and colleagues, who observed higher mean cortisol levels in men under 50 years of age [12,16]. Emerging tissue-level biomarkers may offer improved resolution in assessing glucocorticoid status by better reflecting functional glucocorticoid activity at the cellular level. Clarke and colleagues advocate for the use of putative tissue-level biomarkers such as FKBP5 methylation, osteocalcin, and GDF-15 as proxies for glucocorticoid activity, though these remain investigational [2].

Reduced sensitivity to glucocorticoids along with altered cortisol metabolism have been described as key pathophysiological factors in critical Illness-related corticosteroid insufficiency [17]. Although GS varies in chronic inflammatory states, its assessment and variability in critical illness using the *in vitro* monocyte dexamethasone suppression of cytokine production (DSCP) assay has only recently been reported with one study revealing a significant link between disease severity and GS in 21 patients with septic shock [18,19]. Although median tissue GS did not differ between patients with septic shock and control group participants in this study, patients with septic shock exhibited greater variability in GS with evidence of an association between illness severity and reduced GS [19].

Furthermore, prior studies have demonstrated a positive correlation between PFC elevation following corticotrophin and illness severity [20–23], and the PFC fraction is suggested by previous investigators to provide insights into individual variability in GS [19]. Given known variations in TC and PFC responses to corticotrophin, alongside differences in GS among healthy individuals, evaluating comparative normative data for the PFC response to corticotrophin and GS in healthy populations enhances the interpretation of adrenal function tests across various clinical contexts, as well as facilitating future comparative analyses with critically ill populations. We thus aimed to evaluate GS and the plasma total and free cortisol responses to the administration of synthetic ACTH (1-24) in healthy adults.

MATERIALS AND METHODS

Study design and participants:

This prospective, single-centre observational study was conducted from the 2017 to 2020 and was approved by the Metro North Hospital Health Service Ethics Committee (HREC/15/QRBW/609). Written informed consent was obtained from all participants prior to inclusion in the study and the study was performed in accordance with the Declaration of Helsinki. Recruitment was conducted through the Royal Brisbane and Women's Hospital (RBWH) and University of Queensland newsletters, as well as via social media posts. Exclusion criteria included age under 18 years, documented hypoadrenalism, or recent treatment with glucocorticoids (oral long-term use within 6 months or short-term use within the last four weeks). Although no participants were on corticosteroids, participants using inhaled or topical steroids, as well as those with well-controlled chronic conditions such as essential hypertension, were eligible for inclusion. In this study, one

participant had essential hypertension, and another had asthma managed with salbutamol pro re nata.

Study procedures:

Participants underwent a medical assessment at study entry, including the collection of demographic data (age, sex, body mass index (BMI), medical history, and a physical examination. Once enrolment criteria were confirmed, a peripheral venous cannula was inserted for blood sampling. The standard short synacthen test (250 µg of synthetic ACTH (1-24), Novartis, NSW, Australia) was performed in an outpatient setting between 7:30 and 10:00 am. Blood samples were collected at baseline, and at 30- and 60- minutes post-synthetic ACTH (1-24) administration. Samples were processed for serum and ultra-filtrate, which were stored at −20°C until analysis. Plasma TC and PFC concentrations were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [24]. The highest cortisol value at either 30- or 60- minutes post-synthetic ACTH (1-24) was recorded as the peak cortisol concentration, with the difference between the peak and the baseline calculated as the cortisol increment.

Glucocorticoid sensitivity assessment:

Glucocorticoid sensitivity was assessed using the dexamethasone suppression of cytokine production (DSCP) test. This assay evaluates the ability of exogenous dexamethasone to suppress lipopolysaccharide (LPS)-stimulated monocyte production of IL-6 and TNF-α, by measuring the *in vitro* suppression of interleukin (IL)-6 and tumour necrosis factor (TNF-α) production from lipopolysaccharide (LPS)-stimulated leukocytes following dexamethasone administration. Lithium heparinised whole blood (1 mL) was separated into three portions and incubated in wells containing buffer (baseline), LPS with (LPS+Dex) or without dexamethasone (LPS) to a final concentration of 1000 nmol/L[18,19]. After a 3-hour incubation, at 37°C the samples were centrifuged,

to separate the plasma and the plasma was stored at −20°C until analysis on a Luminex analyser for IL-6 and TNF-α [18]. TNF-α and IL-6 production by monocytes was measured in the supernatants (ng/L). The buffer portion served as the baseline, LPS represented maximum cytokine stimulation, and LPS + Dex indicated glucocorticoid suppression. Results are expressed as a ratio. The ratio of change in cytokine levels from baseline between LPS + Dex and LPS indicated GS, with 95% reference intervals of 0.244–0.472 for IL-6 and 0.372–0.693 for TNF-α, with values outside these ranges indicating altered GS [18]. A lower ratio (values below these ranges) indicated increased GS (greater cytokine suppression), while a higher ratio (values above these ranges) suggested reduced sensitivity (less suppression)] [18,19]. The DSCP assay is a novel *in vitro* test, distinct from the standard dexamethasone suppression test used for Cushing's syndrome [18]. Compared to previous methods like the thymidine assay, the DSCP assay offers superior sensitivity in detecting tissue glucocorticoid responsiveness [18].

DATA ANALYSIS

Statistical Methods:

Patient characteristics, cortisol and GS ratio measures of interest were summarised as median (IQR) and compared between sexes using Wilcoxon's rank sum test. The distributions of variables of interest were assessed graphically using boxplots, histograms and scatterplots and outliers were identified and checked. Generalised linear mixed-effects models with a log-link and patient-level random intercepts were used to assess changes in cortisol levels over time. Overall associations between GS ratio measures, patient characteristics (age, sex BMI) and PFC and TC levels were tested in these models.

Peak TC and PFC values were derived as the maximum of the 30-minute or 60-minute values. Three missing values for peak PFC and one biologically implausible baseline value for PFC were replaced by the model-based predicted values. Correlations between IL-6 and TNF-α and cortisol measures were assessed using Pearson's correlation coefficient and linear regression analyses. (Statistical analyses were conducted using Stata software, version 18 (StataCorp. 2023. Stata Statistical Software: Release 18. College Station, TX: StataCorp LLC.) [25].

RESULTS

Safety and tolerability: Of the 48 healthy volunteers (24 males and 24 females) included in the study, eight (16.7%) reported minor self-limiting adverse effects following synthetic ACTH (1-24) administration. Four participants described experiencing a sensation of epigastric warmth immediately after injection and one reported a sensation of warmth spreading from head to toe. Two participants reported transient nausea lasting a few seconds and one developed a minor bruise at the cannula insertion site.

Participant characteristics: Of 48 patients, 45 (aged 20-64 years) had sufficient data for inclusion in analyses. Summary statistics are shown in **Table 1.** The median BMI was significantly higher in males compared to females (26 vs. 22; p = 0.028). No significant differences in age were observed by sex.

Cortisol response to synthetic ACTH (1-24): The distribution of TC and PFC levels over time following synthetic ACTH (1-24) stimulation is shown in **Figure 1**. Peak total cortisol levels ranged from 391-1570 nmol/L (Median [IQR] 618 nmol/L, [555-729 nmol/L], while PFC ranged from 27-133 nmol/L, (Median [IQR] 62 nmol/L, [53-73 nmol/L]. No significant differences in baseline PFC, or TC levels were observed by sex. However, a trend was observed at 30 minutes post-synthetic ACTH

(1-24) stimulation, where males had higher plasma free cortisol levels compared to females (Median [IQR]: 49 [41-54] vs. 41 [34-49] nmol/L; p = 0.074), although this difference did not reach statistical significance. In response to synthetic ACTH (1-24), participants exhibited an average 4-fold increase in PFC levels from baseline to 30 minutes and an average 5.5 times increase from baseline to 60-minutes **(Table 2)**. Corresponding increases for total cortisol were 1.7 and 1.9 times for total cortisol. There was no evidence for associations between cortisol measures and GS.

Glucocorticoid sensitivity:

A strong positive correlation was observed between the IL-6 and TNF-α ratio measures derived from the dexamethasone suppression tests (Pearson correlation coefficient = 0.82, p < 0.001).

There was considerable variation in ratio measures. Dex-IL-6 ratio values ranged from 0.02 to 0.78 with a mean (SD) and coefficient of variation (CoV) values of 0.32 (0.17) and 0.55 respectively. TNF-alpha ratio values ranged from 0.08-0.98 (mean: 0.34, SD:0.16; CoV: 0.47). Based on the cut-points for the IL-6 assay, 16/45 (36%) displayed increased GS, while for the TNF-α assay 30 (66%) were classified as having increased sensitivity. Decreased GS was observed for 10 (22%) for the IL-6 assay and one (2%) for the TNF-α assay. Only 6 (13%) patients had “normal” values on both assays.

Relationship Between Cortisol Levels and Glucocorticoid Sensitivity

Scatterplots revealed wide dispersion of IL-6 and TNF-α ratio values around the best fitting linear regression lines **(Figure 2).** On linear regression analyses, no significant associations were found between glucocorticoid ratio measures and cortisol measures **(Table 3)**.

DISCUSSION

The core finding of this study is the lack of a significant relationship between free and total cortisol responses to synthetic ACTH (1-24) and GS in healthy adults. Following synthetic ACTH (1-24)

stimulation, there were no significant associations between peak TC or PFC levels and GS, as measured by IL-6 and TNF-α ratios.

The lack of a significant relationship between cortisol responses to synthetic ACTH (1-24) to GS observed in this study highlights findings by previous investigators that GS is associated with factors beyond circulating cortisol levels [26]. Although adrenal responsiveness to ACTH and peripheral GS are mechanistically distinct, tissue-level resistance may indirectly influence HPA axis dynamics. Reduced glucocorticoid signalling at tissue level may attenuate negative feedback, leading to increased ACTH drive and, over time, altered adrenal responsiveness [27,28]. This has been observed in obesity, where impaired glucocorticoid feedback correlates with elevated ACTH or enhanced adrenal output despite normal cortisol level [28,29]. This perspective aligns with the proposed concept of an "extended hypothalamic-pituitary-adrenal-tissue axis," whereby tissue-level glucocorticoid sensitivity can modulate central HPA axis regulation. In this model, reduced peripheral glucocorticoid sensitivity results in impaired negative feedback and increased ACTH drive, potentially influencing adrenal responsiveness over time. This tissue-led feedback mechanism, demonstrating altered ACTH dynamics despite normal circulating cortisol has been demonstrated by various authors [28–30]. While speculative in healthy individuals, such mechanisms could contribute to inter-individual variation in ACTH-stimulated cortisol responses

The considerable variability in GS indices observed among participants in this study likely reflects underlying biological heterogeneity in the general population. Glucocorticoid sensitivity is modulated by a variety of mechanisms, including the density and affinity of glucocorticoid receptors, co-chaperone proteins, and downstream signalling pathways that are not directly influenced by cortisol levels alone [26,31,32].

Genetic polymorphisms in the glucocorticoid receptor gene are also known to significantly alter sensitivity to glucocorticoids, independent of cortisol concentrations [33]. Patients with glucocorticoid resistance or insensitivity can present with normal or elevated cortisol levels, suggesting that circulating cortisol is not always a reliable predictor (or cause) of glucocorticoid effects[34]. This is particularly evident in conditions such as glucocorticoid resistance syndrome, where therapeutic outcomes are not aligned with cortisol concentrations [35]. Furthermore, cortisol responses to stress, including ACTH stimulation, have been demonstrated to be highly variable across individuals, influenced by factors such as chronic stress, psychological conditions, and overall health status, without consistently correlating with GS [19]. Findings of the current study support the understanding that GS is a multifaceted phenomenon requiring a broader assessment framework that goes beyond simple cortisol measurements.

Male participants exhibited significantly higher body mass index (BMI) compared to females (26 vs. 22; $p = 0.031$), likely due to physiological differences in muscle mass, fat distribution, and hormonal factors such as higher testosterone levels [36–38]. No significant differences were observed in age between sexes. While baseline total cortisol levels tended to be higher in males, this difference did not reach statistical significance ($p = 0.093$).

Independence of glucocorticoid sensitivity from cortisol measures and BMI:

IL-6 and TNF-α ratio measures were independent of PFC and BMI in this population, suggesting that variations in these parameters do not significantly affect GS. Participants in this study had a normal BMI, indicating that baseline cortisol levels and BMI may not need to be heavily factored into GS assessments in adults within a normal BMI range, although this relationship might differ in overweight or obese populations [3].

Interrelationship between IL-6 and TNF-α suppression ratios:

Another key finding of this study is the strong positive correlation between IL-6 and TNF-α suppression ratios (Pearson correlation coefficient = 0.82, $p < 0.001$), indicating that these ratio measures reflect a similar biological response in the DSCP assay. Both cytokines respond similarly when exposed to an inflammatory stimulus with their production being effectively suppressed by dexamethasone, highlighting a coordinated glucocorticoid effect on cytokine regulation. This high correlation reinforces the robustness of using these cytokine ratios in clinical and research settings to evaluate GS.

It is important to note the wide range of values for both ratios, with many falling outside the proposed reference range. This variability suggests that while the ratios capture a general suppression response, their ability to reliably differentiate between individuals with normal or impaired sensitivity may be limited. Despite this, the high correlation between IL-6 and TNF-α suppression suggests that these ratios still provide meaningful insights into GS , though further refinement of reference ranges may be necessary to enhance their discriminatory power in clinical and research settings.

Study limitations and future directions:

The relatively small sample size of the current study may have limited the power to detect more subtle associations between cortisol dynamics and GS. While we acknowledge the absence of a prospective power calculation, our sample size (n=45) is comparable to previous similar exploratory studies [19,39]. Future studies should explore mechanisms underlying observed sex differences and their potential clinical implications. explore these relationships in larger, more diverse populations and investigate additional factors such as genetic polymorphisms, receptor expression levels, and other markers of immune function. Additionally, while the DSCP assay has shown promise as a

biologically grounded method for evaluating tissue-level glucocorticoid sensitivity, we acknowledge its limited standardisation in broader clinical settings. The reference ranges for GS (IL-6 and TNF-α suppression ratios) were derived from a cohort of 12 healthy volunteers, as described by Cardinal and colleagues [18]. While these ranges provide valuable insight, their derivation from a relatively small sample may impact the generalisability of these findings [18].

Nonetheless, subsequent studies, such as Cohen and colleague's investigation in septic shock, have replicated its use in larger and more diverse populations [19]. Our study applied the same methodology and laboratory procedures. We recognise that the lack of universally accepted thresholds for defining 'normal' versus 'abnormal' glucocorticoid sensitivity is a constraint. Standardisation across laboratories and larger reference datasets would strengthen the assay's utility for comparative and clinical studies. Despite these limitations, the DSCP assay currently represents one of the few available methods to assess functional tissue-level glucocorticoid effects in vitro, particularly in healthy populations where few alternatives exist.

Future studies with larger sample sizes are needed to further validate these reference ranges and confirm their applicability across broader populations and clinical settings.

CONCLUSION

The standard short synthetic ACTH (1–24) stimulation test, while useful for evaluating adrenal function, did not demonstrate any significant correlation with GS as measured by the DSCP assay test in the current study, Differences in cortisol responses did not result in significant variations in GS as measured by IL-6 and TNF-α ratios.

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AUTHORS CONTRIBUTIONS

Study concept and design: Gladness Dakalo Nethathe, Jeremy Cohen, Jeffrey Lipman, Ronald Anderson, Charles Feldman

Acquisition of data: Gladness Dakalo Nethathe

Analysis and interpretation of data: Karen Elizabeth Hay

Drafting of manuscript: Writing – original draft; Gladness Dakalo Nethathe. Writing – review & editing. Gladness Dakalo Nethathe, Jeremy Cohen, Jeffrey Lipman, Ronald Anderson, Karen Elizabeth Hay, Carel Pretorius, Charles Feldman

Critical revision of manuscript for important intellectual content: Jeremy Cohen, Jeffrey Lipman, Ronald Anderson, Carel Pretorius, Charles Feldman

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Study supervision: Gladness Dakalo Nethathe, Jeffrey Lipman, Ronald Anderson, Charles Feldman

Data Availability: Some or all datasets generated during and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

COMPETING INTERESTS: N/A

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FIGURES AND TABLES

TABLES

Table 1. Summary statistics by group and sex

Table description: Variables are presented as median (interquartile range [IQR]). The number of participants for whom data was available is indicated where applicable (e.g., n=41). Abbreviations: TNF-α = Tumour Necrosis Factor-alpha, IL-6 = Interleukin-6, Dex = Dexamethasone, Ratio_Dex_IL-6 = Ratio of IL-6 after dexamethasone suppression, Ratio_Dex_TNF-α = Ratio of TNF-α after dexamethasone suppression.

Variable	Total group (n=45)	Female (n=23)	Male (n=22)	p-value
Age	33 (27-42)	32 (26-49)	33 (30-35)	0.94
Body mass index	24 (22-27)	22 (21-25)	26 (23-27)	0.028
Baseline plasma free cortisol (n=44)	10 (6-17)	10 (8-17)	9 (5-17)	0.35
Plasma free cortisol at 30 min (n=41)	46 (38-52)	41 (34-49)	49 (41-54)	0.074
Plasma free cortisol at 60 min (n=42)	62 (53-71)	58 (52-70)	64 (54-71)	0.69
Baseline total cortisol	305 (225-391)	312 (269-458)	246 (206-391)	0.093
Total cortisol at 30 min (n=44)	535 (493-574)	543 (493-820)	529 (493-553)	0.16
Total cortisol at 60 min	622 (555-729)	623 (568-874)	586 (546-690)	0.17
Ratio_Dex_IL6	0.31 (0.18-0.40)	0.30 (0.22-0.37)	0.35 (0.18-0.52)	0.31
Ratio_Dex_TNF-α	0.33 (0.26-0.43)	0.33 (0.25-0.39)	0.33 (0.27-0.48)	0.44

Table 2: Results of regression modelling of cortisol response to ACTH stimulation testing.

Variable	Category	RR[a] (95% CI)	*p*-value	Marginal mean (95% CI)	Change from baseline mean (95% CI)
Plasma free cortisol					
Time	Baseline			11.7 (8.9-14.4)	
	30 minutes	4.0 (3.2-5.1)	<0.001	46.8 (42.7-51)	35.1 (30.6-39.6)
	60 minutes	5.5 (4.4-6.9)	<0.001	64.3 (59.3-69.3)	52.6 (47.4-57.8)
Ratio_Dex_IL-6		1.2 (0.8-1.8)	0.76		
Ratio_Dex_TNF-α		1.0 (0.6-1.6)	0.65		
Total cortisol					
Time	Baseline			356 (312-399)	
	30 minutes	1.7 (1.5-1.9)	<0.001	600 (540-659)	244 (194-294)
	60 minutes	1.9 (1.7-2.1)	<0.001	662 (598-725)	306 (254-358)
Ratio_Dex_IL-6		0.2 (-0.4-0.7)	0.57		
Ratio_Dex_TNF-α		1.2 (0.7-2.1)	0.57		

[a] Estimates derived from generalised linear mixed effects models fitted with a log link and patient-level random intercept.

Table 3: Results of linear regression analyses assessing associations between glucocorticoid sensitivity ratio measures and plasma free cortisol and total cortisol following ACTH stimulation (n=45).

Outcome	Variable	R^2	Coefficient (95% CI)	*p*-value
IL-6 Ratio	**Baseline PFC**	0.059	0.006 (-0.001-0.013)	0.11
	Peak PFC	0.003	0.001 (-0.003-0.004)	0.73
	Proportion change PFC	0.024	0.00 (0.00-0.00)	0.31
	Baseline TC	0.005	0.00 (0.00-0.00)	0.63
	Peak TC	0.000	0.00 (0.00-0.00)	0.99
	Proportion change TC	0.016	-0.02 (-0.07-0.03)	0.41
TNF-α ratio	**Baseline PFC**	0.016	0.003 (-0.004-0.01)	0.41
	Peak PFC	0.000	0.00 (-0.003-0.003)	0.88
	Proportion change PFC	0.006	0.00 (-0.01-0.01)	0.62
	Baseline TC	0.000	0.00 (0.00-0.00)	0.93
	Peak TC	0.015	0.00 (0.00-0.00))	0.43
	Proportion change TC	0.009	0.01 (-0.03-0.06)	0.54

PFC: Plasma free cortisol (per nmol/L); TC Total cortisol (per nmol/L)

FIGURES

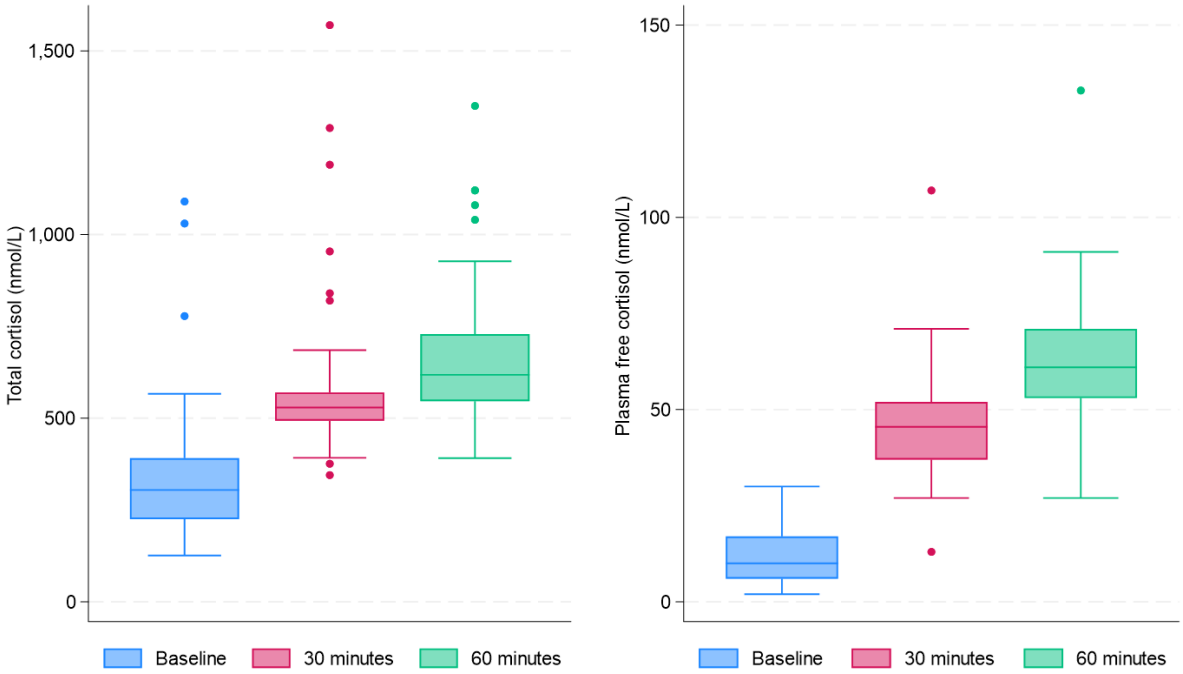

Figure 1: Boxplots showing the distribution of total cortisol and plasma free cortisol levels over time following administration of corticotrophin. Cortisol measurements are shown at baseline, 30 minutes, and 60 minutes post-baseline.

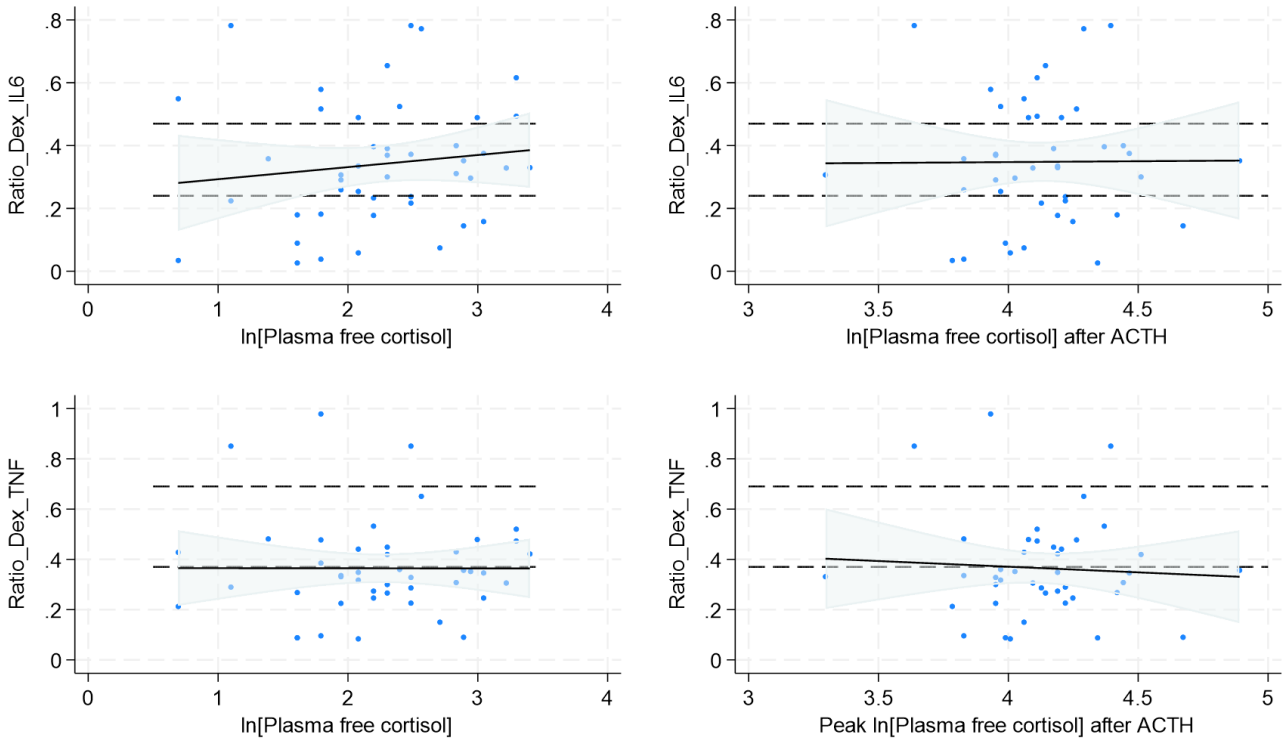

Figure 2: Relationship between Peak Plasma Free Cortisol (ln_peak_PFC) and Glucocorticoid Sensitivity (Ratios of TNF-α and IL-6). Scatter plots illustrate the relationships between the natural logarithm of peak plasma free cortisol (ln_peak_PFC) and glucocorticoid sensitivity, measured by the ratios of cytokine levels in dexamethasone-suppressed (LPS + Dex) versus LPS-stimulated conditions for TNF-α (Ratio_Dex_TNF-α) and IL-6 (Ratio_Dex_IL6). In both plots, each blue dot represents an individual data point. The red lines indicate the fitted linear regression lines, and the shaded grey areas show the 95% confidence intervals (CIs) for the fitted values. There is a wide variation in ratio values and no evidence of an association.

