CORTICOSTEROID-BINDING GLOBULIN IN INFLAMMATION AND SEPTIC SHOCK.

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PUBLICATIONS

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The author acknowledges the copyright of published works contained within this thesis including:

Chapter 1:

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Meyer E. Supplemental Data - Corticosteroid-binding globulin deficiency independently predicts mortality in septic shock. *Dryad, Dataset,*. 2022. doi:10.5061/dryad.7pvmcvdvd.

Authorisation to publish each paper has been given and provided in print for each chapter containing copyright and co-authored work, including acknowledgement of contribution to the work from each author.

All supplementary material can be found online using the citation information as described above for each published article.

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ABSTRACT

Septic shock is a life-threatening organ dysfunction due to a dysregulated host response to pathogenic infection and is clinically defined by persistent hypotension requiring vasopressors to maintain a mean arterial pressure > 65 mmHg despite fluid resuscitation. Septic shock is common and has a mortality of 25–40%. Despite large randomised controlled trials, the benefits of hydrocortisone administration to patients with septic shock remain uncertain.

Corticosteroid-binding globulin (CBG) is the principal transport protein for cortisol in the circulation. Neutrophil elastase (NE) cleavage of the reactive centre loop (RCL), irreversibly converts high affinity (ha) CBG to a low cortisol-binding affinity (la) state, providing the targeted release of immunomodulatory cortisol at inflammatory sites. CBG is consumed and production in septic shock is reduced. Serum CBG concentrations fall substantially in proportion to sepsis severity and in survivors levels normalise within days. An acquired CBG deficiency in septic shock may reduce the capacity of cortisol to prevent clinical deterioration thus predicting organ failure and mortality.

CBG transports cortisol and other steroids. Development of a novel homogenous ligand bind assay using surface plasmon resonance (SPR) technology allowed for the determination of binding affinities of CBG to cortisol and 18 other steroid ligands at temperatures and acidic conditions that mimic the pathophysiological conditions of septic shock. The most potent binding was recorded for haCBG binding to cortisol (K_D 42 nM) and corticosterone (K_D 45 nM). An expected 4–8-fold reduction in affinity for cortisol, cortisone, corticosterone, 11-deoxycortisol, progesterone, 17-hydroxyprogesterone and prednisolone occurred with NE-mediated haCBG-to-laCBG conversion. CBG:cortisol binding affinity was further reduced 3.5-fold at 39 °C relative to 37 °C; binding affinity was further reduced by acidosis for both haCBG and laCBG. Using a conformational antibody (9G12) generated against the RCL, confirmed RCL antibody binding was eliminated by NE cleavage, but preserved in pyrexia and acidosis.

In silico molecular dynamic simulation studies performed at 40 °C identified the thermocoupling mechanism of cortisol release is mediated through the destabilisation of Trp371, a tryptophan residue located within the cortisol-binding pocket, suggesting an additional non-RCL mechanism for the delivery of anti-inflammatory cortisol in pyrexia. CBG has a cortisol tissue delivery role in inflammation, with pyrexia and acidosis reversibly reducing cortisol binding affinity. Taken together the synergy of NE cleavage, pyrexia and acidosis on CBG:cortisol binding may serve to enhance cortisol delivery to the interstitial space in inflammation.