CHAPTER 6: Mineralocorticoid dysfunction during critical illness

ANESTHESIOLOGY

Mineralocorticoid Dysfunction during Critical Illness

A Review of the Evidence

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ABSTRACT

The recent demonstration of the significant reduction in mortality in patients with septic shock treated with adjunctive glucocorticoids combined with fludrocortisone and the effectiveness of angiotensin II in treating vasodilatory shock have renewed interest in the role of the mineralocorticoid axis in critical illness. Glucocorticoids have variable interactions at the mineralocorticoid receptor. Similarly, mineralocorticoid receptor–aldosterone interactions differ from mineralocorticoid receptor–glucocorticoid interactions and predicate receptor–ligand interactions that differ with respect to cellular effects. Hyperreninemic hypoaldosteronism or selective hypoaldosteronism, an impaired adrenal response to increasing renin levels, occurs in a subgroup of hemodynamically unstable critically ill patients. The suggestion is that there is a defect at the level of the adrenal zona glomerulosa associated with a high mortality rate that may represent an adaptive response aimed at increasing cortisol levels. Furthermore, cross-talk exists between angiotensin II and aldosterone, which needs to be considered when employing therapeutic strategies.

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Two recent large-scale clinical trials have demonstrated a beneficial effect of adjunctive glucocorticoid treatment in septic shock on shock reversal, weaning from mechanical ventilation, and duration of intensive care stay, with, however, divergent effects on mortality.[1,2] A possible explanation for the discrepancy is that adjunctive fludrocortisone was added to the treatment regimen in the trial that reported a mortality benefit.[3]

Additionally, a phase II clinical trial of angiotensin has demonstrated that angiotensin II is effective in treating vasodilatory shock.[4] These recent demonstrations of significant reductions in mortality in patients with septic shock treated with adjunctive glucocorticoid combined with fludrocortisone, as well as the effectiveness of angiotensin II in treating vasodilatory shock, have renewed interest in the role of the mineralocorticoid axis in critical illness.[1,5–7]

This narrative review aims to summarize current insights into the pathophysiologic mechanisms and clinical implications of the renin–angiotensin–aldosterone system in critical illness. We focus on the issue of fludrocortisone supplementation in critical illness, as well as current understanding of the role of mineralocorticoid replacement in this group of patients. Other aspects of mineralocorticoid dysfunction, such as the presentation of a different approach to the understanding of mineralocorticoid dysfunction with a focus on hyperreninemic hypoaldosteronism, are additional sections that are included in this review.

Physiology of Mineralocorticoid Secretion and Metabolism

Adrenal Steroid Biosynthesis

The acute effect of corticotropin (adrenocorticotropic hormone) released from the pituitary gland is to stimulate early steps in the process of adrenal steroidogenesis by promoting the transport of cholesterol into mitochondria. By binding to a cell-surface receptor on the adrenal cortex, the melanocortin receptor-2, adrenocorticotropic hormone leads to activation of adenylyl cyclase, which, in turn, leads to an increase in cyclic adenosine monophosphate production, stimulation of protein kinase A, and protein phosphorylation.[8–11] More chronically, adrenocorticotropic hormone causes an overall increase in all adrenal steroid production and secretion, primarily through increasing the synthesis of most of the enzymes of the steroidogenic pathway.[12–14]

The mammalian adrenal gland consists of the adrenal cortex and medulla. The adrenal cortex consists of three zones; the zona fasciculata, the zona reticularis, and the zona glomerulosa, which secrete glucocorticoids, androgenic precursors, and mineralocorticoids, respectively.[15]

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All steroid hormones are produced from modifications of cholesterol (fig. 1).[16] Steroidogenesis entails regulation of cellular uptake and conversion of cholesterol to biologically active steroid hormones and occurs in the adrenal glands, placenta, brain, and gonads. Subsequent chemical modifications of cholesterol include a series of hydroxylations that require cytochrome P-450 enzymes.[14,17] There are zonal differences in enzymatic concentrations. Notably, 17-hydroxylase is present in low concentrations in the zona glomerulosa. The zona glomerulosa is the only zone that has the enzyme required to convert deoxycorticosterone to aldosterone.[18] This zonal difference in enzyme concentrations results in compartmentalization of enzymatic reactions. Adrenal cortical cells take up cholesterol from the circulation (as lipoproteins) or synthesize cholesterol *de novo*.[19] In the mitochondria, cholesterol is converted to Δ5-pregnenolone and then subsequently to progesterone.[17,20]

The main glucocorticoid secreted in response to adrenocorticotropic hormone is cortisol. Cortisol plays a role in carbohydrate metabolism and promotes protein catabolism to provide gluconeogenesis substrates. It also plays a role in immune and cardiovascular function.[21] In addition, corticotropin secretion stimulates aldosterone release through responses mediated by melanocortin receptor-2.[22]

Androgens play a role in secondary reproductive development and are secreted as precursors by the zona reticularis. They include dehydroepiandrosterone, dehydroepiandrosterone sulfate, and androstenedione. Androstenedione is subsequently converted peripherally to testosterone.[23,24]

Aldosterone is the primary mineralocorticoid secreted by the adrenal cortex and is essential in the maintenance of sodium, potassium, and extracellular fluid balance. Other sites of aldosterone synthesis include the brain, blood vessels, and the heart.[25,26] The primary regulator of aldosterone synthesis is the renin–angiotensin–aldosterone system. This is mediated through adrenal angiotensin II receptors and results in sodium retention and renal potassium loss. Corticotropin release stimulates production of aldosterone, but compared with cortisol, aldosterone is relatively independent of adrenocorticotropic hormone secretion. Aldosterone synthase catalyzes synthesis by converting deoxycorticosterone to corticosterone and then to aldosterone.[22] Aldosterone synthase expression is restricted to the zona glomerulosa. Unlike cortisol, aldosterone is not specifically bound to plasma proteins, making it less affected by plasma protein concentrations and binding alterations that occur in critical illness.[26]

Regulation of Aldosterone Secretion

In healthy subjects, angiotensin II, together with a rise in serum potassium concentration, are the primary secretagogues that stimulate aldosterone release.[22,26] Levels of aldosterone also fluctuate with changes in adrenocorticotropic hormone and renin levels.[26]

Renin is a proteolytic enzyme that is cleaved from its precursor, prorenin, and stored in the granules of renal macula densa and juxtaglomerular cells.[27] Prorenin production also occurs in a number of extrarenal sites such as the adrenal glands, gonads, and placenta.[27] The release of renin is triggered by a number of mechanisms, most importantly changes in blood pressure, sympathetic nervous system activity, and in response to hyponatremia.[28] A fall in plasma volume or blood pressure causes renin release, which in turn activates angiotensinogen synthesis from the liver to form angiotensin I. Angiotensin I is converted to angiotensin II by angiotensin-converting enzyme, an enzyme located in many tissues.[26] Angiotensin II, a potent vasoconstrictor, stimulates aldosterone release from the adrenal cortex.[29]

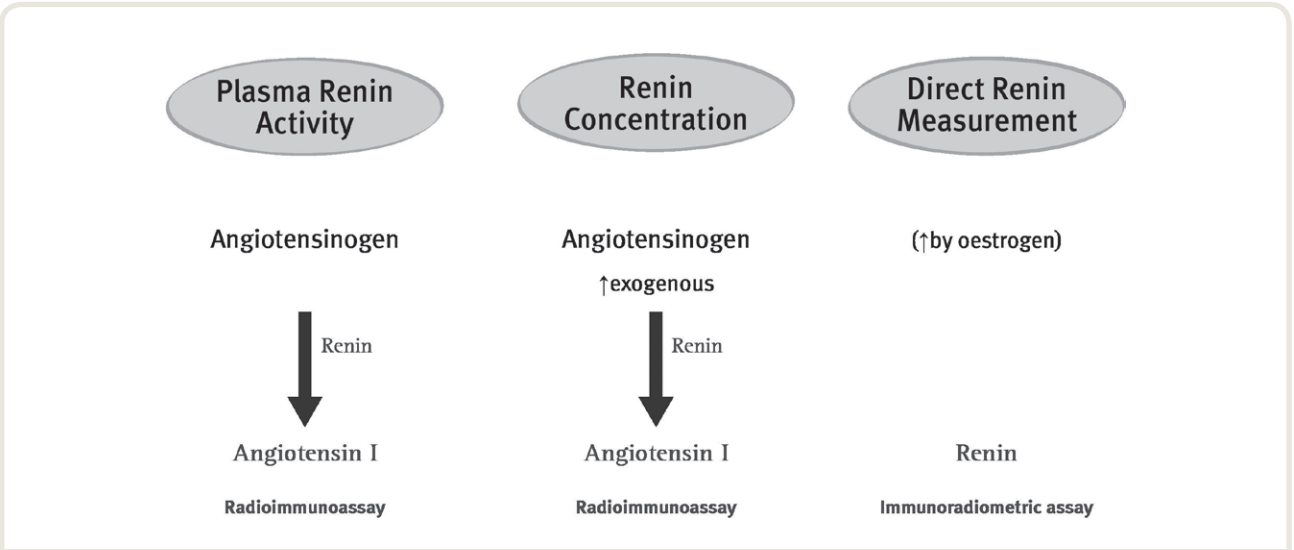

Fig. 1. Adrenal steroid biosynthetic pathway. Figure modified from Payne and Hales,[16] with permission from Oxford University Press and the Endocrine Society.

The resultant increase in blood pressure causes a decline in renin release, inhibiting further aldosterone production (fig. 2).[30] Angiotensin is currently understood to be one of a number of drivers of aldosterone secretion.

Recently, in a murine model, the hormone leptin has been found to be a new direct regulator of aldosterone secretion.[31] Obesity is associated with higher levels of aldosterone, and in both humans and murine models, obesity has been demonstrated to increase plasma renin activity and angiotensin II levels.[32–35] Although the clinical relevance of these findings requires further testing, the added effect of increased aldosterone secretion mediated by leptin may contribute to the increased cardiovascular risk associated with obesity.[29] More importantly, this suggests that leptin-mediated cardiovascular and endothelial dysfunction may be ameliorated pharmacologically by mineralocorticoid receptor antagonists. In the acute critical care setting, we postulate that increased levels of aldosterone in obesity may confer potential benefit through hemodynamic and vascular tone effects as much as increased levels of aldosterone in pregnancy are postulated to be adaptive and to mediate vascular tone.[36–38] Given the rising incidence of obesity

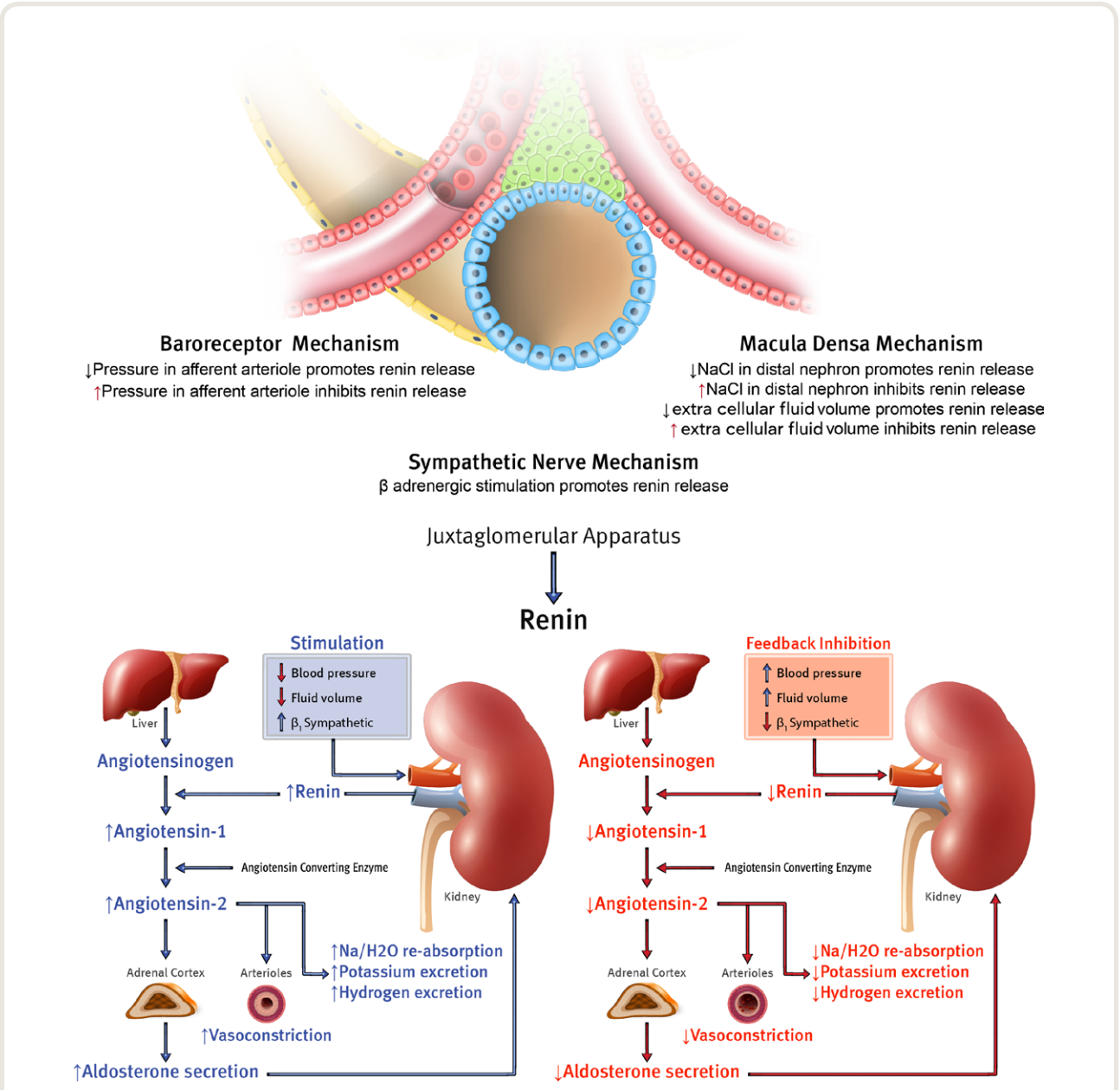

Fig. 2. Mechanisms of renin release and aldosterone secretion. Figure modified from Raffa *et al.*,[30] with permission from Elsevier. Na, sodium; H2O, water.

worldwide and the association of obesity with outcomes in critical illness, further investigations are warranted.[39]

Plasma/serum levels of aldosterone also vary according to population, age, sex, posture, and acute salt loading, as well as among different laboratories because of variations in assay procedures.[40,41] As a result, there is currently no standard reference range that applies to all populations. Serum levels (sitting position) have been found to range between 35 and 827 pmol/l and 15 and 408 pmol/l (before and after sodium-loading test, respectively) in some populations.[40] Reference values between 12 and 140 ng/l (males) and 5.0 and 134.0 ng/l (females) in the adult population have also been described.[42]

Extraadrenal Synthesis of Aldosterone

With the exception of the brain, evidence suggesting local aldosterone synthesis in the cardiovascular and renal systems, as well as in adipose tissue, has been proposed but is conflicting and unconvincing.[32,34] Adipocytes contain angiotensin I and angiotensin II receptors, mineralocorticoid receptors, 11β-hydroxysteroid dehydrogenase type 2, as well as evidence of selected steroidogenic enzyme messenger RNA.[32] 11β-Hydroxysteroid dehydrogenase, type 2, is an intracellular isoenzyme that converts cortisol to cortisone, thus preventing its binding to mineralocorticoid receptors. The significance of the presence of these enzymes has yet to be elucidated.

The summation of evidence from murine and human models suggests that aldosterone production in the brain, however, may be possible. Mineralocorticoid receptors are present in high density in certain regions of the brain, even though the blood–brain barrier significantly reduces exposure of the brain to circulating aldosterone. Furthermore, the presence of messenger RNA encoding corticosteroidogenic enzymes in certain regions of the human brain suggests that specific areas of the brain are able to produce low levels of aldosterone. The ligand for the mineralocorticoid receptor in the brain has not been proven to be necessarily aldosterone (glucocorticoid presence in the brain is at higher concentration).[43]

Evidence supporting the extraadrenal synthesis of aldosterone challenges the conventional understanding of aldosterone action, because aldosterone synthesis is thought to be secreted exclusively by the adrenal gland. Locally synthesized aldosterone has been shown to regulate blood pressure in murine models and to confer high spatial specificity of aldosterone action. Exploration of these pathways in the critical care setting may reveal previously unexplained physiologic and therapeutic avenues.[44–47]

Aldosterone Mode of Action and Cellular Effects

The essential role of mineralocorticoids in regulating sodium and water homeostasis has been demonstrated in adrenalectomized rats. Adrenalectomy results in weight loss, severe dehydration as a result of sodium loss, hyperkalemia, and hyponatremia, which resolve on treatment with mineralocorticoids.[48,49] The action of aldosterone is primarily on the kidney, where it causes sodium and water retention and active excretion of potassium and hydrogen ions. Its action on luminal cortical tubular cells results in an increased number of open sodium channels promoting sodium reabsorption.[50] Removal of sodium from the tubular fluid creates an electrical gradient that favors the secretion of potassium and hydrogen ions into the lumen. Aldosterone effects are purportedly mediated by genomic interaction with the Na,K-ATPase pump, as well as by nongenomic increases in the permeability of cells to sodium and protons.[49]

Glucocorticoids and mineralocorticoids have similar affinity for mineralocorticoid receptors. In conditions such as congestive heart failure, cortisol has been shown to activate the mineralocorticoid receptor.[51] Differences in the interaction of various ligands with the mineralocorticoid receptor have been previously demonstrated, and a number of factors confer mineralocorticoid action of glucocorticoids.[52] Mineralocorticoid receptors bound to aldosterone have been shown to be more resistant to proteolysis than mineralocorticoid receptors bound to either spironolactone or cortisol.[53] Notably, mineralocorticoid receptor activation by glucocorticoids is not applicable to all glucocorticoids. On a molecular level, glucocorticoid conformational mobility conveys specificity to the mineralocorticoid receptor. Steroids are compounds with a molecular skeleton consisting of four rings of carbon atoms designated A, B, C, and D.[54] Steroid ring A is required for glucocorticoid action and is rigid in the "pure" glucocorticoids (*e.g.*, dexamethasone) and flexible in aldosterone. Ring C conformation is required for mineralocorticoid action and is rigid in aldosterone (but is relatively flexible in the pure glucocorticoids). The exception is 11-deoxycorticosterone and its synthetic derivative, δ11,12-deoxycorticosterone, which are specific mineralocorticoids with marked glucocorticoid activity and a flexible C ring.[55,56] A putative mechanism for this difference is the lack of C-11 oxygenation of 11-deoxycorticosterone, which may confer versatility.[55,57] Additionally, the action of 11β-hydroxysteroid dehydrogenase, type 2, an intracellular isoenzyme that converts cortisol to cortisone thus preventing its binding to mineralocorticoid receptors, further renders mineralocorticoid responsive tissues, such as the kidney, sensitive to mineralocorticoids only.[49,58,59]

Multiple new members of the renin–angiotensin–aldosterone system have been shown to exist. The current and continuously evolving understanding is that the renin–angiotensin–aldosterone system consists of at least three additional local or tissue axes that regulate renal function, blood pressure, and the cardiovascular and nervous systems. These axes include a number of multifunctional enzymes, mediators, and their receptors; angiotensin-converting enzyme 2 (an angiotensin-converting enzyme homolog), angiotensin-(1–7) heptapeptide, and its G protein–coupled receptor, Mas. Other tissue or local axes include the precursor prorenin, the enzyme renin, and the (pro)renin receptor, which activates mitogen-activated protein kinases and the extracellular signal–related protein kinase 1/2, and last the angiotensin

IV hexapeptide, angiotensin 4 receptor, and insulin-regulated aminopeptidase axes.[60,61] This is in contrast to the classical endocrine system and its circulating mediators. Thus the understanding that blood pressure is under the sole control of the classical renin–angiotensin–aldosterone system is debatable.[60] Importantly, cross-talk between the renin–angiotensin–aldosterone system and aldosterone production is well established, and thus these two systems should not be seen in isolation.[62] Experimentally, aldosterone has been demonstrated to potentiate the constrictor effect of angiotensin II on vascular smooth muscle and to have a combined role with angiotensin II on vascular remodeling, endothelial dysfunction, and oxidative stress. The apparent involvement of angiotensin II with the additional axes of the renin–angiotensin–aldosterone system clearly merits further intensive investigations.

Causes of Hypoaldosteronism and Clinical Implications in Critical Illness

Causes of reduced levels of aldosterone include inherited and acquired disorders that may lead to a reduction in aldosterone synthesis, reduced renin secretion, or an impairment of the renal response to aldosterone.[63] Hyporeninemia, pharmacologic inhibition of aldosterone, renin, or angiotensin II, and primary adrenal insufficiency are important examples of such factors. Hypoaldosteronism can be classified as hyporeninemic or hyperreninemic.[64]

Hyporeninemic Hypoaldosteronism

Hyporeninemic hypoaldosteronism represents the most common cause of secondary mineralocorticoid insufficiency and is characterized by reduced aldosterone production secondary to reduced renin release or action and reduced angiotensin II production.[65] It is most commonly seen secondary to renal dysfunction or as an effect of pharmacologic agents.[65]

Primary Adrenal Insufficiency. Primary adrenal insufficiency is associated with a lack of both aldosterone and cortisol and is characterized by hyponatremia, hyperkalemia, worsening fatigue, muscle weakness, volume depletion, and hypotension.[66] In secondary adrenal insufficiency, because of a lack of adrenocorticotropic hormone, hypoaldosteronism is not a feature because adrenocorticotropic hormone does not play a significant role in the regulation of aldosterone release in this condition. A lack of aldosterone or reduced aldosterone results in an increase in plasma renin activity except in cases of hyporeninemic hypoaldosteronism.

Hyporeninemic Hypoaldosteronism in Renal Dysfunction: Is Prorenin–Renin Conversion Impaired? More than 50% of patients in the intensive care unit (ICU) develop acute kidney injury during the course of their ICU stay, with an associated mortality over 50%.[67] Patients with mild-to-moderate renal insufficiency (glomerular filtration rate of 45 to 89 ml/min)[68] have been previously shown to exhibit reduced angiotensin II production, which in turn contributes to reduced aldosterone secretion.[65,69–71]

Prorenin–renin conversion classically occurs in the kidney and has more recently been shown to have extrarenal sites of conversion.[72] It is currently accepted that the number of renin-producing cells varies according to demand.[72,73] Previously thought to be an inactive precursor of renin, prorenin is currently understood to be functional; to be elevated in certain conditions, such as pregnancy and diabetes mellitus; and to have extrarenal sites of production (notably adrenal).[72] Increasing evidence also suggests that prorenin plays a role in the regulation of sodium and water balance.[74] In critically ill ICU patients, evidence describing the association of raised renin levels with acute kidney injury has been conflicting, with some suggesting the use of renin as a marker of tissue perfusion and as a risk stratification tool in septic shock.[75] The concept of hyperreninemic hypoaldosteronism as an etiological factor in acute kidney injury associated with septic shock has also been proposed.[75,76] What is also apparent is that mechanisms responsible for renin release in the acute setting (cyclic adenosine monophosphate, calcium signaling pathways) differ from mechanisms responsible for chronic renin stimulation (metaplastic transformation of renin cell precursors).[73,75] The significance of this, and the degree to which renin and prorenin exert identical effects, are currently unknown and present knowledge gaps that warrant further investigation.[72] Available studies to date have included heterogeneous populations with acute and chronic pathophysiologic states, probably associated with variable mechanisms of renin release.

Hyporeninemic Hypoaldosteronism in Traumatic Head Injury and Subarachnoid Hemorrhage: Is Fludrocortisone Beneficial in Cerebral Salt Wasting? Hyponatremia and perturbations of water balance are common in traumatic head pathology or subarachnoid hemorrhage.[77] Cerebral salt wasting and the syndrome of inappropriate antidiuretic hormone secretion are potential causes of hyponatremia in this population. The pathophysiologic mechanisms involved have been studied in both conditions, more so in subarachnoid hemorrhage.[77] Although not a consistent finding in all studies, likely because of the heterogeneous populations included in various studies, high levels of atrial natriuretic peptide and brain natriuretic peptide in patients with cerebral salt wasting are purported to be contributory.[77–79]

Hyporeninemic hypoaldosteronism in cerebral salt wasting and normal or high aldosterone levels in the syndrome of inappropriate antidiuretic hormone secretion have been demonstrated previously.[80] Treatment for cerebral salt wasting in traumatic brain injury and in subarachnoid hemorrhage is not, however, well defined. Although the evidence for the role of fludrocortisone in cerebral salt wasting states is poor, hyponatremia in cerebral salt wasting associated

with trauma or traumatic brain injury has previously been successfully treated with fludrocortisone.[77,80]

In subarachnoid hemorrhage, management of cerebral salt wasting by hypovolemia and fluid restriction may increase the risk of cerebral vasospasm, and fludrocortisone has been advocated as a therapeutic intervention in the management of plasma volume and hyponatremia.[81,82] The two clinical trials conducted to date that have investigated the role of mineralocorticoid supplementation in the management of subarachnoid hemorrhage have found a significant reduction of sodium excretion after treatment with fludrocortisone.[83,84] In a controlled randomized trial involving three centers and consisting of 91 patients, treatment with fludrocortisone at a dose of 400 μg/day (orally or intravenously) administered for a maximum of 12 days was found to reduce excessive natriuresis in the first 6 days of admission compared with controls. Fludrocortisone was administered to 46 of the 91 patients in the study. Mean daily fluid intake was 3,261 ml during the first 6 days and 3,352 ml during the 12-day study period in the treatment group and 3,341 and 3,264 ml, respectively in the control group. Mean daily sodium intake was 219 mmol during the first 6 days and 222 mmol during the 12-day study period in the treatment group and 208 and 202 mmol, respectively, in the control group. The frequency of a negative sodium balance was reduced from 63 to 38% in the first 6 days ($P = 0.041$) and 70 to 29% ($P = 0.0023$) during the 12-day study period.[83] This study confirms the presence of natriuresis in patients with subarachnoid hemorrhage that was significantly reduced with fludrocortisone treatment.

Similar findings were noted by Mori *et al.*[84] in a randomized controlled trial consisting of 30 patients with subarachnoid hemorrhage who were within 1 to 2 days of surgery for aneurysmal clipping. Of these 30 patients, 15 received intravenous hypervolemic therapy (group 1), and 15 received oral or nasogastric fludrocortisone acetate at a total divided dose of 300 μg/day for 8 days in addition to hypervolemic therapy (group 2). Oral salt and hypertonic saline were also administered to manage hyponatremia, whereas water replacement was administered at 8-h intervals to match losses, and 6% hydroxyethyl starch was administered additionally from days 2 to 14 for volume expansion. The mean daily sodium intake was found to be significantly lower for group 2 (487 ± 34.52 mEq/day compared with 634.2 ± 42.86 mEq/day; $P < 0.05$). In addition, group 1 patients demonstrated a significant decline in serum sodium levels from days 4 to 7, which was not evident in group 2 ($P < 0.01$).[84]

The management of subarachnoid hemorrhage, however, has since evolved, with less focus on hypervolemia and a substantial focus on endovascular techniques. Furthermore, fludrocortisone dosing and timing, as well as sodium intake, differed between these two studies. The bioavailability of oral fludrocortisone has been shown to be variable in critical illness, and oral sodium intake may influence sodium balance.[85] In the study by Hasan *et al.*,[83] fludrocortisone given within 72 h of the hemorrhage was administered intravenously or orally at a dose of 0.4 mg in two doses for 12 days, whereas in the study by Mori *et al.*,[84] fludrocortisone was given orally only at a dose of 0.3 mg/day in three divided doses from days 1 to 8. Patients who were able to tolerate an oral diet were placed on a controlled low-sodium diet to reduce variability in sodium intake. This was not controlled for in the study by Mori *et al.*[84]

Although also limited by factors such as sample size, these studies nonetheless highlight the potentially beneficial effects of early treatment with fludrocortisone on sodium and water balance in patients with subarachnoid hemorrhage.[86] In addition, they also raise the question of potential hypoaldosteronism in this group of patients. There was, however, no neurologic outcome benefit in either study. The contribution of serum sodium to complications such as cerebral edema and seizures in individual patients with subarachnoid hemorrhage remains difficult to determine.[86] The role of therapies aimed at reversing hyponatremia remains debatable because the evidence that hyponatremia influences prognosis in patients with subarachnoid hemorrhage is inconclusive.[86] It is, however, considered reasonable practice (as per consensus guidelines) to use fludrocortisone and hypertonic saline to prevent and correct hyponatremia in the management of subarachnoid hemorrhage.[81]

Pharmacologic Causes of Hyporeninemic Aldosteronism. Angiotensin-converting enzyme inhibitors inhibit aldosterone release by reducing the conversion of angiotensin I to angiotensin II. Angiotensin II receptor blockers interfere with angiotensin II action at the receptor level, whereas direct renin inhibitors act by binding competitively to the active site of the enzyme.[87] Renin secretion is, however, increased with angiotensin-converting enzyme inhibitors.[88] Potassium load and potassium-sparing diuretics are another type of medications that are also known to interfere with aldosterone metabolism.[89]

Nonsteroidal antiinflammatory drugs have been shown to impair both renal renin secretion and angiotensin II–induced release of aldosterone. Interestingly, agents such as diclofenac, mefenamic acid, and naproxen have also been shown to inhibit the glucuronidation of aldosterone by human kidney cells and liver microsomes and to increase plasma and renal aldosterone concentrations by as much as 320%, potentially counteracting the effects of mineralocorticoid antagonists[90,91] and posing a considerable cardiovascular risk in patients with conditions such as moderate or severe heart failure.[91] This mechanism of action of nonsteroidal antiinflammatory drugs (NSAIDs) appears to vary between different NSAIDs and may be a potential mechanism for cardiovascular adverse events related to NSAID use.[90,92,93]

Increased sympathetic flow to the kidneys, through β1-adrenergic stimulation, directly activates a blood-pressure mechanism of renin secretion by the juxtaglomerular

cells.[73] Evidently, direct β1-adrenergic receptor stimulation of aldosterone secretion in bovine zona glomerulosa cells has been observed.[94,95] Additionally, cyclosporine has also been shown to cause reduced plasma renin activity and reduced tubular sensitivity to aldosterone, leading to reduced secretion and responsiveness to aldosterone, an effect possibly mediated by reduced mineralocorticoid receptor expression.[96,97]

Cyclosporine has been associated with a hyporeninemic hypoaldosteronism state that leads to hyperkalemia and metabolic acidosis in transplant patients caused by tubular insensitivity to aldosterone mediated by downregulation of mineralocorticoid receptor expression.[97,98] Normal or near normal aldosterone levels have been described in this condition, and treatment with fludrocortisone has been shown to be beneficial.[97,98]

Other pharmacologic effects, which require further exploration, include that of heparin therapy. Heparin has a direct toxic effect on the adrenal zona glomerulosa cells and has been shown to lead to a substantial reduction in plasma aldosterone concentrations, even at low doses.[89] Heparin-induced hyperkalemia is a potentially life-threatening complication that may occur in patients who have concurrent renal dysfunction, type 4 renal tubular acidosis, metabolic acidosis, and diabetes.[89] Serum potassium and sodium should be regularly monitored in these patients on initiation of heparin therapy. Heparin-induced hyperkalemia has been successfully treated using oral fludrocortisone.[89] The underlying mechanism is unclear and may be mediated by reduced numbers and affinity of adrenal angiotensin II receptors.[89,99,100] Collectively, such routinely prescribed agents may significantly alter renal and plasma aldosterone concentrations, potentially counteracting effects of mineralocorticoids and their antagonists in some instances (moderate or severe heart failure) or modifying compensatory mechanisms in conditions such as septic shock.

Aldosterone Resistance and Hyperaldosteronism

The administration of potassium-sparing diuretics is the most common cause of aldosterone resistance. These diuretics act by antagonizing the action of aldosterone on the collecting tubules, by competing for the aldosterone receptor (spironolactone and eplerenone), or by closing the sodium channels in the luminal membrane (amiloride and probably triamterene). Other pharmacologic agents implicated in aldosterone resistance include antibiotics such as trimethoprim, which inhibit the collecting tubule sodium channel, the site of action of aldosterone.[89] More rarely, genetic disorders are the cause.[101]

Hyperaldosteronism is a hallmark of chronic heart failure.[102–105] The value of mineralocorticoid receptor antagonism in moderate-to-severe heart failure has been demonstrated to reduce morbidity and mortality and is supported by well conducted trials, including the Randomized ALdactone Evaluation Study (RALES) and the EPlerenone neuroHormonal Efficacy and the SUrvival Study (EPHESUS) trials.[106] The persistent and evidently detrimental effects of chronic renin-angiotensin-aldosterone system activation seen in chronic heart failure should be distinguished from the acute renin-angiotensin-aldosterone system activation seen in critical illness from trauma and/or sepsis.[107] The initial beneficial effects of renin-angiotensin-aldosterone system activation on sodium and water balance, as seen in acute states such as trauma and sepsis, become deleterious and maladaptive when sustained, as seen in heart failure, because the counterregulatory mediators (natriuretic peptides types A and B) become overwhelmed.[102,108] In addition, long-term renin-angiotensin-aldosterone system activation as seen in congestive heart failure results in cytokine- and growth factor–mediated remodeling of the heart and vasculature.[109,110]

Mineralocorticoid Dysfunction during Critical Illness

Physiologic responses to shock include increased secretion of catecholamines, vasopressin, and activation of the renin–angiotensin–aldosterone system. Decreased adrenal production of aldosterone and stress-induced hypersecretion of adrenocorticotropic hormone are additional features that have been demonstrated in some patients in earlier reports.[111]

More recently, plasma adrenocorticotropic hormone in critical illness has been shown to be mostly low during the early phase of ICU stay, correlating with higher than normal plasma free-cortisol levels.[112] The rise in adrenocorticotropic hormone and cortisol to supranormal levels was a feature seen 1 week after ICU discharge in one study.[112] A concurrent adrenocorticotropic hormone/aldosterone response was not demonstrated in this study. Feedback inhibition mediated by high free-cortisol levels explains the low adrenocorticotropic hormone in association with high free-cortisol levels. Evidently hypothalamus–pituitary–adrenal axis alterations vary, depending on the etiology as well as the phase of critical illness.[113,114] Concurrent changes in aldosterone levels during acute and prolonged critical illness warrant further investigation because they may help clarify the role of mineralocorticoid supplementation in these patients.

Plasma renin activity has been shown to be frequently elevated in critical illness. The associated inappropriately low aldosterone levels have resulted in the changes being described as hyperreninemic hypoaldosteronism.[115,116] Such a state of dissociation between plasma renin and aldosterone levels has been previously associated with hypotension and is interpreted to represent a state of aldosterone deficiency.[115,117]

The first description of selective hypoaldosteronism (hyperreninism with hypoaldosteronism) in hemodynamically unstable critically ill patients was in a subset of 18 critically ill patients.[117] In this single-center study,

which enrolled 28 patients, a spectrum of aldosterone responsiveness was demonstrated in 18 patients with persistent hypotension. Although plasma renin activity was increased in all participants (21.6 ± 7.2 ng · ml^{-1} · h^{-1}), plasma aldosterone levels were inappropriately low in 18 patients (1 to 9 ng/dl). This subgroup had a high mortality rate (78% *vs.* 30% for the high-aldosterone group, $P < 0.001$). A defect at the level of the zona glomerulosa was suggested by the lack of an aldosterone response to an infusion of angiotensin II or adrenocorticotropic hormone in this group.[117] Although the study was small and the methods used to measure both aldosterone and renin have since been improved, it nevertheless highlighted that there exists a population of hemodynamically unstable critically ill patients with hypoaldosteronism despite high plasma renin activity.[117] These findings suggest that as much as there is a subset of critically ill hemodynamically unstable patients with inappropriately low cortisol levels, similar (or corresponding) changes affecting aldosterone may coexist in this population.

In a similar study, which included 83 critically ill patients, plasma renin activity, angiotensin II, potassium, and adrenocorticotropic hormone levels were measured. Plasma renin activity was found to be greater than 2.0 ng · ml^{-1} · h^{-1} in 59 patients, and plasma renin and aldosterone were found to be dissociated in 24 patients.[118] Those with an impaired aldosterone response to renin (plasma aldosterone–to–plasma renin activity ratio less than 2) had mean aldosterone levels of 19 ± 5 ng/dl compared with significantly higher mean aldosterone levels of 48 ± 6 ng/dl ($P < 0.5$) in those with an appropriate renin response to aldosterone.[118] Patients with a plasma aldosterone–to–plasma renin activity ratio less than 2 (defined as inappropriately low) had a significantly higher mortality rate (75%) than those with an plasma aldosterone–to–plasma renin activity ratio of 2 or more (46%; $P < 0.001$).[118]

Similar findings have been described in pediatric septic shock.[119,120] Inappropriately low aldosterone levels were demonstrated in a pediatric critically ill population encompassing 60 children. Of these patients, 31 were admitted with acute meningococcal disease, and 29 had other diagnoses (major surgery, severe respiratory infection). Plasma renin activity was measured in 15 participants with meningococcal disease, 80% of whom (12 of 15) had demonstrable aldosterone/plasma renin activity ratios of less than 2 on admission.[119]

Patients with meningococcal sepsis had mean plasma aldosterone levels of 427.5 ± 88.1 pg/ml (96.7% of values within the normal range for age for healthy children), whereas the levels were 1,489.2 ± 244.2 pg/ml ($P < 0.0001$) for the group with other diagnoses (59.3% of values above the normal range for age for healthy children).[119] The meningococcal sepsis group had a higher predicted risk of mortality compared with the nonmeningococcal sepsis group (32.3% *vs.* 9.4%) and had higher levels of serum cortisol.[119] Interestingly, those with the highest plasma aldosterone levels had the lowest cortisol measurements on admission. All participants survived.

Taken together, these studies suggest that inappropriately low aldosterone levels despite high plasma renin activity occur, much like evidence of inappropriately low cortisol and adrenal androgen secretion[118] may occur in the critically ill, potentially as an adaptive response aimed at increasing cortisol production. Recent studies suggesting the presence of this entity in the critically ill and its association with organ dysfunction have since been published.[76,121] Renin has been suggested as a marker of tissue perfusion and prognosis in the critically ill.[75]

The Role of Mineralocorticoid Supplementation during Critical Illness

Several clinical trials have examined the role of mineralocorticoid supplementation in critical illness. However, an adequately powered study comparing fludrocortisone to a matched control group has not yet been performed. Notably, the role of mineralocorticoid replacement in patients with septic shock is not discussed in recent consensus statements (Society of Critical Care Medicine, European Society of Intensive Care Medicine 2017).[58,122,123]

Annane *et al.*[124] investigated 300 patients with septic shock who had undergone a short corticotropin test, allocated to a 7-day period of treatment with a 50-mg intravenous bolus of hydrocortisone administered every 6 h and a 50-μg daily dose of nasogastrically delivered fludrocortisone or placebo. The patients were characterized as either responders or nonresponders to the corticotropin test. Of these, 229 were classified as nonresponders and 70 as responders (1 patient withdrew consent). Of the responders, 115 were randomized to the placebo group, and 114 were randomized to the corticosteroid group. Of the nonresponders, 34 were randomized to the placebo group, and 36 were randomized to the corticosteroid group. Treatment with low doses of hydrocortisone and fludrocortisone was found to significantly reduce the risk of death in patients with septic shock and relative adrenal insufficiency (so-called nonresponders; 73 deaths (63%) in the placebo group and 60 deaths (53%) in the corticosteroid group; hazard ratio, 0.67; 95% CI, 0.47 to 0.95; $P = 0.02$), suggesting that mineralocorticoid therapy combined with glucocorticoid therapy confers a mortality benefit in patients with septic shock. Apart from the methodologic concerns that have been raised regarding the utility and accuracy of the short synacthen test in critical illness, the bioavailability of a single dose of fludrocortisone administered in this study (50-μg tablet daily) is debatable. A single dose (50 μg) was shown to lead to undetectable plasma levels in one third of patients with septic shock in one study.[85] Mineralocorticoid insufficiency was not specifically reported.

Corticosteroid Treatment and Intensive Insulin Therapy for Septic Shock in Adults (COIITSS) was a randomized, 2 × 2 factorial trial with a primary objective of assessing the

efficacy of intensive insulin therapy in patients with septic shock treated with hydrocortisone and, as a secondary objective, assessing the benefit of fludrocortisone as an oral dose of 50 μg administered for 7 days. The study consisted of 509 patients with septic shock presenting with multiple organ dysfunction (Sequential Organ Failure Assessment score of 8 or more) who received hydrocortisone (50-mg bolus at 6-h intervals for 7 days). Of these, 245 received fludrocortisone in combination with hydrocortisone administered as a daily single oral dose (50 μg).

With regards to the secondary objective, 105 (42.9%) of 245 patients treated with fludrocortisone died, and 121 of 264 (45.8%) in the control group died (relative risk, 0.94; 95% CI, 0.77 to 1.14; $P = 0.50$). Neither aldosterone levels nor plasma renin levels were assessed in this study. However, the study was not adequately powered to detect a clinically relevant treatment effect, and no significant effect was observed.[125]

More recently, 1,241 patients from 34 centers were enrolled over a 7-yr period in the Activated Protein C and Corticosteroids for Human Septic Shock (APROCCHSS) trial. Overall, 614 patients were randomized to receive a combination of a 60-mg hydrocortisone IV bolus every 6 h and 50 μg of fludrocortisone *via* nasogastric tube for 7 days, whereas 627 patients received placebo.[1] Ninety day all-cause mortality at ICU discharge was lower among those who received hydrocortisone plus fludrocortisone than among those who received placebo (35.4% *vs.* 41.0%; $P = 0.04$). The hydrocortisone-fludrocortisone group also had more vasopressor-free and organ failure–free days. However, in this study, the role of fludrocortisone in improving outcomes was not well delineated for a number of reasons. Fludrocortisone was administered concurrently with high doses of hydrocortisone (240 mg daily). Such a dose of hydrocortisone would have sufficient mineralocorticoid activity. The relative mineralocorticoid potencies of hydrocortisone and fludrocortisone are 1 and 12, respectively, and a dose of 20 mg of hydrocortisone provides a mineralocorticoid effect equivalent to 0.1 mg of fludrocortisone. The mineralocorticoid effect of the dose of hydrocortisone given in the study would therefore have amounted to an equivalent of a total of 1.2 mg (1,200 μg) per day of fludrocortisone.[126] Clinical evidence for activation of the mineralocorticoid receptor at high doses of hydrocortisone also exists.[51,126] Additionally, few data exist on the bioavailability of multiple oral fludrocortisone dosing in patients with septic shock.[85]

The combined evidence from the above-mentioned studies indicates that the additive beneficial role of fludrocortisone to hydrocortisone is clearly challenging to delineate.[127] Notably, the mineralocorticoid receptor is permissive of the binding of various ligands. The action of cortisol on the mineralocorticoid receptor can be both as an agonist and an antagonist, depending on the underlying pathophysiology.[128] Under normal conditions, cortisol binds to the mineralocorticoid receptor but does not lead to its activation (mineralocorticoid antagonistic effect). In conditions of tissue damage and in the presence of reactive oxygen species, cortisol activates the receptor and acts as a mineralocorticoid agonist.[128]

Such variable effects of cortisol at receptor level may support the argument for concurrent fludrocortisone administration in critical illness; however, this contention requires support through investigations that will take into account the assessment of mineralocorticoid (dys)function (defined pathology), the bioavailability of orally administered fludrocortisone (effectively administered treatment), and more proximal endpoints such as shock resolution (well defined response to treatment).

Hyperreninemic Hypoaldosteronism in Trauma: Are Trauma Patients Mineralocorticoid-deficient?

The first demonstration of mineralocorticoid deficiency in a cohort of trauma patients was in a study consisting of 32 trauma patients admitted in hemorrhagic shock.[120] In this study, mineralocorticoid deficiency (plasma aldosterone–to–plasma renin activity ratio less than 2) was found in 48% of trauma patients at ICU admission.[120] Normal aldosterone levels were demonstrated in patients with and without mineralocorticoid deficiency; however, markedly elevated renin levels were demonstrable only in the mineralocorticoid-deficiency cohort ($P < 0.0033$).[120] Patients with mineralocorticoid deficiency were more likely to be hypotensive during the study period, required more blood products and crystalloids, and were at higher risk of acute kidney injury.[120] This was a single-center study limited by methodologic concerns in addition to the small sample size. All patients were administered etomidate, an adrenal mitochondrial 11β-hydroxylase inhibitor, for intubation. The enzyme 11β-hydroxylase is required for the conversion of both 11-deoxycortisol to cortisol and 11-deoxycorticosterone to corticosterone and subsequently to aldosterone and has been shown to contribute significantly to adrenal insufficiency in critical illness.[129]

Apart from sepsis and trauma, hyperreninemic hypoaldosteronism has since been described in murine models of hypoxia, chronic physiologic stress, and acute hemorrhage. These conditions have also been associated with a reduction in plasma aldosterone levels despite rising adrenocorticotropic hormone and renin levels.[130,131] The failure of aldosterone levels to increase after angiotensin II or adrenocorticotropic hormone infusions suggests that damage to the zona glomerulus is a cause of the hyperreninemic hypoaldosteronism syndrome, with recovery of normal plasma renin and aldosterone ratios in survivors, suggesting a reversible cause.[116,117] The clinical significance of pharmacologic reversal of these changes is not yet validated; however, early identification of these patients may help identify potential therapeutic avenues and improve prognostication.

Additional Theoretical Considerations in Critical Illness

The Role of Adrenocorticotropic Hormone

The role of adrenocorticotropic hormone in the regulation of aldosterone production is normally minimal but may become important under specific conditions.[22,132,133] Hypersecretion of adrenocorticotropic hormone has been demonstrated *in vitro* (cultured bovine cells) and may theoretically lead to reduced aldosterone synthesis by diverting precursors in this pathway to the production of cortisol in conditions of physiologic stress.[111] Using bovine zona glomerulosa cells exposed to various concentrations of adrenocorticotropic hormone, Braley *et al.*[111] demonstrated increased 17α-hydroxylase activity in zona glomerulosa cells, suggesting a shift from aldosterone to cortisol production. Histological and immunohistochemical studies suggest that a difference exists between bovine and human adrenal morphology and function, making the extrapolation of such data to humans debatable.[134] High levels of adrenocorticotropic hormone have been described in patients undergoing surgery and are seen in the acute phase of critical illness.[132,133] More recently, in critical illness, adrenocorticotropic hormone–cortisol dissociation has been described. Adrenocorticotropic hormone levels have been shown to be variable in various phases of disease, with the initial rise in acute disease being subsequently followed by low–adrenocorticotropic hormone levels in chronic critical illness.[114] Mechanisms responsible for this may include negative feedback inhibition mediated by increased *free* cortisol availability and suppression of adrenocorticotropic hormone release mediated by circulating bile acids in the acute phase of critical illness.[114]

Effects of Electrolytes and Fluid Shifts

Volume expansion may theoretically contribute to decreased adrenal production of aldosterone in some patients with critical illness. The stimulation of aldosterone release by potassium should also be considered.[22,26] A concurrent lack or relative lack of cortisol in some patients with adrenal insufficiency may stimulate ADH secretion, as a result of loss of negative feedback control, leading to further water retention and hyponatremia.[135–138]

Challenges Relating to the Diagnosis of Mineralocorticoid Insufficiency in Critical Illness

Previous investigators have shown that 40 to 65% of critically ill patients have high plasma renin activity and low plasma aldosterone concentrations.[117,118] The currently well accepted definition of critical illness–associated hyperreninemic hypoaldosteronism is defined by a plasma aldosterone–to–plasma renin activity ratio of less than 2, which corresponds to the 98th percentile of the control population.[115] This current definition is based on values used in non–critically ill patients with mineralocorticoid deficiency. There are currently no internationally accepted standardized methodologies of assessment, and reference material and reporting units for both aldosterone and renin also differ.[66,139,140] The diagnosis of hypoaldosteronism based on renin and aldosterone measurements using reference ranges obtained from non–critically ill populations poses a number of challenges.

Measurement of Plasma Renin Activity

The measurement of plasma renin activity is based on: (a) indirect measurement of the combined effect of angiotensinogen and renin; (b) indirect renin concentration measurement, which involves the *ex vivo* addition of angiotensinogen to the assay; and (c) more recently, direct immunoradiometric measurements (fig. 3).[141–143] Plasma renin activity has previously relied on the quantification of the cumulative generation of angiotensin I and not the direct quantification of renin *per se*.[142,143] Plasma renin activity is, in turn, reliant on the available levels of renin substrate (angiotensinogen) and measures the combined effect of angiotensinogen levels and renin.

Not much is known about levels of angiotensinogen in critical illness. Angiotensinogen, an α2-globulin, released primarily by the liver, has been shown to be reduced in liver dysfunction and congestive heart failure and to be elevated by factors such as adrenal insufficiency, corticosteroid therapy, and estrogen levels.[144,145] Higher circulating levels of angiotensinogen are found in women and in those on estrogen-replacement therapy and contraceptives.[142] This indicates that measurements of plasma renin activity overestimate circulating renin levels in the face of high levels of angiotensinogen in the assay.[145]

Low renin levels found in conditions of low angiotensinogen levels may underestimate the active renin concentration when plasma renin activity is measured.[146] When measured using direct assays, renin levels are shown to be suppressed in women compared with men, in premenopausal women more than postmenopausal women, and in postmenopausal women using estrogen therapy than in postmenopausal women without estrogen replacement.[145] Direct immunometric measurements of renin are currently used, although not widely.[141,142]

The implications of varying methods of plasma renin activity measurement in the critically ill setting are currently unclear; however, this variability in measurement methods highlights potential concerns with the comparative use of data from older studies where plasma renin activity was measured indirectly, as well as the effect that changes in the plasma concentration of angiotensinogen may have on angiotensin II synthesis and as a result, indirect renin concentration measurements.[117,147]

Plasma renin activity has been validated for use in non–critically ill patients for the investigation of primary

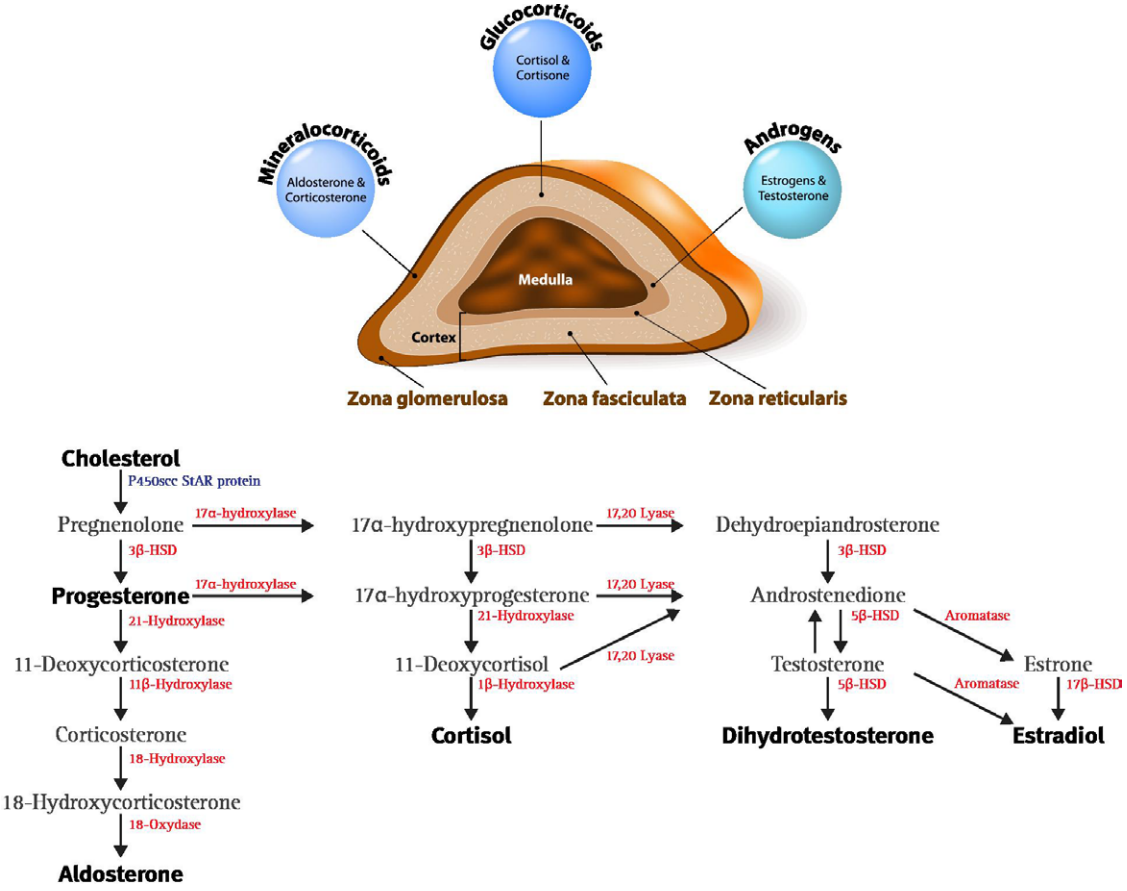

Fig. 3. Methods for renin measurement. Figure modified from Fischer *et al.*,[142] with permission from Oxford University Press and the European Society of Cardiology. HSD, hydroxysteroid dehydrogenase.

and secondary hypoaldosteronism. In the non–critical care setting, plasma renin activity and serum aldosterone measurements should be performed after 3 h in the upright or seated position, which increases renin and aldosterone release in normal individuals.[143] The normal concentration of plasma renin activity has been shown to be 1 pmol/l.[143] Currently, no reference ranges exist for the critically ill population.