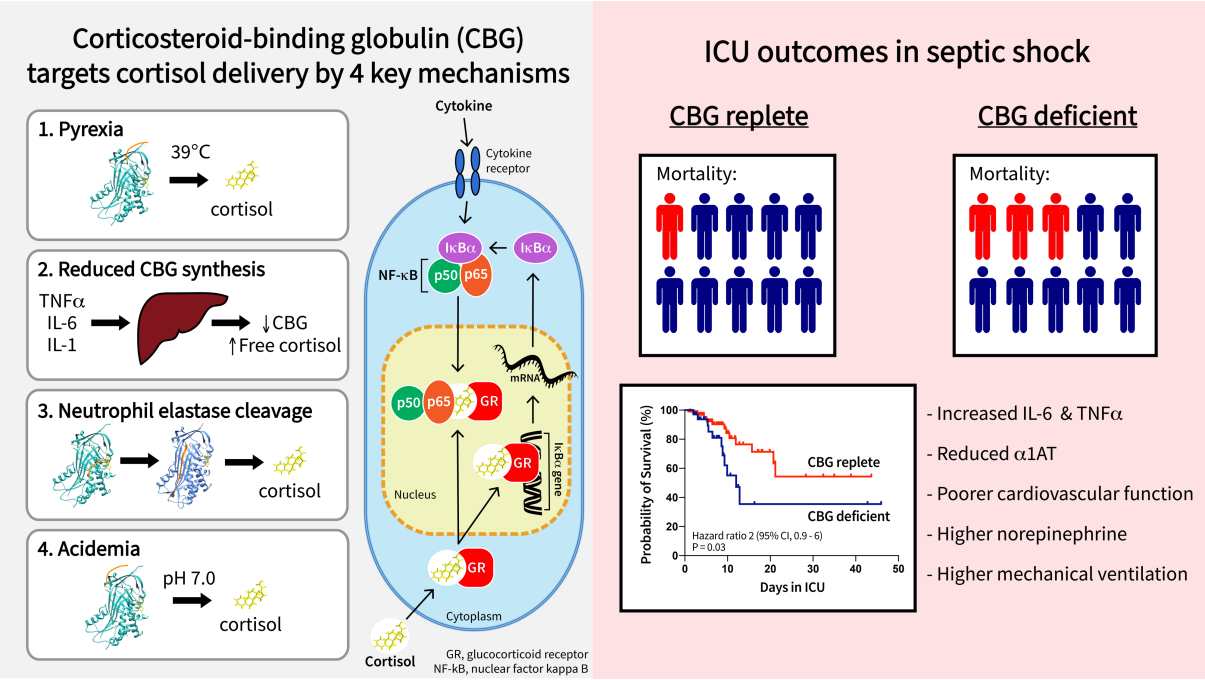
Novel measurement of total CBG and haCBG by enzyme-linked immunosorbent assay is at the forefront of CBG research and this thesis is predicated on this methodology. I hypothesised that severe deficiency in CBG (<200 nmol/L [Reference interval 269–641 nmol/L]) may independently predict mortality and is associated with key disease severity markers in patients with septic shock.

An initial study of 30 patients with septic shock showed an association between CBG and mortality. Total CBG and haCBG were significantly lower in septic shock patients who died than in those who survived ($P < 0.009$, $P = 0.021$, respectively). Total and free cortisol were higher in septic than non-septic individuals, however cortisol levels were not associated with mortality. Albumin levels fell in sepsis but were not related to mortality. Approximately one-third of septic shock patients have admission CBG concentrations below 200 nmol/L.

A subsequent large single centre prospective observational study of 135 patients with septic shock revealed the novel finding that CBG deficiency (<200 nmol/L) on Intensive Care Unit (ICU) admission independently predicted ICU mortality (3.2-fold risk ratio) and was associated with the need for ventilator and vasopressor support. Mortality rates in ICU were higher in the CBG <200 nmol/L *vs* the CBG ≥200 nmol/L group: 32.4% vs 13.9%; Odds ratio (OR), 2.97, (95% confidence interval (CI) 1.19,7.41); P=0.02: 28-days; OR 2.25 (95% CI 0.99,5.11): 90-days; OR 2.21 (95% CI 0.99,4.91). Multivariate analysis revealed 4 factors independently associated with ICU mortality: CBG <200 nmol/L (OR 3.23, 95% CI 1.06,9.88), Acute Physiology and Chronic Health Evaluation II >25 (OR 3.58, 95% CI 1.20,10.68); SOFA liver score (OR 1.98, 95%

CI 1.04,3.72); and renal-replacement therapy (OR 6.59, 95% CI 2.17,20.01). CBG concentration was the only directly reversible independent mortality risk factor. Nadir CBG levels were associated with higher SOFA cardiovascular scores, norepinephrine total dose (μg) P<0.01 and duration (days) P<0.01. Plasma cortisol concentrations, serum albumin concentrations and hydrocortisone administration did not relate to ICU mortality.

Septic shock is a public health emergency and represents 20% of all global deaths. Standard treatments including fluids, vasopressors and antimicrobials often fail, however despite this there has been no new therapeutic advances in over 20 years. Using SPR technology were able to demonstrate the importance of temperature and pH in CBG:cortisol binding affinity, supporting its role in the delivery of cortisol in states of pyrexia and acidaemia. Our clinical studies in septic shock demonstrate a strong relationship between CBG deficiency and disease severity, cardiac dysfunction and increased mortality. This accords with CBG's role in targeted delivery of immunomodulatory cortisol to tissues to quell systemic inflammatory processes. Given the 3.2-fold increase in mortality, replenishing CBG may have a large therapeutic effect in those with CBG <200 nmol/L in the treatment of septic shock, avoiding the multiorgan failure cascade that accompanies severe illness and death. Human clinical trials investigating CBG supplementation in septic shock may be of practical benefit and warrants further investigation.