Aldosterone Levels Are Difficult to Interpret in Isolation

In the setting of renal impairment, the use of homogenous immunoassays (which do not require a separation step to distinguish bound from unbound labeled antibody or antigen) has been shown to result in marked overestimation of aldosterone levels, possibly because of antibody cross-reactivity with uncleared aldosterone metabolites.[140] Aldosterone levels determined by high-performance liquid chromatography and tandem mass spectrometry are more accurate and should be used.[140,148] However, because of the cost implications and technical demands associated with high-performance liquid chromatography and tandem mass spectrometry systems, radioimmunoassays and automated immunoassays are still used in many laboratories, and radioimmunoassays are the method of choice.[139,149] Most importantly, there is currently no published standardized high-performance liquid chromatography and tandem mass spectrometry reference method or standard reference material available.[140] Such variability in analytic methods presents challenges to diagnosis and screening, as well as leading to uncertainty such as in the interpretation of outcomes from clinical investigations of aldosterone disorders.

Effect of Concurrent Pharmacotherapy

The use of drugs that impair aldosterone release, such as nonsteroidal antiinflammatory drugs, angiotensin inhibitors, calcineurin inhibitors, heparin, or β-adrenergic blockers has been discussed and should be considered as possible causes of hypoaldosteronism in the critically ill. The presence of preexisting disease such as chronic kidney disease, diabetes-associated hyporeninemic hypoaldosteronism, and HIV infection are other potential confounders of measurements occurring in critical illness.[150]

Other factors that may interfere with the assessment include differentiating preexisting hyporeninemic hypoaldosteronism from hyperreninemic hypoaldosteronism associated with critical illness and the prognostic and clinical implications of low renin levels in this population, as well as the effects of volume depletion or hypotension on plasma renin activity measurements. Patients with primary adrenal insufficiency are characterized by both low serum aldosterone and low cortisol concentrations but may have a high plasma renin activity because of concurrent volume depletion and/or hypotension.

Pathophysiology and Clinical Manifestations of Hypoaldosteronism

In patients without critical illness, a loss of mineralocorticoid activity results in dehydration associated with increased renal sodium excretion, metabolic acidosis, and hyperkalemia. Despite the role of aldosterone in sodium retention, hypoaldosteronism is not typically associated with prominent sodium wasting because there are compensatory mechanisms that help retain sodium, such as the effect of angiotensin II and norepinephrine.[69]

Unlike isolated aldosterone deficiency, there are a number of unique factors that affect aldosterone release, as well as sodium and water balance, in the critically ill population. These include the effects of drug therapy, such as pharmacologic inhibition of angiotensin II and impairment of aldosterone release by heparin therapy, calcineurin inhibitors, and nonsteroidal antiinflammatory drugs.

Therapeutic Implications

Fludrocortisone Replacement

Therapy with both glucocorticoids and mineralocorticoids is routinely instituted for primary adrenal insufficiency because these patients have low serum aldosterone and cortisol concentrations.[66] Either hydrocortisone, cortisone acetate, or dexamethasone can be used and should be titrated to clinical response (blood pressure, body weight, signs of glucocorticoid excess). Mineralocorticoid replacement should be instituted in those with a confirmed deficiency state with fludrocortisone (50 to 100 μg in adults).[66]

The benefits of fludrocortisone replacement in patients with septic shock are debatable and not yet well described. The pharmacokinetics of a single oral dose (50 μg) of fludrocortisone in adult patients with septic shock were investigated in a single-center study consisting of 21 adults.[85] Fludrocortisone plasma concentrations were measured by liquid chromatography–mass spectrometry tandem analysis, before and repeatedly until 18 h after the oral dose. Plasma levels were found to be highly variable and undetectable in one third of patients in this study. The pharmacokinetics of daily dosing and of higher doses of fludrocortisone, as well as other factors that may influence fludrocortisone bioavailability in the critically ill, still requires further investigation.

Novel Therapeutic Options

A number of therapeutic agents for the management of catecholamine-resistant shock are currently under investigation.

Angiotensin II Infusion Therapy. Currently, no pharmacologic agents in routine clinical use support the renin–angiotensin–aldosterone system in the treatment of septic shock. The addition of angiotensin II may be beneficial, however.[151] Angiotensin II for the Treatment of High-Output Shock 3 (ATHOS-3) was a phase III, multicenter, double-blind, randomized controlled trial, which aimed to compare the efficacy and safety of angiotensin II *versus* placebo in catecholamine-resistant shock.[151] The trial consisted of 321 patients.[4] The primary endpoint was a mean arterial pressure (MAP) of 75 mmHg or an increase in MAP by 10 mmHg within 3 h of infusion initiation. The average MAP was 66 mmHg. Patients with a low cardiac output were excluded from the study. Of the patients in the angiotensin group, 70% reached the primary endpoint compared with 23% of the placebo group. The angiotensin group had a reduced mortality (46%) compared with the placebo group (54%; $P = 0.12$) but was not powered for this endpoint. There was no difference in the frequency of adverse events between the groups.[4]

Because of the exclusion of patients with a low cardiac output, as well as the fact that the study was not powered to detect a mortality benefit, the mortality benefit and safety profile of angiotensin II infusion therapy in low cardiac output states is thus currently unknown. Angiotensin II as a potent vasoconstrictor will likely be appropriate because cotherapy in conditions in which cardiac contractility is reduced.[152,153] Additionally, as discussed, cross-talk exists between angiotensin II and aldosterone beyond the role of the latter in mineralocorticoid effects, the implications and clinical relevance of which remain uncertain. Angiotensin acts on the zona glomerulosa, stimulating the release of aldosterone, which leads to increased sodium reabsorption and a subsequent increase in extracellular and blood fluid volume.[154] Further cross-talk mechanisms between aldosterone and angiotensin II also appear to be important in the development of endothelial dysfunction, cellular proliferation, and hypertrophy and in diseases such as hypertension.[155]

Currently, the results of a randomized, placebo-controlled trial that aims to evaluate the effect of LJPC-501 (angiotensin II) infusion on mean arterial pressure, as well as the safety and tolerability of LJPC-501 in pediatric patients, are awaited.[156] Additionally, a number of experimental and clinical studies assessing novel agents that modulate the renin–angiotensin–aldosterone system in the management

of diseases such as congestive heart failure, myocardial ischemia, and hypertension are currently underway.[157]

A substantial change in our understanding of the renin–angiotensin–aldosterone system has occurred over the last decade. The classical components of the system have now been expanded to include new enzymes and receptors, bringing into perspective the possibility of new avenues for investigation.[61]

Conclusions

Hyperreninemic hypoaldosteronism associated with a high mortality rate has been previously described in critically ill patients with shock. Variable plasma levels of aldosterone are seen in critical illness, as well as an impaired adrenal aldosterone response to increased levels of renin. However, the assessment of hypoaldosteronism, as well as the role of mineralocorticoid replacement in the critically ill, remains a challenge, while the effect of angiotensin II in shock states remains untested.

Summary of Key Points

There is renewed interest in the role of renin–angiotensin–aldosterone in critical illness. The available evidence is limited compared with what is known about the glucocorticoid axis. Whether mineralocorticoid deficiency exists as a relevant pathophysiologic entity in critical illness and the role of fludrocortisone treatment remain unknown. Further studies investigating these issues are warranted.

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Competing Interests

The authors declare no competing interests.

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CHAPTER 7: Critical illness related mineralocorticoid insufficiency (CIRMI) - a new term for a concept worthy of further exploration: a narrative review

Review Article

CIRMI—a new term for a concept worthy of further exploration: a narrative review

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Background and Objective: Critical illness-related corticosteroid insufficiency (CIRCI) describes hypothalamic-pituitary-axis impairment during critical illness associated with three major pathophysiological events; dysregulation of the hypothalamic-pituitary-axis, altered cortisol metabolism, and tissue corticosteroid resistance. Similar changes are evident with regard to mineralocorticoid dysfunction in critical illness. Hyperreninemic hypoaldosteronism describes a sub-population of critically ill patients with an impaired adrenal aldosterone response to increased levels of renin. In the light of the recent demonstration of significant mortality improvements associated with adjunctive glucocorticoid treatment in combination with fludrocortisone in septic shock, and the suggestion that angiotensin II is effective in treating vasodilatory shock, the clinical relevance of mineralocorticoid dysfunction in critical illness requires further exploration. This interpretative review considers hyperreninemic hypoaldosteronism, a concept worth re-examining in the light of the potential mortality benefit of mineralocorticoid supplementation in critical illness. We compare the pathophysiological and clinical characteristics of CIRCI and hyperreninemic hypoaldosteronism, two syndromes that represent corticosteroid and mineralocorticoid dysfunction in critical illness. We highlight gaps in the literature and give novel insights into the limitations of assessment, diagnosis and treatment.

Methods: English language abstracts and articles published before June 2021 were identified through PubMed and Google Scholar. Randomized trials, observational studies, basic sciences studies, systematic and narrative reviews were considered. Reference lists of articles were searched for further relevant material.

Key Content and Findings: Difficulties are encountered in interpreting measures of gluco- and mineralocorticoid activity in critical illness. Aldosterone levels, like cortisol, have been shown to be increased in sepsis and hemorrhagic shock. The finding of hyperreninemia and hyperaldosteronism with an aldosterone/plasma renin activity ratio below 2 should prompt consideration of hyperreninemic hypoaldosteronism, a finding, which likely signifies the loss of negative feedback control of the renin-angiotensin-aldosterone system.

Conclusions: As there is evidence to suggest that in acute critical illness, hyperreninemic hypoaldosteronism, is associated with poor outcomes, co-administration of hydrocortisone with fludrocortisone in patients with septic shock should be considered. In keeping with the concept of CIRCI, we suggest the term critical illness-related mineralocorticoid insufficiency as a more appropriate description

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of the impaired aldosterone response to increased levels of renin seen in this group of patients.

Keywords: Adrenal insufficiency; critical illness; glucocorticoids; mineralocorticoids; corticosteroid insufficiency

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Background

Critical illness-related corticosteroid insufficiency (CIRCI) describes hypothalamic-pituitary-axis impairment during critical illness, arising from a concern that global tissue availability of cortisol is altered, which influences outcomes in the critically ill (1-4). A recent task team has described three major pathophysiological events in CIRCI; dysregulation of the hypothalamic-pituitary-axis, altered cortisol metabolism, and tissue corticosteroid resistance (1). Although there is no single test that can reliably diagnose CIRCI, a delta cortisol (change in baseline cortisol at 60 min of <9 µg/dL) after intravenous cosyntropin (250 µg) and a random plasma cortisol of <10 µg/dL may be used (5). Similar changes are evident regarding mineralocorticoid dysfunction in critical illness (6,7). One such aldosterone dysfunction syndrome is selective hypoaldosteronism, which describes a sub-population of critically ill patients with an impaired adrenal aldosterone response to increased levels of renin, and is defined by the finding of hyperreninemia and hyperaldosteronism with an aldosterone/plasma renin activity (ALDO/PRA) ratio below 2. Alternatively termed hyperreninemic hypoaldosteronism, selective hypoaldosteronism has been described in both hemodynamically unstable critically ill adult and pediatric patients with severe trauma and septic shock (8-11). Aldosterone deficiency/dysfunction syndromes have, however, not been well characterized in critical illness, and like corticosteroid replacement in CIRCI, the indication for mineralocorticoid replacement or modulation of the renin-angiotensin-aldosterone system in this group remains controversial.

In this interpretative review, we consider hyperreninemic hypoaldosteronism, a concept worth re-examining given the recent demonstration of significant mortality improvements associated with adjunctive glucocorticoid treatment in combination with fludrocortisone in septic shock (12). The suggestion that angiotensin II is effective in treating vasodilatory shock further highlights the potential role of therapeutic approaches targeting the renin-angiotensin-aldosterone system in septic shock (13).

Objective

We summarise and compare the pathophysiological and clinical characteristics of CIRCI and hyperreninemic hypoaldosteronism, two syndromes that represent glucocorticoid and mineralocorticoid dysfunction in critical illness. We explore diagnostic challenges encountered in interpreting measures of glucocorticoid and mineralocorticoid activity in the critically ill. Current gaps in the literature are highlighted and novel insights into the limitations of assessment, diagnosis and treatment are presented (11).

Furthermore, the term CIRCI is well established despite the associated diagnostic challenges. In keeping with the concept of CIRCI, we thus suggest the term critical illness-related mineralocorticoid insufficiency (CIRMI) as a more appropriate description of the impaired aldosterone response to increased levels of renin seen in this group of patients. We present the following article in accordance with the Narrative Review reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-5572/rc).

Methods

We identified source references through searches of PubMed and Google Scholar for English language abstracts and articles published before June 2021. Randomized trials, observational studies, basic sciences studies and systematic and narrative reviews were considered. We focused on mineralocorticoid dysfunction and critical illness. The keywords used include: the terms “adrenal”, “adrenal insufficiency”, “mineralocorticoid insufficiency”, “critical illness”, “aldosterone”, “critical illness corticosteroid insufficiency”, “stress response”, “renin-angiotensin-aldosterone-system”, “hyperreninaemia”, “hypoaldosteronism”, “hyperreninaemic hypoaldosteronism”, “glucocorticoid receptors” and “mineralocorticoid

Table 1 The search strategy summary

Items	Specification
Date of Search	The initial search was conducted on 12/10/2017
Databases and other sources searched	Google Scholar, PubMed
Search terms used	Terms used included "adrenal", "adrenal insufficiency", "mineralocorticoid insufficiency", "critical illness", "aldosterone", "critical illness corticosteroid insufficiency", "stress response", "renin-angiotensin-aldosterone-system", "hyperreninaemia", "hypoaldosteronism", "hyperreninaemic hypoaldosteronism", "glucocorticoid receptors" and "mineralocorticoid receptors"
Timeframe	English language abstracts and articles published before June, 2021
Inclusion and exclusion criteria	English language abstracts and articles
Selection process	GDN identified source references. Randomized trials, observational studies, basic sciences studies and systematic and narrative reviews were considered. Reference lists of original articles, narrative reviews, clinical guidelines, and previous systematic reviews and meta-analyses were searched for further relevant material. Citations from articles identified in these searches were also reviewed and included, where appropriate

receptors". These keywords were used as single search terms and in combinations. Reference lists of original articles, narrative reviews, clinical guidelines, and previous systematic reviews and meta-analyses were searched for further relevant material. Citations from articles identified in these searches were also reviewed and included, where appropriate. The search strategy summary is presented in *Table 1*.

Selected basic physiology

Challenges to the concept of adrenal functional zonation

The standard description of the mammalian adrenal is that of a gland that consists of a cortex and medulla (14). The adrenal cortex consists of the zona fasciculata, zona reticularis and zona glomerulosa. Each zone is understood to have a distinct role in secreting glucocorticoids, androgens and aldosterone respectively, an observation referred to as 'functional zonation' of the adrenal cortex (15,16). Such zonal differences in enzyme concentrations are generally understood to result in compartmentalization of enzymatic reactions (17). Individual zones are, however, not distinctly autonomous in their production of steroids (18,19). The zona glomerulosa; for instance, does not appear to have all the enzymes required for aldosterone biosynthesis (18). Aldosterone synthase expression is understood to be restricted to the zona glomerulosa; however, expression of the enzyme by a cell population of zona glomerulosa cells, as well as by the brain, blood vessels, and the heart, has been described (14,20-23). Such integrated functions imply that pathology affecting an adrenal zone such as that may occur in critical illness due to widespread inflammation, is more likely to have widespread effects affecting the entire adrenal cortex and its mediators, rather than be localised to one specific zone (18).

The zona reticularis, the innermost adrenal cortical layer, secretes androgens (dehydroepiandrosterone, dehydroepiandrosterone sulphate and androstenedione), which are not the focus of this review (24,25).

The stress response and steroid biosynthesis

Mechanisms involved in regulating the normal hypothalamic axis and the stress response in critical illness have been extensively reviewed (26-32). *Figure 1* illustrates the hypothalamic-pituitary-adrenal axis during acute critical illness.

Corticotrophin leads to increased synthesis of most steroidogenic pathway enzymes, and an increase in production and secretion of all adrenal steroids, a process termed steroidogenesis (3,36-38). With specific reference to aldosterone, aldosterone synthase (CYP11B2), primarily expressed in the zona glomerulosa, converts deoxycorticosterone to aldosterone through the intermediates, corticosterone and 18-hydroxycorticosterone (16,28,39,40). Further corticotrophin release is inhibited by a negative feedback mechanism (41-43). Adrenal stimulation through non-pituitary mechanisms occurs as a result of direct adrenal stimulation by cytokines, adipokines, neuropeptides and other mediators (42,43). Corticotrophin levels are typically low in acute critical illness due to factors

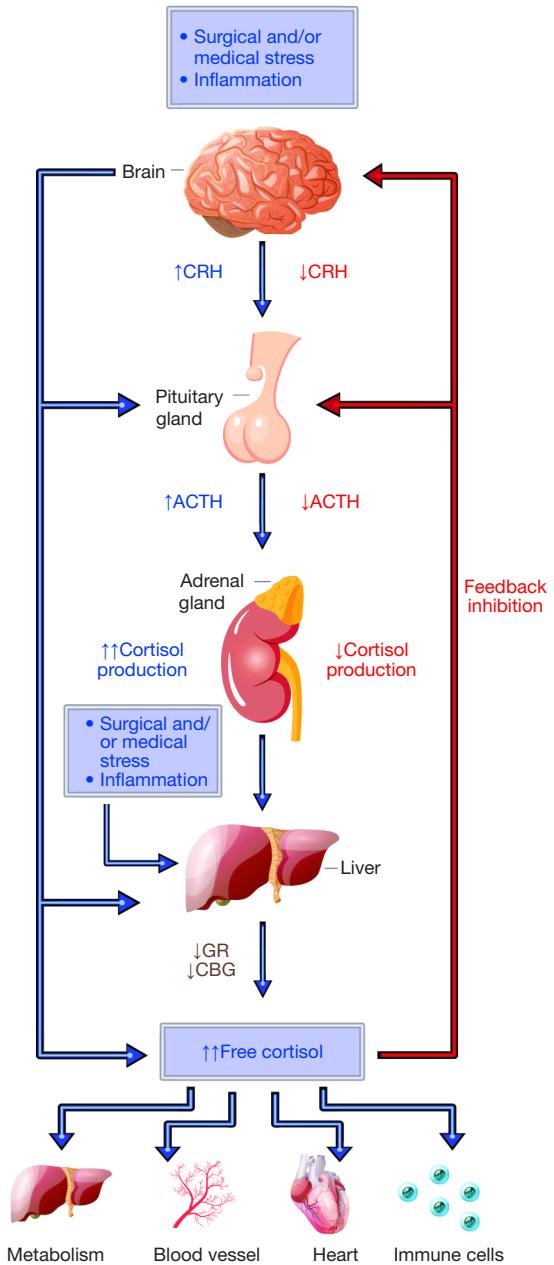

Figure 1 The hypothalamic-pituitary-adrenal axis during acute critical illness. CRH mediates the release of ACTH from the pituitary in response to stress and/or inflammation. ACTH, in turn, results in increased cortisol production from the adrenal gland. The increase in pro-inflammatory mediators during acute inflammation results in a reduction in CBG and plasma albumin and/or downregulation of hepatic GR, with a subsequent increase in free cortisol levels (33-35). CRH, Corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; CBG, cortisol binding globulin; GR, glucocorticoid receptors.

such as negative feedback inhibition because of high free cortisol levels seen in critical illness (44). This has been demonstrated up to day 28 of illness. Importantly in one study, even when cortisol levels begin to decrease if illness persists beyond 4 weeks, corticotrophin levels did not rise until later during recovery (44). The low corticotrophin seen in acute critical illness is of interest as experimental evidence suggests that corticotrophin is required for normal aldosterone production (45). Reduced levels of aldosterone have been shown in a pro-opiomelanocortin-knockout mouse model, suggesting a mechanism through which derangements in cortisol physiology are associated with derangements in aldosterone physiology during critical illness (45).

Chronically, the effects of corticotrophin on aldosterone secretion appear to oppose those of acute corticotrophin secretion (46). Plasma levels of aldosterone decline and, histologically, chronic corticotrophin (≥2 days) results in adrenal hyperplasia, increased conversion of zona glomerulosa cells to zona fasciculata cells, atrophy of the adrenal zona glomerulosa, and the resultant diversion of precursors from the mineralocorticoid pathway to the glucocorticoid pathway (28,46). The duration of the pathophysiological derangement should thus be taken into consideration if mineralocorticoid supplementation or antagonism is to be employed.

Regulation of aldosterone secretion, the mineralocorticoid receptor and mineralocorticoid activity

Classically, aldosterone, angiotensin II and renin regulate extracellular fluid balance, sodium and potassium (46,47). Other peptides and receptors have also been demonstrated to be involved in regulating aldosterone release (48,49). The regulation of the renin-angiotensin-aldosterone system is demonstrated in *Figure 2*. Variations in plasma/serum levels of aldosterone additionally occur within different populations, as well as across age and gender (7,22,47). Interestingly, corticotrophin does not appear to play as significant a role in regulating aldosterone secretion as it does in cortisol secretion (28,46,47,50). Additionally. pharmacological agents such as heparin, adrenaline and dopamine, as well as atrial natriuretic peptide, have been shown to modulate aldosterone secretion (21,46).

The mineralocorticoid receptor is co-expressed with the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in epithelial tissue, and in certain types of non-epithelial tissue such as the human heart (51,52).

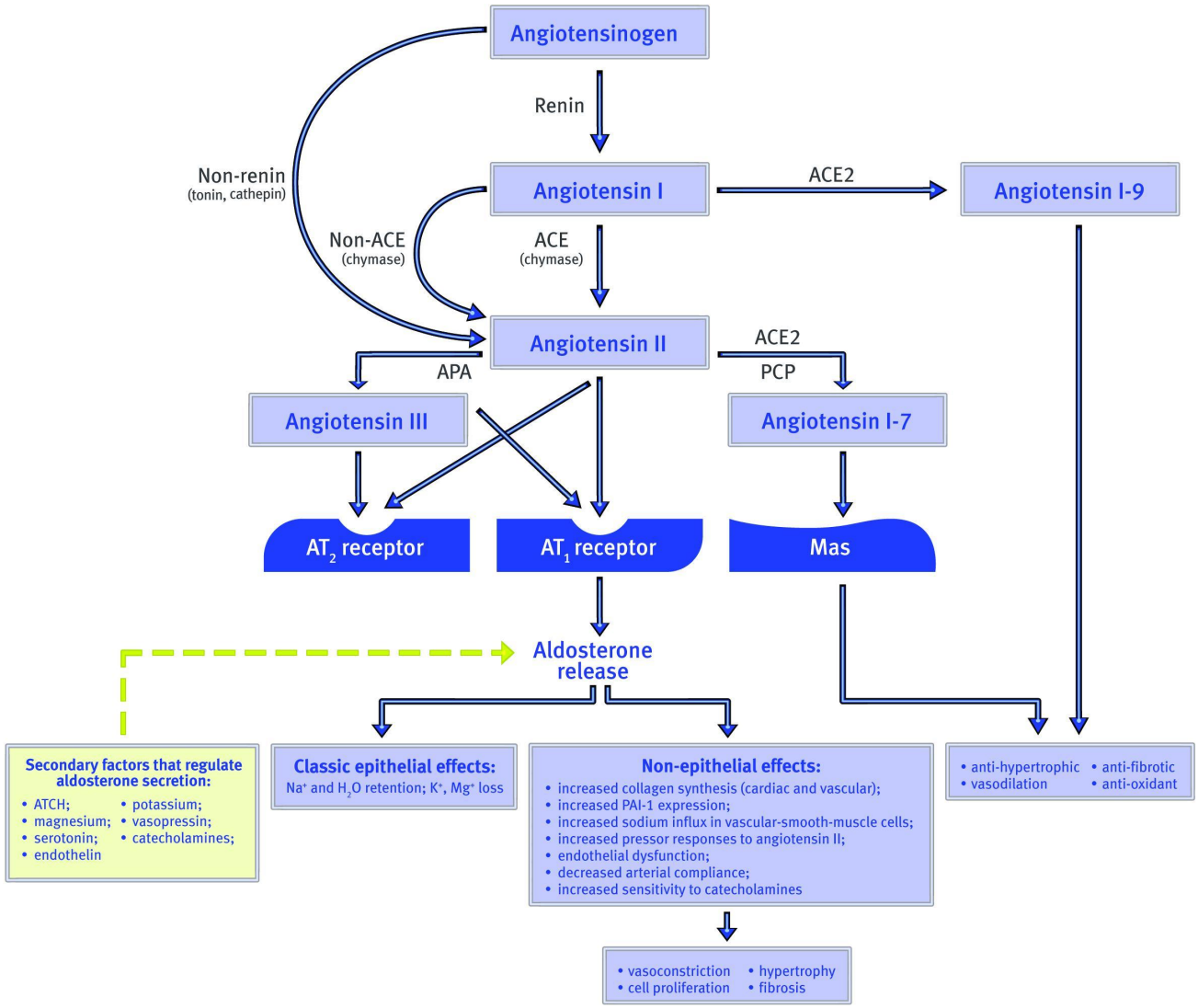

Figure 2 Renin Angiotensin Aldosterone System. Angiotensin I, a result of the conversion of angiotensinogen by the action of renin, is converted to angiotensin II through the action of angiotensin converting enzyme (ACE). Further peptides and receptors have since been demonstrated to be involved in the cascade that regulates aldosterone release. ACE, angiotensin converting enzyme; ADH, antidiuretic hormone; APA, aminopeptidase A; PCP, prolyl carboxypeptidase; Mas, mitochondrial assembly receptor; AT_1 receptor, angiotensin II type 1 receptor; AT_2 receptor, angiotensin II type 2 receptor; K^+, potassium; Mg^{2+}, magnesium.