CBG – corticosteroid-binding globulin, IL-6 – interluken-6, TNFα – tumour necrosis factor alpha, A1AT – alpha-1-antitrypsin.

FIGURE 1. GRAPHICAL ABSTRACT—Four-phase actions of CBG in delivering cortisol to sites of inflammation and outcomes of CBG deficiency in septic shock.

Left panel: CBG's role in targeted delivery of immunomodulatory cortisol to tissues to quell systemic inflammatory processes. CBG targets cortisol delivery via four key mechanisms; 1) CBG releases cortisol in pyrexia independent of cleavage, 2) inflammatory cytokines inhibit CBG mRNA production in hepatocytes, reduction in CBG availability leads to a rise in serum free cortisol, 3) neutrophil elastase cleaves CBG releasing cortisol at inflamed sites, CBG undergoes a permanent conformational change upon NE enzymatic cleavage with the conversion of high cortisol-binding affinity CBG (haCBG) to low cortisol-binding affinity CBG (laCBG) and 4) CBG releases cortisol in acidosis independent of cleavage.

Right panel: Septic shock patients with a deficiency of serum CBG <200 nmol/L had cardiac dysfunction, higher norepinephrine and mechanical ventilation requirements, and 3.2-fold higher ICU mortality. Biochemical factors known to influence CBG levels, α1AT and inflammatory cytokines (IL-6 and TNFα), were shown to directly correlate with serum CBG concentrations in septic shock.

DECLARATION FOR THESIS CONTAINING PUBLISHED WORK

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree. The author acknowledges that copyright of published works contained within the thesis resides with the copyright holder(s) of those works. I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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Chapter 3

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Chapter 4

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Chapter 5

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I dedicate my thesis to my partner Michael and our two children, Hamish and William, for their generous support.



Raoul Dufy (1877-1953),

French. La Cortisone, 1951.

Color lithograph, 18 × 23 in.

Courtesy of Mayo Foundation for Medical Education and Research.

In December 1949 Freddy Homburger MD saw a photograph of French painter Raoul Dufy in *Life* magazine that showed his hands had been deformed by rheumatoid arthritis. Dufy had lost the use of his right hand, struggling to paint with only his left. Homburger, familiar with recent research by Philip Hench MD of corticotrophin (ACTH) and cortisone for arthritis, wrote to Dufy inviting him to participate in clinical research in Boston. Dufy accepted, traveled across the Atlantic Ocean, and commenced treatment in 1950. Dufy saw significant improvements and with his recovery won the grand prize at the Vienna Art Biennial in 1952. In 1950 Hench was the corecipient of the Nobel Prize in Physiology or Medicine for "discoveries related to the hormones of the adrenal cortex, their structure and biological effects," particularly for the treatment of rheumatoid arthritis. Aware of this, Dufy gifted Hench a set of floral prints intitled *La Cortisone*, one of which still hangs today within the patient waiting area of the Mayo Clinic.

COMMUNICATIONS AND PRESENTATIONS

Meyer EJ, Nenke MA, Rankin W, Lewis JG, Hofland H, Feelders R, Torpy DJ. (2017) Low plasma high affinity corticosteroid binding globulin in human sepsis predicts mortality. *Endocrine Society of Australia Annual Scientific Meeting, Perth, Western Australia, Australia.* Oral Presentation.

Meyer EJ. Corticosteroid-binding globulin; role in cortisol transport and inflammation. (2017) *Royal Adelaide Hospital Grand Round, Adelaide, South Australia, Australia*. Oral Presentation.

Meyer EJ. (2017) Low plasma high affinity corticosteroid binding globulin in human sepsis predicts mortality. *RACP South Australian Trainee Research Awards. Adelaide, South Australia, Australia.* Oral Presentation.

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Chernykh A, Nishtala K, Chatterjee S, Tjondro H, Hinneburg H, Sumer-Bayraktar Z, **Meyer EJ**, Torpy DJ, Thaysen-Andersen M. (2019) Site-specific glycoprofiling of recombinant human corticosteroid-binding globulin. *18th Human Proteome Organization World Congress. Adelaide, South Australia, Australia.* Poster Presentation.