The inhibition of 11β-HSD2, an intracellular isoenzyme, which converts cortisol to cortisone, allows cortisol to function as a mineralocorticoid receptor agonist (52,53). In aldosterone-selective tissues, 11β-HSD2, prevents cortisol binding to mineralocorticoid receptors, rendering mineralocorticoid-responsive tissues such as the kidney, sensitive to mineralocorticoids only (54-57). Defective 11β-HSD2 activity has been demonstrated in inflammatory states, and in the syndrome of apparent mineralocorticoid excess, signifying enhanced glucocorticoid activation of renal mineralocorticoid receptors (52,58,59). To the contrary, pro-inflammatory cytokines upregulate 11β-HSD1. This balance of reduced 11β-HSD2 activity with upregulated 11β-HSD1 activity is predicted to increase local glucocorticoid concentrations.

In contrast to cortisol, aldosterone is not bound to plasma proteins, rendering it less affected by alterations in protein binding and plasma protein concentrations that are observed

in critical illness (47). Effects of aldosterone are mediated through mineralocorticoid and non-mineralocorticoid receptors and are understood to be regulated by both genomic and non-genomic mechanisms (53,56,60). Genomic mechanisms were previously understood to be mediated exclusively through the nuclear pathways, translation and transcription, which take several hours to be effective and are mediated via the mineralocorticoid receptor (46,61). Rapid genomic mechanisms, that are activated within minutes, also exist (61,62). Aldosterone additionally operates via non-genomic mechanisms, activated by second messenger pathways (61,63).

Interestingly, aldosterone and estrogens, but not cortisol or corticosterone, have been shown to activate the transmembrane G-protein-coupled receptor 30 (GPR30 receptor), a receptor demonstrated to be involved in non-mineralocorticoid receptor-mediated rapid aldosterone actions (63-66). GPR30 has been postulated to result in the activation of phosphatidylinositol 3-kinase-dependent contraction and extracellular signal-regulated kinase activation in vascular smooth muscle cells (63) More recently, G-protein-coupled estrogen receptor-1 (GPER-1), as well as angiotensin receptor type 1 (AT1), have been demonstrated to play a role in aldosterone-mediated rapid effects (61). Thus aldosterone mediates its rapid actions through both mineralocorticoid and mineralocorticoid-independent pathways (61,63,66). Furthermore, there is evidence to suggest that rapid aldosterone actions are involved in the regulation of classical genomic actions (65). Genomic and non-genomic effects of aldosterone and cortisol are demonstrated in *Figure 3*.

Notably, aldosterone has been demonstrated to achieve greater transactivation than other ligands at the mineralocorticoid receptor (46). On the contrary, the effects of cortisol on the mineralocorticoid receptor depend on the underlying disease process. Cortisol can act as both an agonist and an antagonist at the mineralocorticoid receptor (67). Mineralocorticoid antagonistic effects, which are when cortisol binds to the mineralocorticoid receptor, but does not result in its activation, occur under physiological conditions. In conditions of high oxidant stress, cortisol acts as a mineralocorticoid receptor agonist (67). Therefore, although cortisol has similar affinity for mineralocorticoid receptors as aldosterone, the understanding that the resultant functions thus overlap may be inaccurate due to the molecular structural versatility of cortisol, the variable effects of cortisol at the mineralocorticoid receptor, and the differences in downward signalling, between the two ligands, aldosterone and cortisol, at the mineralocorticoid receptor (64). These variations in receptor utilisation support the need for further investigations on the additive beneficial role of fludrocortisone in combination with hydrocortisone in critical illness (68).

Changes in adrenal physiology with critical illness

Cortisol metabolism, tissue glucocorticoid activity and sensitivity in critical illness

The reduction in plasma albumin and corticosteroid binding globulin, altered protein binding, downregulation of hepatic glucocorticoid receptors, increased volume of distribution and altered 11β-HSD2 activity that occurs in critical illness, results in an increase in the free fraction and bio-availability of cortisol (53,64,69,70). Furthermore, a dissociation between total and free cortisol concentrations occurs over time in acute illness (70). Glucocorticoid tissue action is determined by a combination of tissue cortisol bioavailability, tissue glucocorticoid receptor density and glucocorticoid intracellular metabolism (59,71).

The glucocorticoid receptor is cytosolic in its unbound form, is expressed in almost every human cell and regulates genes that affect metabolism and the immune system (71). The receptor is involved in the adaptation to physiological stress, with the receptor-glucocorticoid complex regulating the expression of anti-inflammatory proteins in the nucleus or suppressing the expression of pro-inflammatory proteins in the cytosol. Inflammation and cytokines significantly affect glucocorticoid receptor number and function (72-74). A decrease in both binding activity and glucocorticoid receptor-mediated transcription, have been shown in acute respiratory distress syndrome (75). On the contrary, however, increased glucocorticoid receptor expression and binding capacity have been demonstrated in burn injury (73). The demonstration of reduced cortisol metabolism in sepsis implies increased exposure to cortisol at tissue level, suggesting that lower doses of hydrocortisone than previously advocated, may be sufficient if steroid supplementation is to be used (4,59,76). A mortality benefit of corticosteroid supplementation was not demonstrated, however, in the *Adjunctive Corticosteroid Treatment in Critically Ill Patients with Septic Shock* (*ADRENAL*) trial (77). In this context, *in vitro* glucocorticoid sensitivity has been shown to be associated with disease severity and to be highly

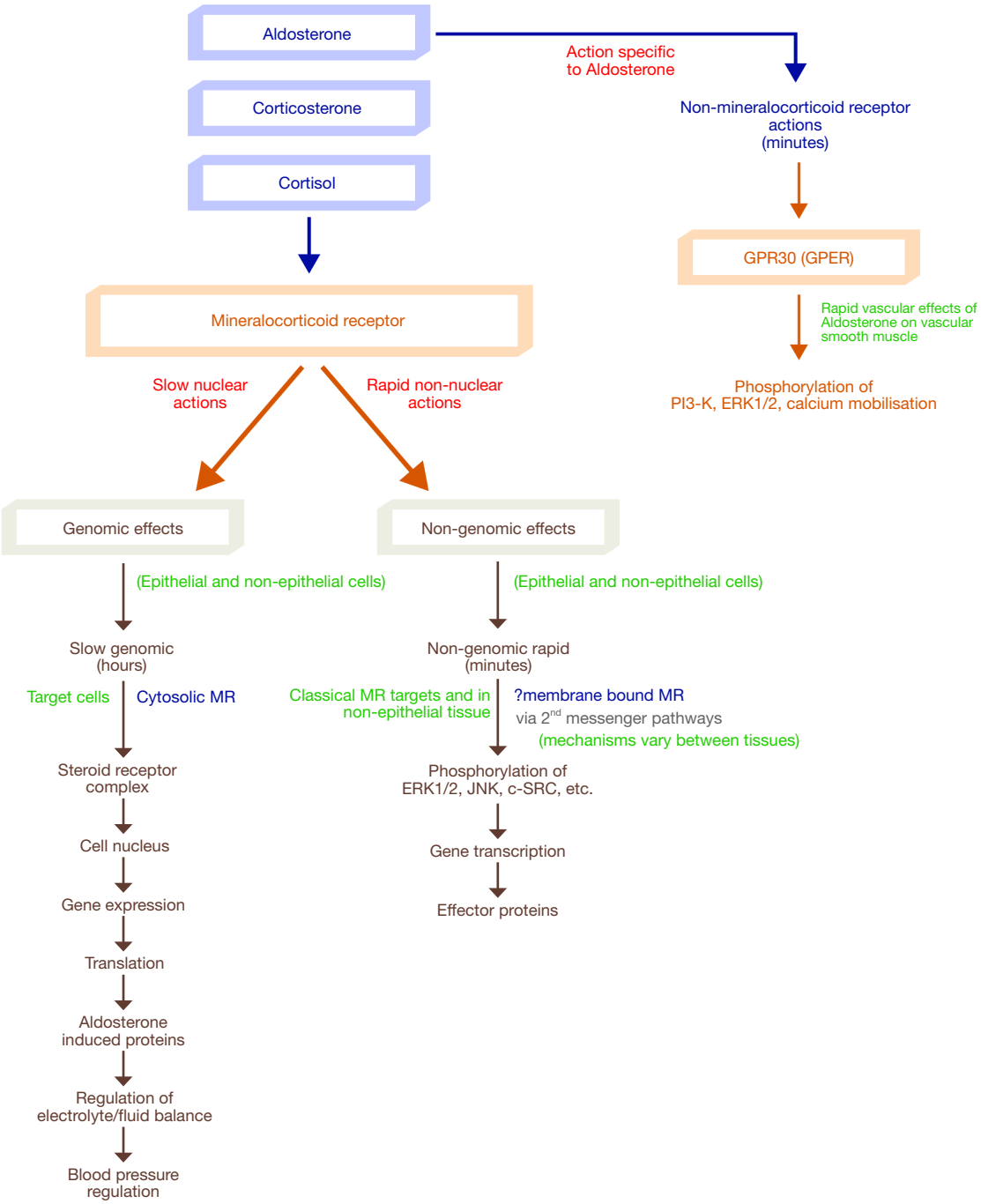

Figure 3 Aldosterone action; genomic and non-genomic effects of aldosterone and cortisol. Classical slow genomic actions mediated through the MR result in gene transcription and the production of effector proteins. Fast actions mediated through a surface receptor, which interact with the classic genomic actions, have been recently described to be mediated through the G-protein coupled receptor GPR30(GPER) and/or possibly through a membrane bound mineralocorticoid receptor. Aldosterone action through GPR30(GPER) is currently understood to be specific for aldosterone. GPR30, G protein-coupled receptor 30; PI3-K, Phosphatidylinositol 3-kinase; ERK1/2, extracellular-signal regulated kinase 1/2; JNK, c-Jun NH2-terminal kinase; c-SRC, non-receptor tyrosine kinase c-SRC protein; BP, blood pressure; MR, mineralocorticoid receptor.

variable in both healthy and critically ill individuals, being influenced by genetic and disease-related factors (78-83). The association of *in vitro* glucocorticoid sensitivity with clinical outcome has not yet been shown, however, regardless of whether glucocorticoid therapy has been employed.

Acute and chronic renin-angiotensin-aldosterone system activation and the role of aldosterone antagonism

A direct correlation between hyperaldosteronism and mortality has been demonstrated and is characteristic of congestive heart failure (84,85). The *Randomized Aldactone Evaluation Study*, was a double-blind randomized controlled trial, which enrolled 1,663 heart failure patients with ejection fraction <35%, New York Heart Association class III or IV, who were already on an angiotensin converting enzyme inhibitor, a loop diuretic, and, in most cases, digoxin therapy (86). In these patients, spironolactone, reduced the risk of death from any cause by 30% (relative risk 0.7, 95% CI: 0.6 to 0.82, P<0.001) and hospitalization from worsening heart failure by 35% (RR 0.65, 95% CI: 54 to 77, P<0.001) (86). Similarly, Pitt and colleagues conducted *The EPHESUS trial*, a large multicenter randomized controlled trial that included patients with an ejection fraction <40% with clinical features of heart failure or diabetes, who were randomized 3–14 days post-myocardial infarction to receive eplerenone or placebo (87). All-cause mortality (RR =0.85, 95% CI: 0.75 to 0.96, P=0.008) and the combined end-point of death from any cause or cardiac hospitalization were significantly reduced in the treatment group (relative risk =0.87, 95% CI: 0.79 to 0.95, P=0.002) (87). Similar and further evidence, linking aldosterone antagonism to mortality improvement in heart failure has led to the generalised assumption that aldosterone antagonism is associated with improved mortality outcomes in critical illness (84,86,88). Chronic mineralocorticoid excess has indeed been demonstrated to lead to hypertension, to promote pro-inflammatory, pro-atherogenic and pro-thrombotic mechanisms leading to cardiac and other visceral fibrosis, fluid retention and congestion (61,84,88,89). In contrast, the acute activation of the renin-angiotensin-aldosterone system as well as the aldosterone neurohormonal mechanisms, which are seen in the acute phase of septic or hemorrhagic shock, are compensatory components of the acute stress response (90-92).

Elevated plasma renin activity associated with relatively low aldosterone levels has been investigated as a marker of perfusion and prognosis (8,93-95). Such a state of selective hypoaldosteronism, featuring a reduction in plasma aldosterone levels, despite rising corticotrophin and renin levels, suggests a dissociation between plasma renin and aldosterone (90,96). Commonly associated with hypotension, hyperreninemic hypoaldosteronism is interpreted to represent a state of aldosterone deficiency (11,93,97).

The etiology of hyperreninemic hypoaldosteronism is hypothesised to be adrenal dysfunction in a subset of patients with CIRCI (11,93,97). As the zona glomerulosa does not appear to have all the enzymes required for aldosterone biosynthesis (18) substrates are likely diverted away from aldosterone production in the promotion of cortisol production during critical illness, with the hyperreninemia likely a subsequent result of relative hypoadrenalism. Proposed etiological mechanisms are discussed in relation to CIRCI. Currently available data from adult and pediatric populations are contrasted and critically synthesised and an interpretation of the findings offered in *Table 2*. Parallels with CIRCI are, however, apparent.

A comparison of critical illness-related corticosteroid insufficiency and hyperreninemic hypoaldosteronism

Altered regulation of cortisol and aldosterone

Dysregulation of the hypothalamic-pituitary-adrenal axis associated with variable cortisol levels, altered cortisol metabolism, and tissue resistance to glucocorticoids are considered the three major constituents of CIRCI (1,5,27). Altered cellular glucocorticoid metabolism and specifically reduced cortisol metabolism in sepsis have been well described elsewhere (1,4,58,99).

Hypoaldosteronism is classified as (I) defective stimulation of aldosterone secretion, (II) primary defects in adrenal synthesis or secretion of aldosterone, and (III) aldosterone resistance (100). Clinically, defective aldosterone action manifests as decreased effective blood volume, orthostatic hypotension and shock. Laboratory characteristics such as hyponatremia, hyperkalemia and metabolic acidosis are likely to be present. Patients with primary adrenal insufficiency exhibit low serum cortisol and aldosterone concentrations, but may have high plasma renin activity in the setting of concurrent volume depletion and/or hypotension.

With regards to hyperreninemic hypoaldosteronism, an impaired adrenal aldosterone response to elevated renin

Table 2 Summary of published data from adult and pediatric populations on hyperreninemic hypoaldosteronism in critical illness

Author, year	Subjects	Summary findings
Zipser *et al.*, 1981 (11).	Twenty-eight critically ill patients with persistent hypotension, hospitalised in a medical intensive care unit (11)	The first description of hyperreninemic hypoaldosteronism in hemodynamically-unstable, critically ill patients
		Plasma renin activity found to be elevated in all participants (21.6±7.2 ng/mL·h), with low plasma aldosterone (1–9 ng/dL) in a subset of 18 patients with septic shock
		A spectrum of aldosterone responsiveness in 18 patients with persistent hypotension and a higher mortality rate (78%) was described
		A defect at the level of the zona glomerulosa was suggested by the lack of an aldosterone response to angiotensin II or corticotrophin in this subset (11). The study is limited, by its small sample size. and the subsequent advancements that have occurred with regard to aldosterone and renin level measurement
Findling and colleagues, 1987	Eighty three critically ill patients	A dissociation between plasma renin and aldosterone levels was found in 24 patients (8). Mean aldosterone levels of 19±5 ng/dL were found in those with an impaired aldosterone response to renin. This, was in comparison to mean aldosterone levels of 48±6 ng/dL in those with an appropriate renin response to aldosterone (8)
		A dissociation between plasma renin and aldosterone levels associated with a higher mortality was found in 24 patients. This may represent adrenal adaptation aimed at promoting cortisol production
		Similar findings have been observed in pediatric patients with septic shock (9,10)
Lichtarowicz-Krynska and colleagues, 2004	Sixty critically ill patients (31 with acute meningococcal disease, 29 twenty-nine with other diagnoses, including major surgery and severe respiratory infection)	Plasma renin activity measured in 15 participants with meningococcal disease. Of these 80% (12 of the 15) had aldosterone/plasma renin activity ratios <2 on admission (9)
		Patients with meningococcal sepsis had mean plasma aldosterone levels of 427.5±88.1 pg/mL (96.7% of values within the normal healthy age range), with levels of 1,489.2±244.2 pg/mL (P<0.0001) for the group with other diagnoses (59.3% of values above the normal healthy age range) (9)
		Compared to the non-meningococcal sepsis group, the meningococcal sepsis group had higher levels of serum cortisol and a higher predicted risk of mortality (32.3% *vs.* 9.4%) (9)
		Low aldosterone levels observed in this pediatric setting
		Of interest, those with the highest plasma aldosterone levels had the lowest cortisol measurements on admission
Tolstoy and colleagues, 2013.	Thirty-two trauma patients with hemorrhagic shock. Prospective observational study aimed at investigating the prevalence and impact of mineralocorticoid deficiency following hemorrhagic shock	Study conducted in an urban level I trauma centre over a 6-month period
		Blood samples for measurement of plasma aldosterone (PA) and renin (PR) (radioimmunoassay) were obtained on admission and at 8, 16, 24, and 48 hours
		Mineralocorticoid deficiency was defined as a plasma aldosterone/PR of ≤2
		Mineralocorticoid deficiency, was observed on admission, in 48% of patients (10)
		Markedly elevated renin levels were observed in the mineralocorticoid deficient cohort (10). In this study, patients with mineralocorticoid deficiency were more likely to be hypotensive, required more blood products and crystalloids and were at higher risk of acute kidney injury (10)

Table 2 (*continued*)

Table 2 (*continued*)

Author, year	Subjects	Summary findings
Chung and colleagues, 2017	Hundred and five patients with septic shock evaluated and observed PRA as a useful prognostic biomarker of 28-day mortality (98)	Blood samples were analysed on days 1, 3, and 7 for plasma aldosterone concentration, PRA and plasma aldosterone concentration/PRA ratio, cortisol and C-reactive protein
		Participants were divided into survivors (n=59) and non-survivors (n=46), according to 28-day mortality
		Lower PRA, plasma aldosterone concentration, Acute Physiologic and Chronic Health Evaluation (APACHE) II scores, and Sequential Organ Failure Assessment (SOFA) scores were observed in the survivor group (all $P<0.05$)
		The group with PRA ≥3.5 $ng \cdot mL^{-1} \cdot h^{-1}$ on day 1 showed significantly higher mortality than the group with PRA <3.5 $ng \cdot mL^{-1} \cdot h^{-1}$ (log-rank test, $P<0.001$) (98)
		Hyperreninemic hypoaldosteronism found in 55.2% of patients with septic shock, was not observed to be correlated with clinical outcome (renal failure, ventilator-free days, ICU-free days, and 28-day mortality)
		Findings support evidence for prolonged plasma renin activation, as a potential prognostic indicator in septic shock (98)
		Failure of aldosterone levels to increase after angiotensin II or corticotrophin infusions suggests damage to the zona glomerulosa
		Changes have been observed to be reversible in survivors (11,97)

levels has been demonstrated in critically ill patients, but not low plasma levels of aldosterone *per se*, with no definitive link of clinical translation to poor outcomes as yet (8,9).

Normal aldosterone levels have been demonstrated in patients with and without mineralocorticoid deficiency, whereas, markedly raised renin levels have been demonstrated only in those that are mineralocorticoid deficient (defined by a PA/PRA ratio of less than 2) (10). In critical illness, hypoaldosteronism is not typically associated with prominent sodium wasting due to compensatory mechanisms that promote sodium retention such as the effect of angiotensin II and norepinephrine (101). The findings of both reduced and elevated plasma aldosterone levels, despite rising corticotrophin and renin levels as seen in hyperreninemic hypoaldosteronism, suggest that hyperreninemia may be a sign of inadequate aldosterone activity for the severity of illness (90,96). Damage to the zona glomerulosa, as suggested by the lack of an aldosterone response to angiotensin II or corticotrophin infusions, the apparent loss of negative feedback regulation and tissue mineralocorticoid resistance may also be contributory (11,47,97).

Clinical features of adrenal insufficiency are often non-specific, and those of mineralocorticoid insufficiency may overlap with those of cortisol insufficiency (18,102). Currently accepted clinical features of CIRCI include fever and asthenia, as well as refractory hypotension, neuromuscular dysfunction, electrolyte and metabolic abnormalities and gastrointestinal dysfunction, which manifests as anorexia and intolerance to enteral feeding (5). Similarly hyperreninemic hypoaldosteronism may manifest as part of hypoadrenalism with similar findings of fever, asthenia and fatigue, as well as orthostatic hypotension and shock, and electrolyte and metabolic abnormalities (10,93).

Clinical characteristics of CIRCI (5) and hyperreninemic hypoaldosteronism (CIRMI) likely reflect the current lack of definitive data and understanding of both of these conditions. The significant overlap that exists between these two conditions is apparent (5). Clinical features of hyperreninemic hypoaldosteronism (CIRMI) are presented in *Tables 2-4*.

Evidence of peripheral resistance

Both glucocorticoid and mineralocorticoid receptors are homologous, compete for the same ligands, form homodimers and heterodimers together, bind a number of the same hormone response elements on DNA, and share co-regulatory proteins required for efficient gene transcription (64). Splicing of the human glucocorticoid receptor gene results in the generation of two glucocorticoid

Table 3 Clinical features of hyperreninemic hypoaldosteronism

System	Recognised clinical features of hyperreninemic hypoaldosteronism
General features	Hypoaldosteronism may manifest as part of hypoadrenalism with findings such as fever and asthenia. Hyperpigmentation is a feature of chronic hypoaldosteronism
Cardiac	Decreased effective blood volume, Orthostatic hypotension and shock (10,93)
	Arrhythmia from hyperkalemia (103)
Electrolyte and Metabolic	Hyponatremia
	Hyperkalemia (102)
	Metabolic acidosis
Neuromuscular	Hypothalamic or pituitary gland necrosis or hemorrhage
	Weakness, muscle cramps and fatigue
Abdominal	Adrenal gland hemorrhage or necrosis
	May manifest as part of hypoadrenalism
	Abdominal discomfort and salt craving (102)

receptor isoforms, namely glucocorticoid receptor-α and glucocorticoid receptor-β. Glucocorticoid receptor-α, the classic glucocorticoid receptor, resides primarily in the cytoplasm where it is expressed ubiquitously. Glucocorticoid receptor-β is expressed ubiquitously, but does not bind glucocorticoid agonists (104). In sepsis, reduced glucocorticoid receptor-α and increased glucocorticoid receptor-β density and transcription in various tissues occurs. The upregulation of glucocorticoid receptor-α has been shown to augment glucocorticoid activity, while glucocorticoid receptor-β has been shown to inhibit glucocorticoid-α activity (105). Even with elevated plasma cortisol concentrations, inadequate tissue glucocorticoid receptor-α-mediated anti-inflammatory activity is postulated to lead to glucocorticoid resistance (5). However, separate mineralocorticoid and glucocorticoid effects exist and their primary ligands, aldosterone and cortisol (or corticosterone), are regulated differently and serve distinct purposes (64).

Little concordance exists between currently available methods of assessing glucocorticoid sensitivity, while inter-individual differences in glucocorticoid receptor sensitivity may be tissue specific (106). Moreover, although changes in cellular glucocorticoid metabolism, in particular decreased cortisol metabolism, have been observed in sepsis, (4) tissue corticosteroid bioavailability has not been consistently shown to predict clinical outcome in critical illness.

Similarly, concerning aldosterone, reduced adrenal aldosterone production and stress-induced hypersecretion of corticotrophin with activation of the renin-angiotensin aldosterone system has been demonstrated in shock (107). Additionally, up to 40% to 65% of critically ill patients have high-plasma renin activity and relatively low-plasma aldosterone concentrations. This dissociation between plasma renin and aldosterone levels is interpreted to represent relative aldosterone deficiency (8,11,27,97). A proposed schematic representation of the hypothalamic-pituitary axis and key mechanisms in hyperreninemic hypoaldosteronism are demonstrated in *Figure 4*.

Challenges with the confirmation of deficiency states

Challenges with the diagnosis of CIRCI, based on the administration of synthetic corticotrophin, have been detailed elsewhere (2,5). Briefly, as baseline total plasma cortisol levels are often variable in critical illness, currently accepted basal and stimulated cortisol levels, which were developed in healthy, non-stressed subjects pose difficulties (108). Thus, a single adrenocorticotropic hormone stimulation test does not reveal adrenal insufficiency in septic shock (109). Additionally, the decline in plasma binding protein that occurs may result in a decrease in total cortisol levels (70,99). Elevated serum cortisol levels in critical illness raise the concern that lack of a total cortisol response to corticotrophin may be an appropriate negative feedback response of a hypercortisolemic state (4,41). Furthermore, considerably

Table 4 Comparison between CIRCI and hyperreninemic hypoaldosteronism

Defining Characteristics	CIRCI	Hyperreninemic hypoaldosteronism-proposed CIRMI
Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis	Loss of negative feedback demonstrated. Reduced corticotropin levels demonstrated likely a result of central adrenocortical suppression	Stress-induced hypersecretion of corticotrophin demonstrated with activation of the renin-angiotensin aldosterone system in shock
		Dissociation of aldosterone and renin shown, implying loss of negative feedback or aldosterone resistance
		Inadequate comparative data
	Challenges of the definition of a deficiency state identified	Challenges of the definition of a deficiency state identified
Altered hormone metabolism	Variable levels of cortisol have been described in critical illness, in different populations and different critical illness diseases	Variable levels of aldosterone and renin action have been described in critical illness, in different populations and different critical illness diseases
	Altered cortisol metabolism has been demonstrated	Dissociation of aldosterone and renin shown, implying loss of negative feedback or aldosterone resistance
	Plasma clearance of cortisol has been shown to be markedly reduced during critical illness	Inadequate comparative data on altered aldosterone metabolism demonstrated
	Confirmatory/diagnostic testing is debatable and is not agreed upon	Confirmatory/diagnostic testing is debatable and is not agreed upon
Tissue resistance to glucocorticoids	Proof of cortisol resistance in critical illness exists- (Changes in glucocorticoid receptor-α and -β expression, decreased clearance of cortisol, 11-β hydroxysteroid dehydrogenase changes)	Minimal evidence of proof of aldosterone resistance in critical illness exists
		Dissociation of aldosterone and renin shown, implying loss of negative feedback or aldosterone resistance

CIRCI, critical illness-related corticosteroid insufficiency; CIRMI, critical illnessrelated mineralocorticoid insufficiency.

raised plasma cortisol levels are generally noted in non-survivors of critical illness and less so in survivors, a finding thought to be inconsistent with the concept of insufficiency (110-114). However, glucocorticoid resistance may be present despite high plasma cortisol levels, much like insulin resistance is tethered to hyperinsulinemia (5,115). Additionally, concerning cortisol measurement, ultra-high frequency tandem mass spectrometry may be more reliable, as variability exists in standard assays (116,117).

As with CIRCI, challenges exist regarding the diagnosis of mineralocorticoid dysfunction in critical illness. Critical illness-associated hyperreninemic hypoaldosteronism is defined by a PA/PRA ratio <2, a definition based on values used in non-critically ill patients with mineralocorticoid deficiency. This corresponds to the 98th percentile of the control population (93). Low aldosterone levels, relative to plasma renin levels, have been demonstrated in pediatric meningococcal septic shock (9). However, reference material and reporting units for both aldosterone and renin differ widely with internationally accepted standardized methodologies not yet in place (96,97,118). Additionally, the use of renin and aldosterone measurements using reference ranges obtained from non-critically ill populations, in the diagnosis of hypoaldosteronism poses a number of on angiotensin II synthesis (11,119). Further methodological concerns include the lack of validation of these tests for use in the critically ill, as well as implications of technical limitations in their performance (plasma renin activity and serum aldosterone measurements should be performed after three hours in the upright or seated position as this increases renin and aldosterone release) (120). Currently, no reference ranges exist for plasma renin activity in critical illness.

Difficulties with the consistent demonstration of hypoaldosteronism in critical illness are compounded by the methods used to measure aldosterone (11). Marked overestimation of aldosterone levels occurs in renal impairment with the use of homogenous immunoassays, likely because of antibody cross-reactivity with uncleared aldosterone metabolites (121). The lack of standard

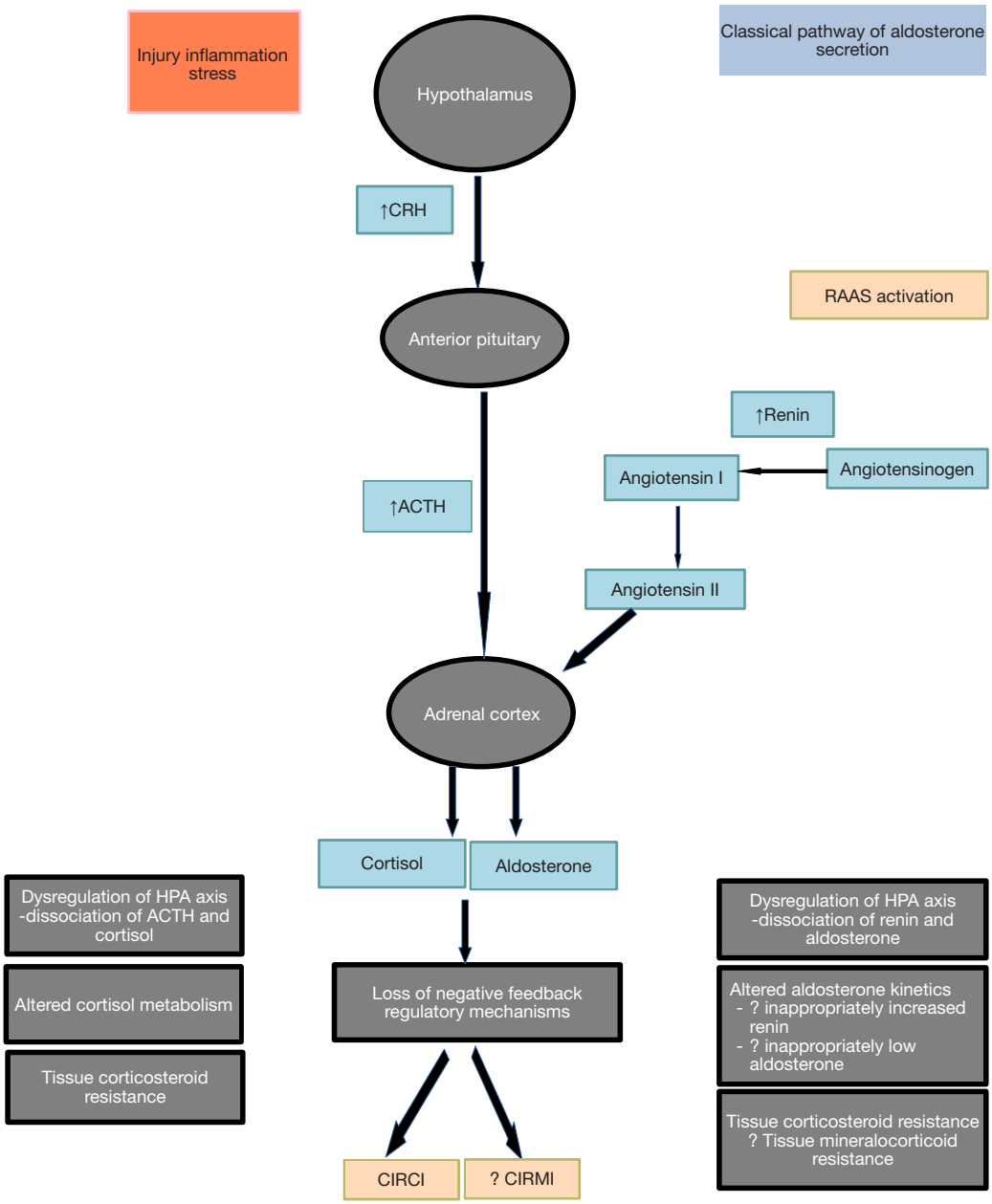

Figure 4 Proposed schematic representation of the hypothalamic-pituitary axis and key mechanisms in hyperreninemic hypoaldosteronism. Renin-angiotensin-aldosterone system activation occurs through the classical pathway of aldosterone secretion. Additional corticotrophin independent pathways include effects of cytokines and vasopressin, which trigger corticotrophin release, independent of hypothalamic control. Critical illness triggers release of corticotrophin-releasing hormone from the hypothalamus. Corticotrophin-releasing hormone stimulates the anterior pituitary to release adrenocorticotropic hormone, which stimulates the release of cortisol and to a lesser extent, aldosterone from the adrenal cortex. In a subset of critically ill patients, the loss of regulatory negative feedback mechanisms results in the dissociation of renin and aldosterone (hyperreninemic hypoaldosteronism) which is characterized by hyperreninemia in the face of inappropriately low aldosterone and is associated with a higher mortality. HPA, hypothalamic-pituitary-adrenal; ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; RAAS, renin-angiotensin-aldosterone system; CIRCI, critical illness-related corticosteroid insufficiency; CIRMI, critical illnessrelated mineralocorticoid insufficiency.

reference ranges for aldosterone across populations, as well as variations in assay procedures among laboratories further compounds aldosterone level assessments (122-124). High performance liquid chromatography and tandem mass spectrometry (LC-MS/MS) is a more accurate method of determining aldosterone levels, however, due to cost implications and technical demands associated with LC-MS/MS systems, radio-immunoassays remain the method of choice (125,126). Currently, no published standardized LC-MS/MS reference method or standard reference materials are available (121).

A comparison of therapeutic interventions

Evidence of an altered adrenal axis in septic shock, associated with a high mortality and characterized by organ dysfunction, and potentially improved by glucocorticoid supplementation, justifies therapy (37,110,111,127). Although the rationale for corticosteroid use in the management of patients with septic shock is well defined (128), there is uncertainty regarding the survival effect of adjunctive corticosteroid supplementation in severe sepsis and septic shock (128-130). Corticosteroids may not be beneficial or may lead to a small reduction in mortality with a possible increase in the risk of hyperglycemia and neuromuscular weakness (12,77,128,130,131).

Four large studies on the use of corticosteroids for the reversal of septic shock reported conflicting results. In the Ger-Inf-05 trial, hydrocortisone therapy in patients with septic shock and adrenal insufficiency was associated with improved survival (110). However, intravenous hydrocortisone did not reverse shock in patients with septic shock and was not associated with improved survival even in patients with adrenal insufficiency in another trial (111). Subsequently, two randomized controlled trials have shown trends in outcomes in favour of the cortisol group (12,77). In the *ADRENAL* trial, which included 3,800 mechanically ventilated patients with septic shock, a continuous infusion of 200 mg of hydrocortisone did not result in a lower 90-day mortality than placebo (27.9% *vs.* 28.8%; OR, 0.95; CI: 0.82 to 1.1; P=0.5) (77,128). Evidence of benefit was demonstrated with regard to time to shock resolution, duration of mechanical ventilation, length of ICU stay and reduced frequency of blood transfusions (77).

The *APROCCHSS* trial evaluated the effect of hydrocortisone, in combination with fludrocortisone, drotrecogin-alfa and respective placebos in 1,241 patients with septic shock. Mortality at intensive care and hospital discharge was significantly lower in the *hydrocortisone in combination with fludrocortisone* group (43.0% *vs.* 49.1%; P=0.03) (12,128). The RR of death in the hydrocortisone in combination with fludrocortisone group was 0.88 (95% CI: 0.78 to 0.99). The number of vasopressor-free days and organ-failure-free days was significantly higher in the hydrocortisone in combination with fludrocortisone group (14 *vs.* 12 days, P=0.003 and 17 *vs.* 15 days, P<0.001 respectively).

A recently suggested treatment strategy for septic shock is intravenous hydrocortisone at a maximum dose of 400 mg/day for at least 3 days in patients with septic shock that is not responsive to fluid and moderate- to high-dose vasopressor therapy (5). A lower dose, of 200 mg/day, for a more defined period of 7 days, commenced within 4 to 6 h of the initiation of vasopressor therapy in patients with persisting shock, has been recommended (128). A comparison of large randomized controlled trials of hydrocortisone therapy in septic shock is presented in *Table 5*.

The role of fludrocortisone, remains unclear. The only two trials demonstrating a decrease in mortality with steroid replacement therapy in septic shock included hydrocortisone in combination with fludrocortisone in the therapeutic group (12,110).

In *COIITSS*, a 2×2 factorial, randomized trial, a secondary objective assessed the benefit of fludrocortisone in septic shock patients who received hydrocortisone (132). Patients were randomly assigned to 1 of 4 groups: hydrocortisone with continuous intravenous insulin infusion, hydrocortisone in combination with fludrocortisone with continuous intravenous insulin infusion, hydrocortisone with conventional insulin therapy, or hydrocortisone in combination with fludrocortisone plus conventional insulin therapy (132). Hydrocortisone in combination with oral fludrocortisone did not result in a statistically significant improvement in in-hospital mortality, however there was a –3% absolute difference in hospital mortality rates in patients treated with hydrocortisone in combination with fludrocortisone (132). Although this result was not statistically significant, the study was not adequately powered to detect a relevant treatment effect (132).

The administration of fludrocortisone in septic shock in the Activated Protein C and Corticosteroids for Human Septic Shock trial was demonstrated to have a mortality benefit at 90 days (12,133). However, on the contrary, the use of renin-angiotensin-aldosterone system antagonism, namely angiotensin converting enzyme inhibitors or angiotensin II receptor blockers, in the setting of sepsis has

Table 5 Comparison of randomized controlled trials investigating hydrocortisone therapy in septic shock

Study	Population	Intervention	Primary outcomes
Annane *et al.*, 2002, JAMA (110). *Ger-Inf-05 trial*	300 adults enrolled after undergoing a corticotropin test (250 µg)	Patients randomized to hydrocortisone 50 mg intravenous (IV) bolus 6 hourly and fludrocortisone 50 µg tablet once daily or placebo for 7 days	Improved survival [53% *vs.* 63% (OR 0.54; 95% CI: 0.31–0.97; P=0.04)]
Sprung *et al.*, 2008, NEJM (111). *CORTICUS*	499 adults within 72 hours of diagnosis of septic shock with hypoperfusion or organ dysfunction after. Enrolled undergoing a corticotrophin test (250 µg)	Patients randomized to hydrocortisone 50 mg IV 6 hourly for 5 days, 12 hourly for 3 days, 24 hourly for 3 days, then stopped or placebo	Intravenous hydrocortisone did not reverse shock in patients with septic shock [76% *vs.* 70.4% (P=0.41)] and was not associated with improved survival [34% *vs.* 32% (P=0.51)] even in patients with adrenal insufficiency
Venkatesh *et al.*, 2018, NEJM (77). *ADRENAL*	3,800 mechanically ventilated adults with septic shock	A continuous infusion of 200 mg of hydrocortisone or placebo daily for 7 days or ICU discharge or death	A continuous infusion of hydrocortisone did not result in a lower 90-day mortality than placebo (27.9% *vs.* 28.8%; OR, 0.95; CI: 0.82–1.1; P=0.5)
Annane, 2018, NEJM (12). *APROCCHSS*	1,241 adults with septic shock	2×2 factorial design: hydrocortisone 50 mg IV 6 hourly and fludrocortisone 50 µg through a nasogastric tube daily (or matching placebo) for 7 days Activated protein C 24 µg/kg/h for 96 hours or matching placebo) (Activated protein C arm discontinued as a result of activated protein C market withdrawal)	Improved survival. All-cause mortality at 90 days [49% *vs.* 43% (RR 0.88; 95% CI: 0.78–0.99; P=0.03)]

yielded conflicting results (92,134).

Fludrocortisone has been administered concurrently with high doses of hydrocortisone, doses purportedly high enough to have sufficient mineralocorticoid activity (12,110).

However, effects of fludrocortisone that are not mediated through the mineralocorticoid receptor should be considered (65,135,136). The implications of the differences in cellular downward signalling between the ligands, cortisol and fludrocortisone, acting on the mineralocorticoid receptor, require further clarification (133). Importantly, the bioavailability of oral fludrocortisone in critical illness requires elucidation (128,137,138).

The currently recruiting *Fludrocortisone Dose Response Relationship and Vascular Responsiveness in Septic Shock* (FluDRes) trial is a phase II, open label randomized controlled trial investigating the biological basis of vascular responsiveness in sepsis, as well as the pharmacokinetics and pharmacodynamics of fludrocortisone in septic shock (139). Results of this trial will, hopefully, help address the role of combination therapy with hydrocortisone and fludrocortisone in the critically ill.

Angiotensin II has recently emerged as a non-catecholamine vasopressor agent in the management of septic shock (13). In the *ATHOS-3* (*Angiotensin II for the Treatment of High-Output Shock*) trial, patients with refractory high output shock were randomised to receive infusions of angiotensin II or placebo within a 48-hour study period. The administration of angiotensin II was associated with a positive vasopressor response to mean arterial pressure at 3 hours when compared to placebo. [114 (69.9%) *vs.* 37 (23.4%) (OR 7.95, P<0.001)]. The measurement of renin levels was not part of the investigation in this trial. We hypothesize that in such populations hyperreninemic hypoaldosteronism may be concurrently present and we advocate for the assessment of hyperreninemic hypoaldosteronism in this population.

Indeed, Bellomo and colleagues recently used renin concentrations to identify patients that may benefit from angiotension II therapy (140). Serum samples from patients enrolled in the *ATHOS-3* trial were assessed for renin, angiotensin I, and angiotensin II concentrations before the start of administration of angiotensin II or placebo and after 3 hours. In those with renin concentrations above the study population median, angiotensin II significantly reduced 28-

day mortality to 28 of 55 (50.9%) patients compared with 51 of 73 patients (69.9%) of the placebo group (unstratified hazard ratio, 0.56; 95% confidence interval, 0.35 to 0.88; P=0.012) (P=0.048 for the interaction). A larger study assessing the response of aldosterone to angiotensin II along with renin measurements and with a patient centred outcome, such as duration of ICU stay or survival, would be of interest. Nonetheless, the finding, in this trial, that patients who had hyperreninemia were more likely to benefit from angiotensin II therapy highlights a population of catecholamine-resistant vasodilatory shock patients who have the potential to benefit from therapy modulating the renin-angiotensin-aldosterone system. As the maintenance of cardiovascular homeostasis is through multiple mechanisms, exploring the concept of synergy with regards to vasoactive medications in refractory vasodilatory shock, through the use of therapeutic approaches targeting both sympathetic tone and endocrine mechanisms, is a rational therapeutic approach.

Conclusion and future directions

Critical illness has been observed to be accompanied by variable degrees of cortisol secretion (33), increased free cortisol, glucocorticoid insensitivity, as well as disruption of glucocorticoid negative feedback. Both cortisol and aldosterone are of an adrenal source, and cortisol is capable of down-regulating both mineralocorticoid and glucocorticoid receptors (141). It is expected that such mechanisms affecting cortisol function in critical illness would be accompanied by a similar disruption in aldosterone function. Aldosterone levels, like cortisol, have been shown to be increased in sepsis and hemorrhagic shock. Furthermore, there is evidence to suggest that in acute critical illness, hyperreninemic hypoaldosteronism, a finding, which likely signifies the loss of negative feedback control of the renin-angiotensin-aldosterone system, is associated with poor outcomes. Indeed, there is evidence to suggest that there exists a population of catecholamine resistant vasodilatory shock patients with high renin levels who have the potential to benefit from therapy modulating the renin-angiotensin-aldosterone system, namely angiotensin II.

Evidence of improved outcomes with cortisol supplementation have been described. Although cortisol has mineralocorticoid activity, aldosterone has clinically relevant non-mineralocorticoid effects.

As with CIRCI, confident consideration and implementation of therapeutic interventions would require some clarifications.

An ALDO/PRA ratio below 2 has been defined as inappropriately low in previous studies and remains a criteria for definition until data from more recent studies becomes available (8). We suggest the assessment of hyperreninemic hypoaldosteronism through the assessment of ALDO/PRA ratio with the consideration of co-administration of hydrocortisone with fludrocortisone in patients with septic shock. Unlike the non-acute setting, cortisol levels are likely to be elevated in critical illness and they are of less diagnostic utility in the diagnosis of hyperreninemic hypoaldosteronism.

A number of questions remain:

(I) Is cortisol the only adrenal hormone with an outcome benefit that is affected in critical illness?

(II) Is there peripheral resistance to aldosterone in critical illness, much like that described with cortisol, and how can it be demonstrated? Peripheral resistance to cortisol has already been described;

(III) Do changes exist that affect the expression and activity of enzymes and receptors involved in aldosterone metabolism and the mineralocorticoid pathway as appears to be the case for cortisol?

(IV) Considering the methodological challenges and the lack of standardisation of diagnostic procedures, how can techniques that assess plasma renin activity, as well as the measurement of plasma aldosterone in the critically ill population, be improved?

(V) Lastly, if critical illness-related mineralocorticoid insufficiency is demonstrated, how should it be treated, as the bioavailability of the current form of fludrocortisone in critical illness is debatable?

In view of the conceptual similarities between the conditions hyperreninemic hypoaldosteronism and CIRCI, we suggest the term critical illness-related mineralocorticoid insufficiency (CIRMI) as a more appropriate description of the impaired aldosterone response to increased levels of renin seen in this group of patients.

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Footnote

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CHAPTER 8: Glucocorticoids with or without fludrocortisone in septic shock – a biochemical and molecular perspective

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REVIEW ARTICLE

Glucocorticoids with or without fludrocortisone in septic shock: a narrative review from a biochemical and molecular perspective

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Summary

Two randomised controlled trials have reported a reduction in mortality when adjunctive hydrocortisone is administered in combination with fludrocortisone compared with placebo in septic shock. A third trial did not support this finding when hydrocortisone administered in combination with fludrocortisone was compared with hydrocortisone alone. The underlying mechanisms for this mortality benefit remain poorly understood. We review the clinical implications and potential mechanisms derived from laboratory and clinical data underlying the beneficial role of adjunctive fludrocortisone with hydrocortisone supplementation in septic shock. Factors including distinct biological effects of glucocorticoids and mineralocorticoids, tissue-specific and mineralocorticoid receptor-independent effects of mineralocorticoids, and differences in downstream signalling pathways between mineralocorticoid and glucocorticoid binding at the mineralocorticoid receptor could contribute to this interaction. Furthermore, pharmacokinetic and pharmacodynamic disparities exist between aldosterone and its synthetic counterpart fludrocortisone, potentially influencing their effects. Pending publication of well-designed, randomised controlled trials, a molecular perspective offers valuable insights and guidance to help inform clinical strategies.

Keywords: adrenal insufficiency; corticosteroid insufficiency; critical illness; glucocorticoids; mineralocorticoids; stress response

Editor s key points

- A reduction in mortality has been reported in septic shock when adjunctive hydrocortisone is administered in combination with fludrocortisone. The mechanisms underpinning this mortality benefit remain unclear.
- The authors explored potential mechanisms derived from laboratory and clinical data, elucidating the distinct biological effects of glucocorticoids and mineralocorticoids, and the tissue-specific and receptor-independent effects.
- An understanding of the interplay between glucocorticoid and mineralocorticoid action in critical illness could help guide future clinical strategies.

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Adrenal insufficiency in critically ill patients is characterised by hypothalamic–pituitary–adrenal axis derangements and involves dysfunction in cortisol handling and the renin–angiotensin–aldosterone system (RAAS). Current guide lines suggest adding corticosteroid therapy for patients with septic shock requiring ongoing vasopressor support.[1,2] Although adjunctive hydrocortisone (i.e. cortisol) alone has not shown improvement in mortality in septic shock, two randomised controlled trials have demonstrated a mortality improvement when fludrocortisone is combined with hydrocortisone.[3–5] However, mechanisms underlying the observed comparative effectiveness of adjunctive fludrocortisone with hydrocortisone over hydrocortisone alone remain poorly understood.[6]

The Ger-Inf-05 trial examined the combination of fludrocortisone and hydrocortisone in septic shock.[3,4] A total of 300 patients were assigned to receive either the combination of a 50-mg i.v. bolus of hydrocortisone 6 hourly with 0.05 mg fludrocortisone daily administered through a nasogastric tube or placebo for 7 days.[3] Patients were classified as responders or non-responders based on a corticotropin test.[3] Combined low-dose hydrocortisone with fludrocortisone was observed to significantly reduce the risk of death in non-responders with septic shock (73 deaths [63] in the placebo group, 60 deaths [53] in the corticosteroid group [hazard ratio, 0.67; 95 confidence interval or CI, 0.47–0.95; *P* 0.02]). No significant mortality benefit was observed in responders (18 deaths [53] in the placebo group and 22 deaths [61] in the corticosteroid group, *P* 0.96). These results suggested that combining mineralocorticoid with glucocorticoid therapy improved mortality outcomes in septic shock.

Recent large, randomised, controlled trials examining the role of hydrocortisone in septic shock have shown divergent effects on mortality with fludrocortisone in combination with hydrocortisone treatment. *Corticosteroid Treatment and Intensive Insulin Therapy for Septic Shock in Adults* (COIITSS) was a randomised, 2×2 factorial trial of 509 patients with septic shock and multiple organ dysfunction assigned randomly to one of four groups: continuous i.v. insulin infusion with hydrocortisone alone, continuous i.v. insulin infusion with hydrocortisone and fludrocortisone, conventional insulin therapy with hydrocortisone alone, or conventional insulin therapy with i.v. hydrocortisone and fludrocortisone.[7] The primary objective was to assess the efficacy of intensive insulin therapy in patients with septic shock treated with hydrocortisone. The secondary objective was to evaluate the additive benefit of fludrocortisone administered for 7 days. Hydrocortisone was administered as a 50-mg i.v. bolus, followed by 50 mg i.v. 6 hourly for 7 days. A total of 245 patients received additional oral fludrocortisone at a dose of 0.05 mg daily. Overall, 105 of 245 patients treated with fludrocortisone died (42.9) and 121 of 264 (45.8) patients in the control group died (risk reduction, 0.94; 95 CI, 0.77–1.14; *P* 0.50). These findings provided evidence against intensive insulin therapy in patients receiving hydrocortisone for septic shock and indicated a lack of significant mortality benefit from the additional fludrocortisone. Notably, the COIITSS trial was underpowered as the mortality in the control group was underestimated and the sample size chosen to detect a large effect size (12.5 risk difference).[5] Furthermore, sample size calculation was based on the estimated effects of the insulin intervention, but not on the effects of the added fludrocortisone.[7] Thus, the trial was not powered to find a mortality difference with respect to the comparison of hydrocortisone with hydrocortisone plus fludrocortisone.

In the *Recombinant Human Activated Protein C and Low Dose of Hydrocortisone and Fludrocortisone in Adult Septic Shock* (APROCCHSS) trial, 614 patients were randomised to receive a combination of a hydrocortisone 50 mg i.v. bolus 6 hourly and fludrocortisone 0.05 mg daily through the nasogastric tube for 7 days, whereas 627 patients received hydrocortisone and fludrocortisone placebos.[4] The 90-day all-cause mortality at ICU discharge was observed to be lower in the hydrocortisone-plus-fludrocortisone group than among those who received placebo rather than hydrocortisone and fludrocortisone (35.4 *vs* 41.0 , *P* 0.04). The hydrocortisone-plus-fludrocortisone group, additionally, had more vasopressor-free and organ failure-free days.

Nonetheless, two randomised, placebo-controlled clinical trials revealed a survival advantage in septic shock through a combination of hydrocortisone and the mineralocorticoid fludrocortisone, compared with placebo. Another smaller trial (*n* 509 patients) contrasted hydrocortisone alone with hydrocortisone in conjunction with fludrocortisone. The findings did not exhibit a significant decrease in mortality with the addition of fludrocortisone but did show a 3 numerically lower mortality with combination therapy (relative risk, 0.94; 95 CI, 0.77–1.14; *P* 0.50). Thus, the unresolved potential value of combining fludrocortisone with hydrocortisone for septic shock treatment persists.

Apparently discrepant findings from these three large trials have led to divergent perspectives on the benefits of adjunctive hydrocortisone in combination with fludrocortisone in septic shock. However, distinctions and the limited power of these studies make direct comparisons difficult with respect to hydrocortisone and fludrocortisone.[1,6,8] Cortisol binds to both the glucocorticoid (GR) and the mineralocorticoid receptor (MR). One perspective suggests that as cortisol binds to the MR, the administration of high doses of hydrocortisone (≥200 mg daily) offers an adequate level of mineralocorticoid activity.[1,6] A dose of 240 mg of hydrocortisone confers a mineralocorticoid effect equivalent to 1.2 mg (1200 μg) daily of fludrocortisone and thus additional fludrocortisone therapy is not required.[9] An alternative perspective is that adjunctive hydrocortisone with fludrocortisone treatment is required in septic shock as although cortisol binds to the MR, the subsequent signalling pathways differ from those mediated by aldosterone (and by implication fludrocortisone).

Pending a well-designed, randomised, controlled trial on the role of fludrocortisone in combination with hydrocortisone in septic shock, a molecular perspective offers valuable guidance for clinical strategies.