Meyer EJ, Torpy DJ, Chernykh A, Thaysen-Andersen M, Lewis JG, Rankin W, Polyak S. (2020) Steroid:Corticosteroid-Binding Globulin Interactions; Effects of Neutrophil Elastase Cleavage, Pyrexia and Acidosis. *ENDO 2020. San Francisco, California, United States of America.* Oral Presentation.

Meyer EJ, Nenke MA, Chapman M, Rankin W, Rushworth LR, Torpy DJ. (2021) Corticosteroid-binding globulin deficiency independently predicts mortality and is associated with norepinephrine requirements in septic shock. *Endocrine Society of Australia Annual Scientific Meeting. Virtual Meeting, Australia.* Oral Presentation.

Selected for the Bryan Hudson Clinical Awards Category.

ABBREVIATIONS

11β-HSD	11β-hydroxysteroid dehydrogenase type 2
α1AT	Alpha-1 antitrypsin
AB	Assay buffer
ACTH	Adrenocorticotropic hormone
AGC	Automatic gain control
ANC	Absolute neutrophil count
ANOVA	Analysis of variance
ANZCTR	Australian New Zealand Clinical Trials Registry
AP-1	Activator Protein 1
APACHE II	Acute Physiology and Chronic Health Evaluation II
BMI	Body mass index
BP	Blood pressure
C	Centigrade
CBG	Corticosteroid-binding globulin
CI	Confidence interval
CID	Collision-induced dissociation
CIRCI	Critical illness-related corticosteroid insufficiency
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CV	Cardiovascular
DHEA	Dehydroepiandrosterone
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ELISA	Enzyme-linked assay
ESR	Erythrocyte sedimentation rate
FWHM	Full width at half maximum
GR	Glucocorticoid receptor
haCBG	High cortisol-binding affinity CBG
HC	Healthy control
HPA	Hypothalamic-pituitary-adrenal

HREC	Human research ethics committee
HRP	Horseradish peroxidase
ICU	Intensive care unit
IL	Interleukin
IQR	Interquartile range
K_D	Dissociation constant
LC-MS	Liquid chromatography
laCBG	Low cortisol-binding affinity CBG
LOS	Length of stay
M	Molar
MD	Molecular dynamic
MS	Mass spectrometry
N	Number
NA	Not applicable
NCE	Normalised collision energy
NE	Neutrophil elastase
NF-κB	Nuclear factor kappa
OR	Odds Ratio
P	Pressure
PBS	Phosphate buffered saline
PGC	Porous graphitised carbon
PNGase F	Peptide:*N*-glycosidase F
RAH	Royal Adelaide Hospital
RCL	Reactive centre loop
REDCap	Research Electronic Data Capture
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
RU	Resonance units
S	Sepsis
SS	Septic shock
SD	Standard deviation
SEM	Standard error of the mean

SERPIN	Serine protease inhibitor
SOFA	Sequential [Sepsis-related] Organ Failure Assessment
SPE	Solid-phase extraction
SPR	Surface plasmon resonance
SS-NS	Septic shock non-survivors
SS-S	Septic shock survivors
T	Temperature
TNFα	Tumour necrosis factor alpha
Trp	Tryptophan
V	Volume
WCC	White cell count
W	Water

CHAPTER 1:

INTRODUCTION – LITERATURE REVIEW.

CHAPTER OUTLINE

This chapter is a published review outlining prior literature about corticosteroid-binding globulin (CBG), the main focus of this thesis. This review provides a background to CBG, focusing on the implications of CBG changes in acute and chronic inflammation, including sepsis; CBG functions; neutrophil elastase cleavage; genetic variants; and glycosylation. This chapter highlights current knowledge regarding CBG and reveals information that is lacking, and which requires investigation to fully understand CBG's role in quelling inflammation.

STATEMENT OF AUTHORSHIP

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Emily J. Meyer		
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Overall percentage (%)	80%		
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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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REVIEW

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Corticosteroid-binding globulin: acute and chronic inflammation

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ABSTRACT

Introduction: Corticosteroid-binding globulin (CBG) is the principal transport protein for cortisol binding 80% in a 1:1 ratio. Since its discovery in 1958, CBG's primary function has been considered to be cortisol transport within the circulation. More recent data indicate a cortisol tissue delivery function, particularly at inflammatory sites. CBG's structure as a non-inhibitory serine protease inhibitor allows allosteric structural change after reactive central loop (RCL) cleavage by neutrophil elastase (NE) and RCL insertion into CBG's protein core. Transition from the high to low affinity CBG form reduces cortisol-binding.