Biological rationale for the combination of hydrocortisone with fludrocortisone in the management of septic shock

Sepsis, which is a life-threatening time-dependent organ dysfunction, arises from a dysregulated host response to pathogens.[10,11] This response manifests as an overwhelming systemic inflammatory response or immune dysfunction, and involves pattern recognition receptors sensing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs).[10,11] The resultant injury occurs at the molecular, cellular, and organ levels and involves the breakdown of both the endothelial and epithelial barriers, and the microvasculature, compromising multiple circulatory beds.[12] Various organ systems, including the cardiovascular and pulmonary systems, renal system, the coagulation cascade, and the immune system become compromised and multiorgan

failure ensues.[10,12] In the blood–brain barrier, it causes perivascular oedema, oxidative stress, leukoencephalopathy, and neurotransmitter alterations.[12] The nervous system additionally plays an anti-inflammatory role, particularly in the early stages.[12] With regard to the immune system, this interaction leads to an excessive innate immune response.[10,12] The persistence of a severe and prolonged compensatory anti-inflammatory innate immune response results in a state of acquired immunodeficiency termed immunoparalysis.[13] Corticosteroids, recognised for their immunomodulatory properties, constitute an integral component of the host response to sepsis.[10,14]

Septic shock initiates a neuro-humoral response, which includes the activation of the RAAS.[15,16] The RAAS is a multi-organ system, which regulates homeostasis, inflammation, apoptosis, and fibrosis.[17] The classical RAAS pathway is considered a cardiovascular circulating hormonal system mediated by angiotensin II (AII), and the non-classical RAAS pathway, a local tissue-based system mediated by angiotensin-(1–7). These two systems can operate synergistically or independently.[18–20] In contrast to the classical RAAS and its circulating mediators, the non-classical RAAS comprises tissue axes, which regulate renal, cardiovascular, and nervous systems.[21,22]

Angiotensin-(1–7) is found in the heart, renal, and brain tissues, and in plasma.[19] Relative contributions of the classical (systemic) *vs* the non-classical (intra-renal) RAAS to blood pressure regulation remain debatable; however, the crosstalk between the RAAS and aldosterone production is well established.[21] As such, these two systems should not be seen in isolation.[23]

Both glucocorticoids and mineralocorticoids are integral components of the stress response.[10,14,24] Notably, steroidogenesis pathways also demonstrate overlap between mineralocorticoid and corticosteroid production and the synthesis of corticosterone, an aldosterone precursor, is exclusively triggered by corticotropin.[25] As such, adrenal corticosteroid insufficiency is likely to occur concurrently with mineralocorticoid dysfunction. Physiologically, daily adrenal production of cortisol oscillates between 5 and 11 mg m^{-2} day^{-1} with a mean of 7 mg m^{-2} day^{-1}, which equates to 20–30 mg daily of hydrocortisone in adults.[26,27] Production rates are highly variable between and within individuals.[26,27] Peak mean cortisol levels have been documented at 400 nmol L^{-1} with nadir levels of 50 nmol L^{-1}. In adrenal insufficiency, the traditional replacement dose from consensus guidelines is 15–25 mg of hydrocortisone daily.[28,29] In septic shock, the currently accepted dose for relative adrenal insufficiency is 100–200 mg daily.[1]

In contrast, aldosterone secretion is 200- to 500-fold lower at a rate of 50–200 μg daily, with plasma levels ranging 40–210 μg L^{-1}.[30,31] Isolated serum aldosterone measurement is of limited clinical value and is often interpreted alongside plasma renin activity.[32]

Aldosterone administration has been demonstrated to restore vascular endothelial alpha-1 receptor expression, improving sensitivity to catecholamines in septic shock models.[14,33] Fludrocortisone, a synthetic mineralocorticoid, may be used as replacement therapy for this purpose. The usual starting dose of fludrocortisone for adults with primary adrenal cortical insufficiency is 100 μg daily with a dose range of 100–500 μg.[34,35] Higher doses may be required in high salt-losing states, in paediatric patients because of relative renal resistance to mineralocorticoids, and during the last trimester of pregnancy because of the counter-regulatory effects of high progesterone levels.[29,36–38]

Mechanisms for the beneficial role of adjunctive use of fludrocortisone with hydrocortisone in septic shock

Reasons for the mortality improvement observed in the APROCCHSS and Ger-Inf-05 trials remain poorly understood.[6] Explanatory mechanisms include the mineralocorticoid activity of fludrocortisone, although high doses of cortisol purportedly possess such activity.[9,39] Furthermore, aldosterone has non-mineralocorticoid effects, which potentially play a role.[40] Although pharmacologically different, a similar implication can be extended to its synthetic counterpart, fludrocortisone.

The additional glucocorticoid effects of fludrocortisone should also be considered.[41,42] However, although fludrocortisone is a potent glucocorticoid,[41] its effects are largely mineralocorticoid at the low dosage used because of its significantly greater potency at the MR. Additionally, various genomic and non-genomic effects of fludrocortisone, and a yet unidentified mechanism resulting from the combination of hydrocortisone and fludrocortisone should also be considered.

Aldosterone has non-mineralocorticoid effects: classical mineralocorticoid receptor–mediated signalling *vs* alternate modes of signalling

Aldosterone has genomic and non-genomic actions (Fig. 1).[45] Genomic actions are the classic nuclear actions occurring over hours in epithelial and non-epithelial cells attributable to activation of the MR, a ligand-dependent transcription factor.[45] Aldosterone also displays rapid actions occurring over minutes. Termed non-genomic or rapid non-nuclear actions, these are independent of gene transcription and occur in classical MR targets and non-epithelial tissue and include actions such as the activation of sodium transport in target cells.[46,47] Rapid actions are clinically relevant in the acute setting as a result of their role in increasing peripheral vascular resistance and blood pressure.[48]

Notably, plasma aldosterone levels, not cortisol, rapidly change with posture as part of normal blood pressure postural control.[49,50] This suggests rapid cellular responses mediated by the acute increase in aldosterone and a role in blood pressure control in the acute setting.[48] Non-genomic actions were thought to be mediated through membrane-bound MR acting through second messenger pathways.[46] The G protein-coupled receptor, GPR30, or the G protein-coupled oestrogen receptor-1 (GPER-1), has been implicated as the receptor that mediates some of the rapid actions of aldosterone on vascular smooth muscle.[45,51–54] Experimentally, aldosterone has also been demonstrated to potentiate the constrictor effect of AII on vascular smooth muscle, which is mediated through the angiotensin receptor type 1 (AT1). Experimental evidence further suggests that GPR30 (GPER-1) has a high affinity for aldosterone but does not bind cortisol.[43]

Clinically, aldosterone (and by implication fludrocortisone) thus has MR-independent actions that are relevant in the acute setting with an effect on vascular smooth muscle and blood pressure control. The clinical rationale for fludrocortisone in combination with hydrocortisone therapy in critical illness should thereby not solely relate to aldosterone or fludrocortisone activation of the renal epithelial MR.[6,8]

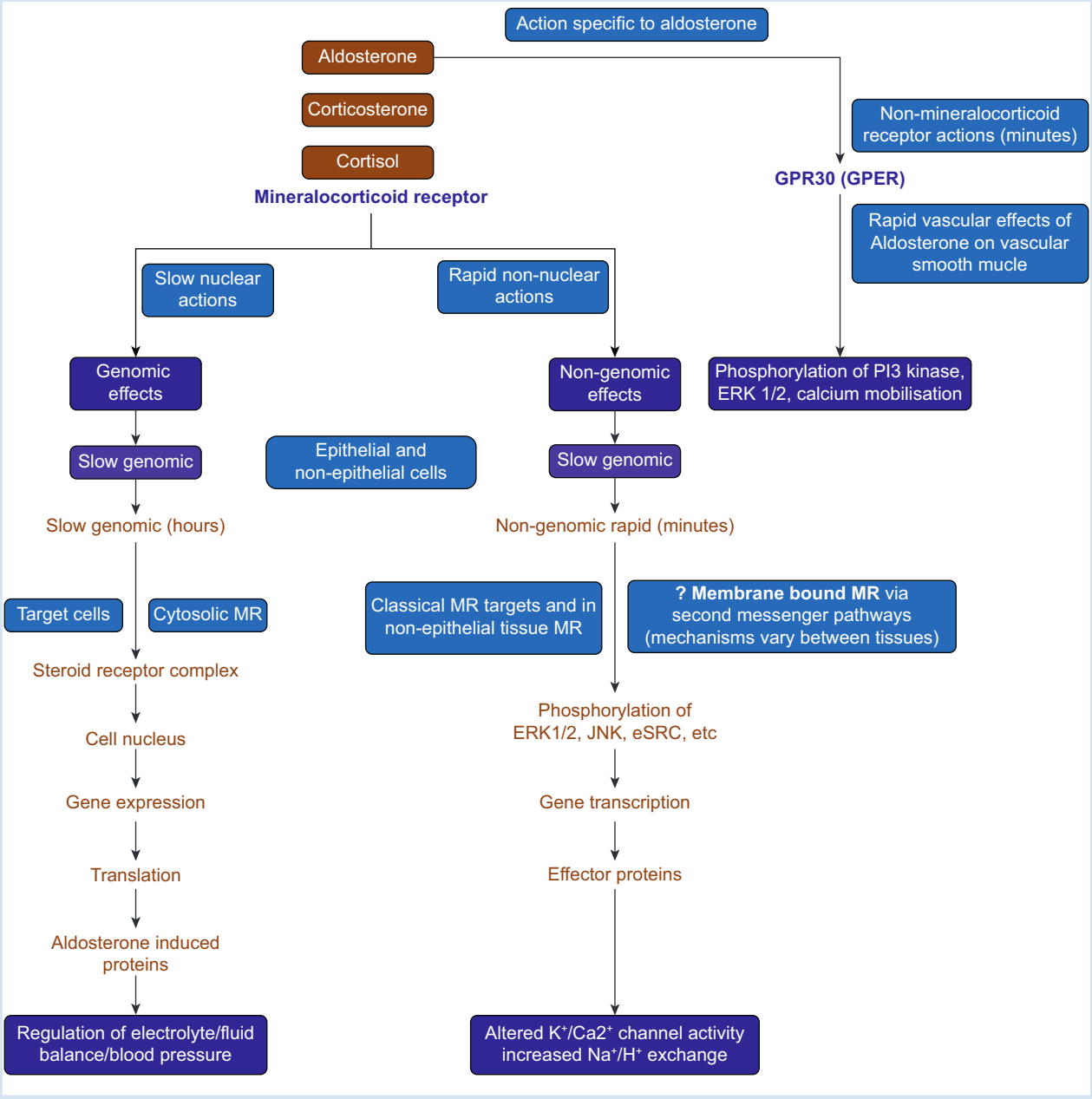

Fig 1. Genomic and non-genomic actions of aldosterone. Key features distinguishing genomic and non-genomic actions of aldosterone. Aldosterone action; genomic and non-genomic effects of aldosterone and cortisol. Classical slow genomic actions mediated through the mineralocorticoid receptor (MR) result in gene transcription and the production of effector proteins. Fast actions mediated through a surface receptor, which interact with the classical genomic actions, have recently been described to be mediated through the G protein-coupled receptor GPR30 (GPER), possibly through a membrane-bound MR, or both. Aldosterone action through GPR30 (GPER) is currently understood to be specific for aldosterone. BP, blood pressure; c-SRC, non-receptor tyrosine kinase c-SRC protein; ERK1/2, extracellular-signal regulated kinase 1/2; JNK, c-Jun NH2-terminal kinase; PI3-K, Phosphatidylinositol 3-kinase. Reused with permission from AME Publishing Company.[43,44]

The multifarious mineralocorticoid receptor

The MR and GR are members of the nuclear receptor super-family, which encompasses seven subfamilies (sub-group 0 to sub-group 6) and 48 members.[55] They act as ligand-activated transcription factors and consist of four structurally distinct domains namely: the amino terminal, a central DNA-binding domain, the hinge region, and the C-terminal ligand-binding domains.[56] Lipophilic ligands including steroids, retinoids, phospholipids, and as yet unidentified ligands (orphan members) regulate their activity.[55,57] Steroid receptors belong to sub-group 3, which includes the GR, MR, androgen receptor, progesterone receptor, and the oestrogen receptors (ERs): ER and ERβ.[55] The MR and GR amino acid sequences exhibit

significant homology being 56 identical in the steroid-binding domain.[56] Distinctively, the MR has the longest amino terminal domain among steroid receptors, sharing less than 15 homology with the GR in this region.[56] Additionally, the MR has affinity for mineralocorticoids, endogenous glucocorticoids, and progesterone.[56,58–60]

In view of higher circulating levels of cortisol and equal affinity of the MR for cortisol and aldosterone, a system for receptor selectivity exists in some tissues. This is mediated at a pre-receptor level in mineralocorticoid target tissues by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzyme system.[58] Two 11β-HSD isoenzymes exist.[61,62] First, 11β-HSD type-1 (11β-HSD1) is a bi-directional enzyme *in vitro* and an activating enzyme *in vivo* by converting receptor-inactive cortisone to cortisol.[62] In contrast, 11β-HSD type-2 (11β-HSD2) is expressed in mineralocorticoid target tissues and is a deactivating enzyme through its conversion of cortisol to cortisone.[50–52,63] The role of 11β-HSD2 is to protect the MR from cortisol in mineralocorticoid target tissues, conferring specificity for aldosterone.[63,64] The observation that both cortisol and aldosterone have a high affinity for the MR indicates a lack of selectivity of the C-terminal ligand-binding domain for aldosterone (or cortisol), but not necessarily comparative activation of the receptor.[56,58–60] Notably, although the GR is activated by cortisol alone, not all glucocorticoids activate the MR. Synthetic glucocorticoids show minimal or no activity at the MR.[65–70]

The observation that different ligands have variable activity at the MR, and that not all glucocorticoids activate the MR, is supportive of the rationale that the choice of corticosteroid supplementation potentially influences clinical outcomes and that cortisol and aldosterone (and by inference their synthetic counterparts) do not necessarily have the same effect as ligands at the MR.

Mineralocorticoid receptors are capable of divergent responses to different ligands

Glucocorticoid conformational mobility is another factor that confers mineralocorticoid action of glucocorticoids on the MR.[71–74] The molecular skeleton of steroids consists of four carbon atom rings defined as rings A, B, C, and D.[75] Steroid ring A conveys glucocorticoid action and is rigid in *pure* glucocorticoids and flexible in aldosterone. The structure of steroid ring C is required for mineralocorticoid action and is rigid in aldosterone (and relatively flexible in *pure* glucocorticoids). The exception is 11-deoxycorticosterone and its synthetic derivative, delta-11,12-deoxycorticosterone, which has a flexible C ring (and thus should be a pure glucocorticoid), but is actually a specific mineralocorticoid with marked glucocorticoid activity.[69,76] The lack of C-11 oxygenation of 11-deoxycorticosterone confers versatility.[76,77]

The MR exhibits ligand- and tissue-specific dichotomy. Cortisol's actions on the MR, although primarily agonist, can be antagonistic depending on the underlying pathophysiology.[78–81] The latter has been reported under certain experimental conditions in tissues in which 11β-HSD2 is not expressed (heart and specific regions in the central nervous system).[78–81] In conditions of tissue damage and in the presence of reactive oxygen species, cortisol acts as MR agonist.[80–82] Thus, functions of the MR are diverse, tightly regulated by ligand-specifying mechanisms, and are tissue-dependent.[61,83,84] Furthermore, MRs bound to aldosterone are demonstrably more resistant to proteolysis than MRs bound to cortisol, a result of ligand-induced conformational changes within the receptor protein.[85] Notably, the MR bound to fludrocortisone, aldosterone's synthetic counterpart, is highly resistant to degradation.[86–88] Thus, the MR is capable of divergent responses to different ligands.[39,89] Furthermore, receptor–ligand interactions differ according to ligand, and mineralocorticoid supplementation may help optimise MR activation.

Downstream signalling pathways between aldosterone and cortisol binding at the mineralocorticoid receptor differ

Although the MR binds multiple ligands, unique mineralocorticoid and glucocorticoid effects are evident. Their main ligands, aldosterone and cortisol, perform diverse roles and are governed by different regulatory mechanisms.[90–92] The effects of cortisol are influenced by various factors, including whether the signalling occurs through the GR, MR, or perhaps both.[93] Furthermore, therapeutic agents such as spironolactone, a competitive antagonist with weak agonist activity at the MR, are able to exert their protective effects not only through the exclusion of cortisol or aldosterone from the MR but potentially through the induction of protective factors.[94] Differences in differential downstream signalling pathways that have been observed when the MR is bound to aldosterone compared with cortisol are also likely to be cell- and tissue-specific.

Mineralocorticoid activity in the regulation of salt and water balance

Aldosterone release is the final step in the classical RAAS signalling system. In renal 11β-HSD2-expressing cells, cortisol is metabolised to bio-inactive cortisone. This pre-receptor exclusion of cortisol in 11β-HSD2-expressing tissues renders the MR aldosterone-selective, suggesting that cortisol and aldosterone may have similar or overlapping effects on the MR 11β-HSD2-expressing tissues.[95] This implicates the utilisation of similar signalling pathways between cortisol and aldosterone, specifically with respect to sodium and potassium regulation. The process of filtered sodium (Na^+) reabsorption in collecting duct cells begins with luminal Na^+ crossing the apical plasma membrane through epithelial sodium channels (ENaC) driven by its electrochemical gradient.[96] Subsequently, intracellular Na^+ is actively transported into the interstitial space by the basolateral Na^+/K^+-ATPase. Aldosterone-induced genes modulate ENaC subunit turnover, enhance ENaC synthesis, ENaC activity, and stimulate Na^+/K^+-ATPase activity.[71,96–98] The interaction of aldosterone with the MR leads to induction or repression of additional aldosterone-regulated genes, including serum and glucocorticoid-induced kinase (SGK1), which also play a key role in the regulation of Na^+ transport in distal kidney nephron epithelia.[99] SGK1 is the main regulator of ENaC.[100] Additionally, aldosterone has been found to have acute, non-mineralocorticoid effects on GPR30, which are not observed with cortisol.[51,53]

Mineralocorticoid activity in the brain

Neuroinflammation, along with ischaemic injury, neuronal cell death, and neuroanatomy changes, are major factors associated with cerebral dysfunction secondary to sepsis and septic shock.[101] Inflammation associated with sepsis and

septic shock has a direct impact on the structural integrity of the hippocampus and prefrontal cortex.[102,103] No effective therapeutic interventions exist; however, there is evidence to suggest that elevated levels of glucocorticoids may exacerbate neural inflammation, and evidence to the contrary.[102–105] The role of glucocorticoids, acting through both MR and GR, in modulating the immune response has been well studied.[10] Although, interest in the potential immune effects of mineralocorticoids has only recently emerged, the general consensus is that there is a role for both GR and MR in modulating the neuroinflammation associated with sepsis and septic shock.[10,102]

Compared with the GR, which is expressed ubiquitously in neurones and glial cells, the MR has a more restricted distribution, with the highest expression in limbic structures.[102,106] Aldosterone-selective neurones are limited to areas (periventricular, brain stem nucleus tractus solitarii) that regulate haemodynamic, fluid, and electrolyte homeostasis.[90]

Brain aldosterone levels are normally low because of the absence of aldosterone synthase and poor penetration across the blood–brain barrier caused by the presence of P-glycoprotein, an ATP-driven, drug efflux pump, which transports certain substrates across the cerebral vascular endothelium and into the circulation.[106–108] In contrast, cortisol (or corticosterone in rodents) circulates at significantly higher levels (100–1000 fold), acting as the main ligand for brain MR and GR.[109] Although MR signalling in the brain has been demonstrated to induce a pro-inflammatory response, the results of MR activation in the brain can vary depending on the ligand.[10,110]

Numerous studies show the diverse impacts of cortisol and aldosterone on brain MR.[111,112] Despite this variability, experimental data indicate that both hormones, when interacting with the brain cortisol-responsive MR, can influence cellular activity in similar ways through both genomic and non-genomic pathways.[113] Notably, these neurones also contain 11β-HSD1, which regenerates cortisol. However, it is essential to consider that poor penetration of the blood–brain barrier by aldosterone may be a limiting factor in its effects on brain MR activation.

Indirect evidence of downstream signalling differences is seen in murine models, where corticosterone antagonises aldosterone-mediated hypertension at the brain MR.[114] The brain shows widespread 11β-HSD-1 expression, whereas 11β-HSD-2 is limited to neurones in the fasciculus solitarius.[61,70,106] Aldosterone interactions with these neurones regulate salt appetite.[112] The subsequent blood pressure response to dietary sodium is inhibited by spironolactone.[115] The presence of 11β-HSD-1 and lack of 11β-HSD-2 imply significant roles for both cortisol and aldosterone in the brain, beyond salt regulation. In these cells, salt regulation is not the primary function, providing indirect evidence of the requirement for both cortisol and aldosterone in those tissues, although the specific role of aldosterone is these tissues is not well defined.

Our hypothesis is that in aldosterone-selective tissues, downstream signalling mechanisms are similar between the two ligands, hence the pre-receptor exclusion of cortisol to prevent MR activation by cortisol, which is ubiquitous. In non-aldosterone selective tissues, downstream signalling mechanisms activated by the MR differ between ligands, and pre-receptor modulation of access is therefore not required. These variable downstream actions resulting from aldosterone and cortisol binding at the MR may extend to their synthetic counterparts.

Mineralocorticoid activity in the heart

Differences in aldosterone and corticosterone signalling have been described in murine cardiomyocytes.[116,117] Both GR and MR are present in the heart. In a murine model, the activation of MR and GR resulted in both common and distinct responses, suggesting similarities and differences in signalling. In a study using isolated neonate cardiomyocyte cells *in vitro*, both aldosterone and corticosterone induced acceleration of spontaneous cardiomyocyte contractions with a chronotropic response to corticosteroids mediated by both MR and GR.[116,117]

Increased chronotropy was dependent on low threshold T-type calcium channel expression, which was disrupted by GR antagonism, but not by MR antagonism. Aldosterone's chronotropic action was further found to be additive to forskolin's effect on intracellular cyclic adenosine monophosphate (cAMP) levels, an additive response selectively abolished by GR inhibition.[118] Moreover, GR antagonism prevented cardiomyocyte hypertrophy induced by aldosterone, whereas MR antagonism (spironolactone) had limited effect. This suggests that, in murine cardiomyocytes, complete electrical remodelling and the maximal chronotropic response to corticosteroids require both GR and MR activation, with the GR alone involved in sensitising cells to aldosterone's chronotropic regulation through the cAMP pathway and in the hypertrophic response.[117]

Further evidence suggestive of signalling differences between cortisol and aldosterone in cardiomyocytes include well-established recognition of rapid non-genomic effects unique to aldosterone, not mimicked by cortisol.[119] Acute non-genomic effects of aldosterone in cardiac and other tissues mimicked by classical mineralocorticoid agonists, fludrocortisone, and deoxycorticosterone, but not by cortisol, have been demonstrated.[119] These effects are not blocked by spironolactone.[119] In a murine cardiomyocyte model, aldosterone specifically increased [^{3}H]leucine incorporation into protein, which was not observed with MR occupancy by corticosterone.[120]

The observation that reduced GR signalling and increased MR signalling are associated with an increased risk of heart disease, whereas alterations that favour increased GR signalling and diminished MR signalling exhibit cardioprotective effects, provides further indirect evidence of differences in signalling between glucocorticoids and mineralocorticoids.[121] GR and MR have complementary roles in regulating the hypothalamic–pituitary axis. The balance of glucocorticoid signalling through GR and MR has been demonstrated to play a role in disease.[90,92,121] Increased GR signalling and decreased MR signalling is considered cardioprotective, with unopposed MR signalling, irrespective of ligand (cortisol or aldosterone), associated with accelerated cardiovascular disease in experimental models. These responses can also be viewed as being anti-inflammatory or pro-inflammatory, respectively.[94,122]

Molecular interactions of the MR that differ by ligand have been described, providing compelling evidence of distinct MR conformation when bound to aldosterone *vs* cortisol.[91,109,123–125]

Collectively, these findings support the view that downstream signalling pathways vary depending on whether the MR is bound to aldosterone or cortisol and emphasise the

clinical implications of unique signalling pathways for cortisol, aldosterone, and their synthetic analogues. Although these represent potential mechanisms and, in a sense, proof-of-concept, a link to specific responses or outputs remains to be fully elucidated in humans.

Aldosterone *v rsus* fludrocortisone mechanism of action: is there a difference?

Synthetic hormones structurally vary from physiological steroid hormones, leading to differing effects.[126–128] Although they may mimic selected biological actions of endogenous hormones, they may not exert identical effects at a molecular level.[90,126,127,129–133]

Pharmacokinetic differences between synthetic and physiological steroid hormones are evident in their binding to corticosteroid-binding globulin (CBG). The majority of cortisol (>90) is bound to CBG, whereas synthetic glucocorticoids (prednisone, prednisolone, dexamethasone, fludrocortisone) have lower CBG affinity, leading to increased free fractions.[67,134] This difference in bioavailability supports additive *glucocorticoid* activity as another mechanism explaining the mortality improvement in patients administered hydrocortisone in combination with fludrocortisone in septic shock. In contrast to fludrocortisone, aldosterone plays a minimal role in glycogen deposition, glycogen phosphorylation, glycogenolysis, insulin resistance, or sensitivity.[76] Thus, additive fludrocortisone may exacerbate hyperglycaemia compared with hydrocortisone alone.

Moreover, fludrocortisone is a synthetic steroid with potent mineralocorticoid and substantial glucocorticoid activity (Fig. 2a) which was developed from the addition of a fluorine molecule at the 9-alpha-position to hydrocortisone (Fig. 2b and c).[76,87,136–139] It is a structural analogue of hydrocortisone with the same A ring, but is functionally similar to aldosterone.[140] Fluorination results in inert bonds, a prolonged half-life, improved bioavailability, and increased affinity for GR.[86–88] Fludrocortisone has similar effects to hydrocortisone, but has marked effects on electrolyte balance and carbohydrate metabolism with 10-fold higher glucocorticoid activity than hydrocortisone.[86]

Although human comparative pharmacokinetic and pharmacodynamic data for aldosterone and fludrocortisone dosing are limited, during the characterisation of the novel MR modulator, AZD9977, in a murine model, distinct mineralocorticoid effects of fludrocortisone compared with aldosterone were observed.[76,133] These effects were subsequently extrapolated to humans using preclinical and clinical data modelling. The mechanism of this effect remains unexp lained.

Comparison of aldosterone and fludrocortisone revealed distinct effects.[128] A 1-mg dose of intramuscular aldosterone exhibited an electrolyte regulating effect equivalent to a daily oral dose of 250 μg of fludrocortisone.[128] However, fludrocortisone exerted a more prolonged effect. A 1-mg dose of intramuscular aldosterone showed a greater effect on eosinopenia than 1-mg dose of oral fludrocortisone, indicating that fludrocortisone's effect on immune cell function requires much higher doses than aldosterone.[128,141] Notably, differences in the administration routes of aldosterone and fludrocortisone may have contributed to the observed effects. Fludrocortisone also exhibited pituitary adrenocorticotropic

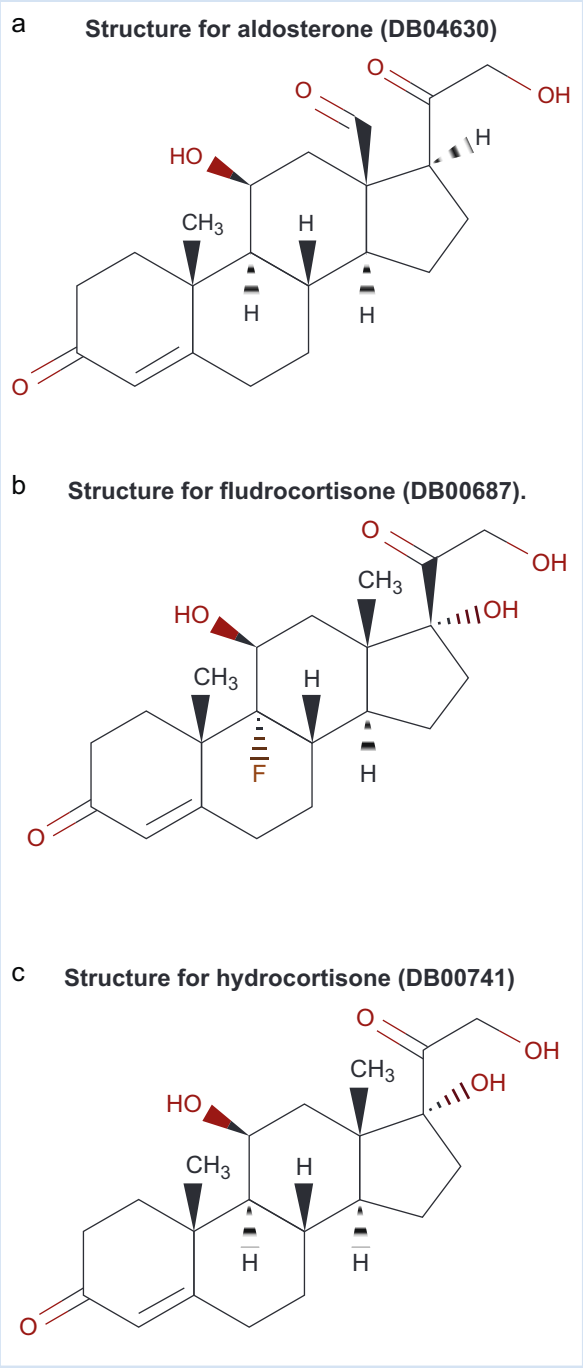

Fig 2. Molecular structure of (a) aldosterone (DB04630), (b) fludrocortisone (DB00687), and (c) hydrocortisone (DB00741).[135] Molecular structures are depicted in a standard format. Distinguishing features between depicted steroids: fludrocortisone has a fluorine atom on C9 replacing hydrogen and aldosterone has an aldehyde group on C18. Bond line notation: double lines indicate a double bond, single arrows indicate a single bond, dashed lines indicate that the bond extends behind the surface plane, bold wedged lines indicate bonds protruding out form the surface plane. DB, DrugBank Accession number.

hormone (ACTH) inhibition, consistent with glucocorticoid activity, an effect not observed with aldosterone, providing further mechanisms whereby fludrocortisone may compound the hyperglycaemia and immune changes observed with hydrocortisone administration.[3,4,128,142]

Thus, although both aldosterone and fludrocortisone are classified as mineralocorticoids, their pharmacological actions differ and may translate into distinct clinical effects. However, although both fludrocortisone and aldosterone have demonstrable non-genomic effects in murine models, evidence for this mechanism in humans as an explanation for the mortality improvement observed with adjunctive fludrocortisone with hydrocortisone in septic shock is minimal.[143]

Role of adrenal age related changes and mineralocorticoid supplementation

The traditional concept of compartmentalised enzymatic reactions within distinct zones of the adrenal cortex, known as functional zonation, has been challenged.[144] Adrenal cortex zones, namely the zona glomerulosa, zona fasciculata, and zona reticularis, were thought to have distinct roles in synthesising aldosterone, glucocorticoids, and androgens, respectively.[145,146] The distribution of enzymes and receptors does not, however, suggest autonomous steroid production by each adrenal zone. This is particularly relevant for the glomerulosa, which seemingly lacks the complete suite of the necessary enzymes for aldosterone biosynthesis.[147,148] Hence, the adrenal gland, seen as an integrated structure, diminishes the likelihood of diseases such as septic shock being restricted to a particular zone.[147]

Furthermore, with progressing age (from about 30 yr), adrenal topographical changes occur, resulting in zona fasciculata enlargement, reduction in zona reticularis and glomerulosa cell masses, and the development of aldosterone-synthase expressing cell clusters at the border of the zona glomerulosa with the zona fasciculata.[147,149,150] Such histological changes suggest reduced functional differentiation between zones. Of clinical relevance is that the reduction in the aldosterone-producing cell mass and observed decrease in aldosterone release with advancing age suggests that adjunctive mineralocorticoid supplementation in septic shock may be more effective in the older population.[149–151] This aligns with the demographic profile of participants in the APROCCHSS trial, where the average age was 66 yr.[4] Thus, adjunctive hydrocortisone and fludrocortisone therapy may hold greater promise for improving outcomes in septic shock in older patients.

Impact of bioavailability of orally administered fludrocortisone

Fludrocortisone differs from other corticosteroids in its 100 bioavailability and long half-life (18–36 h) and duration of action (1–2 days). It is dosed orally and is stable as a solid.[140] However, pharmacokinetic data on the bioavailability of oral fludrocortisone in critical illness is limited. In one-third of patients with septic shock, a single oral dose of 50 μg led to undetectable plasma levels in one study.[152] The usual replacement dose of fludrocortisone in primary adrenal insufficiency is 100–200 μg, a dose significantly higher than the 50 μg administered in studies conducted in septic shock. More recently, an average of 100 μg fludrocortisone has been used in septic shock.[5] Additionally, the different mineralocorticoid effects of synthetic fludrocortisone compared with endogenous aldosterone suggested by preclinical data highlights that translation of the mineralocorticoid effects of fludrocortisone to those of aldosterone requires further exploration.[133,153]

Clinical applications and suggestions for clinical practice

Which population is likely to benefit?

The decline of the aldosterone-producing cell mass with age and the consequent reduction in aldosterone release suggests that adjunctive mineralocorticoid supplementation in septic shock may be more efficacious in the older population. Based on the demographic profile of participants in the APROCCHSS trial, consideration of adjunctive mineralocorticoid therapy in septic shock may be more beneficial in the population group above 60 yr of age.

Dosage and mode of fludrocortisone administration

Current recommendations suggest adjunctive hydrocortisone at a non-tapered dose of 200 mg daily for 7 days if used in septic shock. If adjunctive fludrocortisone therapy is to be commenced in combination with hydrocortisone, we suggest a dose of 50 μg daily for 7 days without tapering. Notably, a recent international survey of clinician preferences for corticosteroid prescription in septic shock suggests a low preference for fludrocortisone.[154] Well-powered RCTs to investigate patient selection, optimal dosage, and treatment regimen are required before any firm recommendations can be made regarding fludrocortisone dosing.

Adverse effects of hydrocortisone and fludrocortisone use

Risks associated with adjunctive corticosteroid therapy in severe sepsis and septic shock include gastroduodenal bleeding, superinfection, neuromuscular weakness, hyperglycaemia, and hypernatraemia.[142] Findings of a systematic review on corticosteroids in the treatment of severe sepsis and septic shock in adults suggested no significant differences in rates of gastroduodenal bleeding, superinfections, or neuromuscular weakness between treated and control patients. However, there were higher risks of hyperglycaemia (nine trials; 51.6 in the treatment group [363 out of 703] *vs* 46 in the control group [308 out of 670]; P 0.001; I^2 0) and hypernatraemia (three trials; 31.4 in the treatment group [127 out of 404] *vs* 19.2 in the control group [77 out of 401]; P 0.001; I^2 0) observed in the treated group.[142] In the APROCCHSS trial, the use of hydrocortisone in combination with fludrocortisone significantly increased the risk of hyperglycaemia (relative risk, 1.07; 95 CI, 1.03–1.12; P 0.002). There were no significant differences in the risks of gastroduodenal bleeding (relative risk, 0.88; 95 CI, 0.58–1.34; P 0.56) or superinfection (relative risk, 1.09; 95 CI, 0.92–1.30; P 0.30) when comparing hydrocortisone plus fludrocortisone with placebo. The relatively minimal adverse implications, along with the substantial potential benefit, would seem to favour adjunctive hydrocortisone and fludrocortisone administration in septic shock.

Conclusions

Human and laboratory data provide insights into potential mechanisms explaining the beneficial role of adjunctive

hydrocortisone with fludrocortisone in septic shock. Cortisol and aldosterone actions at the MR differ and by implication their synthetic counterparts, however, this has not yet translated into compelling clinical evidence of effect. Aldosterone exhibits mineralocorticoid and clinically relevant non-mineralocorticoid effects; thus, when considering potential beneficial mechanisms, the focus should not solely be on the activity of aldosterone or fludrocortisone at the MR. Signalling pathways activated by aldosterone on the MR are likely distinct from those of fludrocortisone. Consequently, specific effects of adjunctive fludrocortisone in combination with hydrocortisone in septic shock are still not well defined.

The precise targets of mineralocorticoid therapy within the context of sepsis require further elucidation. Additionally, the effect of 'pro-inflammatory' mineralocorticoid actions in relation to immunoparalysis in septic shock and on vascular reactivity requires further elucidation.Additionally, there is clearly a need for further investigation into the pharmacokinetics of orally administered fludrocortisone in critical illness.

Authors contributions

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Declaration of interest

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CHAPTER 9: Conclusions

Trauma and sepsis constitute significant global health challenges, with substantial morbidity and mortality (1,2). Traumatic injury consistently ranks as a leading cause of death, hospital admissions, morbidity, and disability (1). Sepsis continues to have a considerable annual incidence, even in high-income countries (2). While accurate estimates of sepsis incidence in developing nations are currently limited due to paucity of reliable data, it is anticipated to be higher, given that infectious diseases remain a prevalent cause of mortality in these regions (2). Advancing insights into the pathophysiology and treatment strategies of both sepsis and trauma has contributed to the decline in mortality rates (3).

The current research framework aimed to improve current understanding of inflammatory and endocrine dynamics in critical illnesses, with a specific focus on sepsis, and traumatic brain injury. Through a combination of clinical, biochemical, and molecular approaches, endocrine dysfunction associated with critical illness is evaluated with the aim of identifying patients with sepsis or trauma likely to have a poor outcome. Across chapters, findings provide novel insights into the pathophysiology of the dysregulated immune and endocrine responses, how these influence disease progression and outcomes, as well as exploring the molecular rationale of therapeutic interventions.

A summary of key findings, insights and core themes that were identified is as follows:

The background and literature review in **Chapters 1 and 2** provided a foundational understanding of the global burden of sepsis and trauma, the physiological basis of the neuroendocrine stress response in these conditions, gaps in the literature and the study rationale.

9.1 The evolving understanding of sepsis and trauma pathophysiology

Over recent decades, the focus in understanding sepsis and trauma pathophysiology has progressively shifted from pathogen-centric to host-centric perspectives with an emphasis on the role of the host's response in disease progression (3,4). Advances in clinical research have increasingly framed sepsis and trauma within an immune-endocrine context, highlighting the intricate interplay of pro- and anti-inflammatory responses (3,4).

The clinical trajectory of sepsis is dynamic and heterogeneous, influenced by pathogen virulence, co-morbid conditions, therapeutic interventions, and genetic predispositions (5). These factors interact to produce distinct inflammatory and immune profiles, resulting in variable patient responses to standard therapeutic strategies (5). Precise identification of high-risk subgroups is critical for optimising sepsis management and improving clinical outcomes (6,7).

Chapter 3 *(Profiles of pro- and anti-inflammatory cytokines and total cortisol levels in severe sepsis and septic shock)* provided an assessment and investigation of profiles of pro- and anti-inflammatory cytokines and total cortisol levels in a population with severe sepsis and septic shock. In patients with severe sepsis and septic shock, elevated pro-inflammatory cytokines were found to strongly correlate with increased 28-day mortality ratios. Key biomarkers, TNFR1, and IL-1Ra, were associated with increased 28-day mortality ratios (and disease severity). These findings highlight the impact of persistent markers of inflammation on sepsis outcomes. Elevated cortisol levels, reflecting the stress response to illness, were not independently associated with an increased mortality ratio, reflecting the multifaceted nature of hormonal regulation in sepsis and highlighting the limitations of total cortisol levels, as an independent predictor of increased mortality.

In the context of trauma, patients with traumatic brain injury demonstrated significantly higher plasma free cortisol levels than healthy controls both at baseline and post-ACTH stimulation (**Chapter 4**: *A comparison of the total and free cortisol response to a standard Synacthen test in patients with traumatic brain injury*), suggesting that plasma free cortisol may be a more accurate marker of adrenal function in traumatic brain injury than total cortisol. The assessment of total cortisol levels appeared to underestimate early adrenal responses in this population. Interestingly, patients with traumatic brain injury also exhibited decreased glucocorticoid sensitivity, evidenced by lower IL-6 and TNF-α suppression ratios as measured by the *in vitro* dexamethasone suppression of LPS-stimulated cytokine production test, although this finding is tempered by the limited sample size of the study.

In healthy controls (**Chapter 5**: *Glucocorticoid sensitivity and plasma total and free cortisol responses to ACTH in healthy male and female volunteers),* the cortisol response to corticotrophin (ACTH) showed substantial variability with no significant association between glucocorticoid sensitivity and cortisol levels demonstrated. The variability in glucocorticoid sensitivity observed, supports the need for more personalised approaches to assessment and therapeutic interventions as well as further adding a comparative baseline for future studies.

9.2 Endocrine dysfunction in critical illness – novel insights

Whether mineralocorticoid deficiency or dysfunction exists as a relevant pathophysiologic entity in critical illness remains an area requiring further investigation. **Chapter 6** *(Mineralocorticoid dysfunction during critical illness)* highlights the renewed interest in the role of the renin–angiotensin–aldosterone system (RAAS) during critical illness, emphasizing the potential under-recognition of mineralocorticoid dysfunction as a pathophysiological

entity. In this context, hyperreninemic hypoaldosteronism associated with a high mortality rate, is explored as a significant entity affecting critically ill patients with shock. Notably, variable plasma levels of aldosterone are seen in critical illness, along with what appears to be an impaired adrenal aldosterone response to elevated levels of renin. It is important to acknowledge that these findings may be influenced by the variability in the methods of measurement. The assessment of hypoaldosteronism in this context, as well as the role of mineralocorticoid replacement in the critically ill, remains a challenge, while the effect of angiotensin II in shock states remains minimally tested.

Hyperreninemic hypoaldosteronism, which is characterised by previous authors as an aldosterone/plasma renin activity (ALDO/PRA) ratio below 2 (8,9) is further explored in **Chapter 7** *(Critical illness related mineralocorticoid insufficiency (CIRMI) - a new term for a concept worthy of further exploration: a narrative review)***.** Hyperreninemic hypoaldosteronism in critical illness, results in the disruption of electrolyte balance and vascular responsiveness, and is associated with worse outcomes, including higher mortality and increased incidence of hypotension and acute kidney injury in shock states (8–11). This study introduces the novel concept of critical illness related mineralocorticoid insufficiency (CIRMI), which draws significant parallels to hyperreninemic hypoaldosteronism—a condition reflecting mineralocorticoid dysfunction—and the more extensively characterised pathophysiology of critical illness-related corticosteroid insufficiency (CIRCI). The abbreviation, CIRMI, reflects conceptual similarities with CIRCI (12) and encompasses a dissociation between renin and aldosterone levels (characterised by disproportionately high renin levels relative to the aldosterone response), reflective of impaired aldosterone synthesis or action. A comprehensive understanding of adrenal dysfunction as a condition affecting both glucocorticoid and mineralocorticoid dynamics is emphasized by this approach.

The diagnostic framework for CIRMI has limitations. The ALDO/PRA ratio is proposed as a diagnostic marker but is hindered by methodological factors. inclusive of variability in aldosterone and renin measurements by various laboratories, lack of standardised diagnostic criteria, and differences in assay methodology. Investigation into aldosterone resistance, alterations in enzyme and receptor expression involved in mineralocorticoid metabolism, and the development of reliable diagnostic tools, requires further elucidation.

However, existing evidence supporting the combined use of hydrocortisone and fludrocortisone in septic shock highlights the potential therapeutic benefit of addressing both glucocorticoid and mineralocorticoid insufficiency in patients with septic shock. By expanding the critical care focus of adjunctive therapy to encompass mineralocorticoid dysfunction, CIRMI provides a rationale for targeted adjunctive therapy inclusive of fludrocortisone in septic shock (13,14).

9.3 Role of treatment modalities – adjunctive fludrocortisone with hydrocortisone

With regards to therapeutic interventions, **Chapter 8** (*Glucocorticoids with or without fludrocortisone- a molecular and biochemical perspective***)** offers a biochemical and molecular perspective on the rationale for the use of hydrocortisone in combination with fludrocortisone in septic shock. Mechanistically, fludrocortisone supports vascular stability, electrolyte homeostasis, and inflammatory regulation, key factors at play in the haemodynamic morbidity associated with septic shock. While high-dose hydrocortisone alone has not consistently reduced mortality in septic shock, the addition of fludrocortisone has been shown to provide a survival advantage (13–15). This combined approach may offer

a more robust strategy for adjunctive therapy in the management of patients with septic shock. Questions do remain, however, regarding the pharmacokinetics of fludrocortisone and its bioavailability in critical illness.

9.4 Future directions

Despite significant progress in elucidating the complexity and heterogeneity of sepsis and trauma, the translation of this knowledge into evidence-based interventions for effective treatment remains an ongoing challenge. This thesis provides the framework and baseline data enabling research aimed at validating the role of free versus total plasma cortisol as a reliable marker of adrenal function in patients with traumatic head injury. Data on inter-individual variability in glucocorticoid sensitivity, particularly in the contexts of traumatic brain injury and sepsis, could enhance risk stratification and provide insights into the negative outcomes previously reported with glucocorticoid supplementation in traumatic brain injury.

The concept of CIRMI introduced in this thesis provides a new framework for understanding mineralocorticoid dysfunction in critically ill patients. Defining CIRMI more precisely, particularly the demonstration of aldosterone resistance, if any, standardising diagnostic criteria and improving assay methodologies for hyperreninemic hypoaldosteronism is essential to identify those with CIRMI.

Lastly, therapeutically, the clinical efficacy of fludrocortisone in combination with hydrocortisone in septic shock warrants expanded trials to optimise dosing, timing, and bioavailability in critically ill populations.

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APPENDICES

1. Ethics clearance certificates

R14/49 Dr Gladness Dakalo Nethathe

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150122

NAME: (Principal Investigator)	Dr Gladness Dakalo Nethathe
DEPARTMENT:	Critical Care Chris Hani Baragwanath Academic Hospital Royal Brisbane and Women's Hospital
PROJECT TITLE:	Aspects of the Neuroendocrine Stress Response in Patients with Severe Sepsis and Multi-trauma
DATE CONSIDERED:	30/01/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Charles Feldman
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	18/02/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. I agree to submit a yearly progress report.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

R14/49 Dr Gladness Dakalo Nethathe

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190888

NAME: (Principal Investigator)	Dr Gladness Dakalo Nethathe
DEPARTMENT:	Critical Care Chris Hani Baragwanath Academic Hospital Royal Brisbane and Women's Hospital
PROJECT TITLE:	Aspects of the Neuroendocrine Stress Response in Patients with Severe Sepsis and Multi-trauma
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Renewal of M150122
SUPERVISOR:	Prof Charles Feldman
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	05/09/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized
to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **August** and will therefore be due in the month of **August** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Queensland Government

Royal Brisbane & Women's Hospital
Human Research Ethics Committee

Enquiries to:	Ann-Maree Gordon A/Coordinator
Telephone:	07 3646 5490
Facsimile:	07 3646 5849
File Ref:	HREC/15/QRBW/609
Email:	RBWH-Ethics@health.qld.gov.au

Dr Gladness Nethathe
Department of Intensive Care Medicine
Level 3, Ned Hanlon Building
Royal Brisbane & Women's Hospital
Herston Qld 4029

Dear Dr Nethathe,

Re: Ref N^{o}: HREC/15/QRBW/609: The free cortisol response to Synacthen and other aspects of endocrine physiology in healthy participants

Thank you for submitting the above research project for single ethical review. This project was considered by the Royal Brisbane & Women's Hospital Human Research Ethics Committee (RBWH HREC) **(EC00172)** at its meeting held on 14 December 2015. This research project meets the requirements of the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007).*

I am pleased to advise that the RBWH Human Research Ethics Committee has granted ethical approval of this research project.

The nominated participating site for this project is:

- Royal Brisbane & Women's Hospital, Qld

This letter constitutes ethical approval only. This project cannot proceed until separate research governance authorisation has been obtained from the CEO or Delegate of the Royal Brisbane & Women's Hospital under whose auspices the research will be conducted.

The approved documents include:

Document	*Version*	*Date*
Covering Letter		26 November 2015
Application: NEAF *(Submission Code: AU/1/A6D228)*	2.2 (2014)	26 November 2015
Advertising Flyer for Healthy Participants		
Curriculum Vitae of Melissa Lassig-Smith	November 2015	
Response to Request for Further Information		10 February 2016
Protocol	2	01 February 2016
Healthy Volunteer Information Sheet & Consent Form	2	05 January 2016

Approval of this project from the RBWH HREC is valid from **12.02.2016** to **12.02.2019** subject to the following conditions being met:

- The Coordinating Principal Investigator will immediately report anything that might warrant review of ethical approval of the project.
- The Coordinating Principal Investigator will notify the RBWH HREC of any event that requires a modification to the protocol or other project documents and submit any required amendments in accordance with the instructions provided by the HREC. These instructions can be found at http://www.health.qld.gov.au/rbwh/research/hrec.asp.
- The Coordinating Principal Investigator will submit any necessary reports related to the safety of research participants in accordance with the RBWH HREC policy and procedures. These instructions can be found at http://www.health.qld.gov.au/rbwh/research/hrec.asp.
- In accordance with Section 3.3.22 (b) of the National Statement the Coordinating Principal Investigator will report to the RBWH HREC annually in the specified format, the first report being due on **12.02.2017** and a final report is to be submitted on completion of the study. These instructions can be found at http://www.health.qld.gov.au/ohmr/html/regu/reporting_templates.asp.
- The Coordinating Principal Investigator will notify the RBWH HREC if the project is discontinued before the expected completion date, with reasons provided.
- The Coordinating Principal Investigator will notify the RBWH HREC of any plan to extend the duration of the project past the approval period listed above and will submit any associated required documentation. Instructions for obtaining an extension of approval can be found at http://www.health.qld.gov.au/rbwh/research/hrec.asp.

- The Coordinating Principal Investigator will notify the RBWH HREC of his or her inability to continue as Coordinating Principal Investigator including the name of and contact information for a replacement.

- A copy of this ethical approval letter together with completed Site Specific Assessment (SSA) and any other requirements must be submitted by the Coordinating Principal Investigator to the Research Governance Office at the Royal Brisbane & Women's Hospital in a timely manner to enable the institution to authorise the commencement of the project at its site.

- Should you have any queries about the RBWH HREC's consideration of your project please contact the HREC Coordinator on 07 3646 5490. The RBWH HREC's Terms of Reference, Standard Operating Procedures, membership and standard forms are available from http://www.health.qld.gov.au/rbwh/research/hrec.asp.

The RBWH HREC wishes you every success in your research.

Yours sincerely,

Dr Conor Brophy
Chairperson RBWH Human Research Ethics Committee
Metro North Hospital and Health Service
12.02.2016

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007)*. The processes used by this HREC to review research proposals have been certified by the National Health and Medical Research Council.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO: Dr GD Nethathe
School of Clinical Medicine
Department of Medicine
Intensive Care Unit
Faculty of Health Sciences

E-mail: gladnessnethathe@gmail.com

CC: Supervisor: Professor C Feldman
<Charles.Feldman@wits.ac.za>
and <HREC-Medical Research Office@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2024/11/06

REF: R14/49

PROTOCOL NO: **M241079** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *Aspects of neuroendocrine stress response in patients with severe sepsis and multi trauma*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

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R49 Dr GD Nethathe

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M241079

NAME: Dr GD Nethathe **DEGREE:** PhD
(Principal Investigator)

SCHOOL: School of Clinical Medicine
Department of Medicine
Intensive Care Unit
Faculty of Health Sciences

PROJECT TITLE: *Aspects of neuroendocrine stress response in patients with severe sepsis and multi trauma*

DATE CONSIDERED: Ad hoc

DECISION: Approved unconditionally

CONDITIONS: Renewal of M19/08/88; expired on 2024/09/04

NOTE: If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>

SUPERVISOR: Professor C Feldman

APPROVED BY:

Professor P Ruff, Chairperson, HREC (Medical)

DATE OF APPROVAL: 2024/11/06 **EXPIRY DATE:** 2029/11/05

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

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I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report** in the format available at https://wits.ac.za/research/researcher-support/research-ethics/ethics-committees/. This report is due annually, for the duration of the Clearance Certificate, beginning on the first anniversary of Date of Approval above. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

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Date

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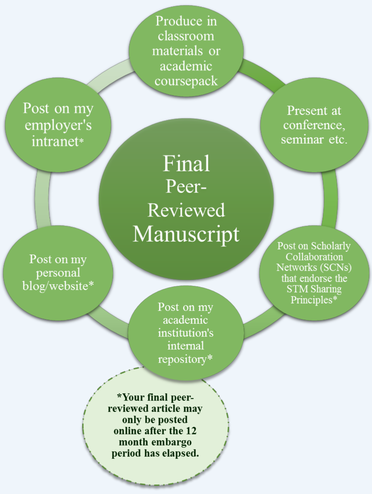

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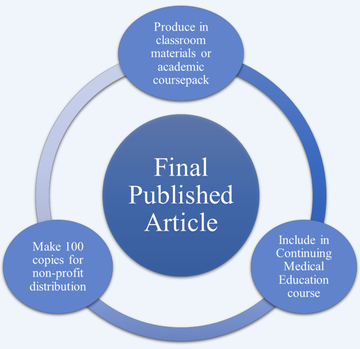

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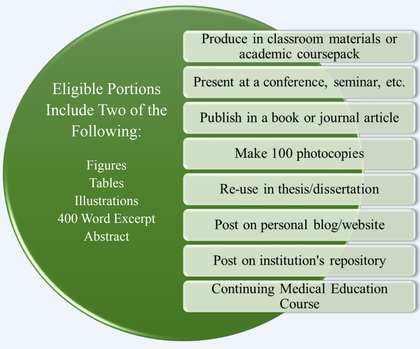

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