Areas covered: In acute systemic inflammation, high affinity CBG (haCBG) is depleted proportionate to sepsis severity, with lowest levels seen in non-survivors. Conversely, in chronic inflammation, CBG cleavage is paradoxically reduced in proportion to disease severity, implying impaired targeted delivery of cortisol. CBG's structure allows thermosensitive release of bound cortisol, by reversible partial insertion of the RCL and loosening of CBG:cortisol binding. Recent studies indicate a significant frequency of function-altering single nucleotide polymorphisms of the SERPINA6 gene which may be important in population risk of inflammatory disease.

Expert commentary: Further exploration of CBG in inflammatory disease may offer new avenues for treatment based on the model of optimal cortisol tissue delivery.

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Cortisol; high-affinity CBG; low-affinity CBG; inflammation; corticosteroid-binding globulin

1. Introduction

1.1. Functions of corticosteroid-binding globulin and relation to its biochemistry

Corticosteroid-binding globulin (CBG) was first discovered in 1958 by Daughaday using equilibrium paper electrophoresis [1]. It was ascribed the role of increasing the solubility of cortisol in plasma, providing circulatory transport, and a buffer function for fluctuating cortisol levels. CBG, a serine proteinase inhibitor (serpin), is a high-affinity (Ka 76 × 10^6 L/mol) cortisol transport protein for cortisol, but it also binds progesterone (Ka 24× 10^6 L/mol) and prednisolone (Ka 56× 10^6 L/mol) with lower affinity [2,3]. In 1988, CBG was found to release cortisol after cleavage by the serine protease neutrophil elastase (NE) [4].

Under basal conditions, 80% of circulating cortisol is CBG bound, ~5% is free, and ~15% is loosely bound to albumin. CBG circulates in concentrations of 300–500 nmol/L, and each CBG molecule can bind only one cortisol molecule. Hence, CBG is readily saturable in the face of rising cortisol concentrations during stress, leading to an exponential free cortisol rise above ambient CBG concentrations [1,5–7].

CBG ultimately circulates as a 383-amino acid protein following removal of a 22-amino acid leader sequence prior to secretion. *SERPINA6*, the CBG gene, is located on chromosome 14 (q31–q32.1) [8,9]. CBG has a molecular size of 42 kDa; however, size varies from 50 to 60 kDa depending on glycosylation status [10]. CBG is primarily synthesized by hepatocytes [11,12]; however, CBG mRNA has been located within other cells or tissues, suggesting the possibility of local steroid-binding function for CBG; for example, CBG mRNA has been localized from adrenocorticotrophs but not in other pituicytes [10,13–18].

CBG allows for the targeted delivery of cortisol to areas of inflammation through NE-mediated proteolysis [4,19]. Cortisol is usually bound to native, high-affinity CBG (haCBG) inside a surface pocket enveloped by β-sheet B, helix H, and helix A [20] (Figure 1). Cleavage of the reactive center loop (RCL) between $Valine^{344}$ and $Threonine^{345}$ by NE converts haCBG to low-affinity CBG (laCBG) through full insertion of the now mobile RCL N-terminal segment into β-sheet A, resulting in a structural change. Helix D then unwinds, and the subsequent allosteric modifications interrupt the cortisol-binding site, reducing CBG:cortisol binding by 90%. This conformational change in CBG after NE is referred to as transition from a stressed 'S' to a relaxed 'R' CBG state [21,22]. The release of cortisol after the action of NE [23] allows for targeted delivery to sites of inflammation, increasing tissue cortisol concentrations three- to fourfold [21,22] (Figure 1) [20,24]. Under basal physiological conditions, laCBG binds 4% of cortisol; however, this increases up to 16% upon complete cleavage of the CBG pool [21,22,25,26] (Figures 1 and 2 and Table 1).

CBG is also susceptible to cleavage by proteinases other than NE, at sites different from Val^{344}–Thr^{345}. Within the RCL,

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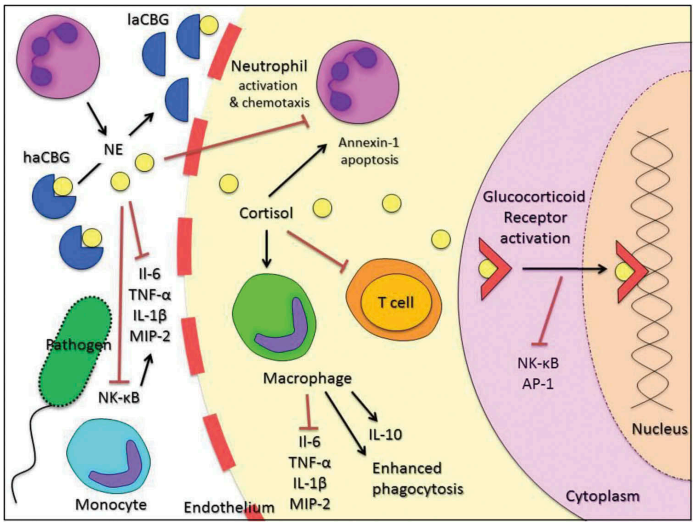


Figure 1. Anti-inflammatory effects of CBG and cortisol. The haCBG:cortisol pool allows targeted delivery cortisol to inflamed sites exerting anti-inflammatory effects. Proteases such as neutrophil elastase secreted by activated neutrophils cleaves haCBG, decreasing cortisol-binding affinity and releasing cortisol. CBG:cortisol acts as a thermocouple with pyrexia decreasing cortisol-binding affinity and releasing cortisol at sites of inflammation. Free cortisol (yellow circle) is able to diffuse into cells and modulate intracellular processes. Within the cell cortisol binds to GR causing GR activation and translocation to the nucleus. Within the nucleus GR represses the pro-inflammatory transcription factors NF-κB (nuclear factor kappa B) and AP-1 (activator protein 1). haCBG – high affinity CBG. GR = glucocorticoid receptor. Cortisol represses the expression of pro-inflammatory cytokines by immune cells, reduces adhesion molecules, induce annexin-1 promoting neutrophil detachment and apoptosis, increase the phagocytic activity of macrophages and block Th1- and Th2 derived cytokine production and induce cell death.

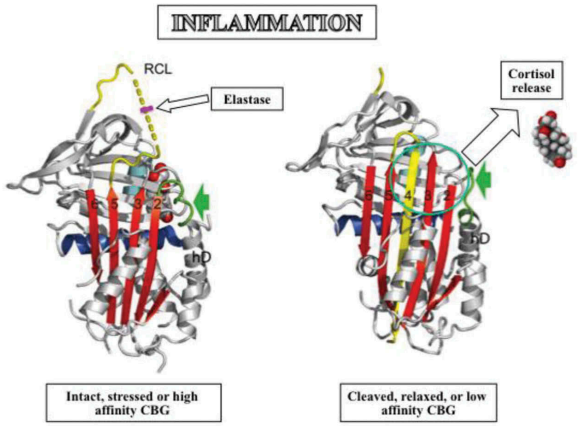


Figure 2. Crystal structure of CBG undergoing conformational change from stressed, haCBG to relaxed laCBG after cleavage and subsequent insertion of the reactive centre loop (RCL; yellow) into the body of the molecule (β sheet A strands numbered 2–6) releasing bound cortisol. The cortisol-binding site is bound by helix H (cyan), helix A (blue) and helix D (hD). Adapted from [25].

LasB, a virulence factor secreted by *Pseudomonas aeruginosa*, cleaves CBG in four places, the primary cleavage site between Asn^{347} and Leu^{348} reducing affinity for and releasing CBG-bound cortisol [29,30]. Chymotrypsin cleaves the RCL of CBG in two places (Table 2) [26,30,31]. The physiological importance of these proteases is unknown.

In addition to the enzymatic-mediated cleavage of the RCL and release of cortisol, cortisol is liberated from CBG with

Table 1. Characteristics of high-affinity corticosteroid-binding globulin (haCBG) and low-affinity corticosteroid-binding globulin (laCBG).

	Reference	haCBG	laCBG
Molecular weight (kDa)	[4]	52	48
Cortisol-binding sites (*n*)		1	1
Cortisol-binding affinity (K_D, nM)	[21]	32.0 ± 1.8	292 ± 27.8
Mean concentration in serum (nmol/L)	[27]	Females: 438	Females: 206
Female: n = 94, male: n = 80		Males: 377	Males: 195
Half-life[a]	[28]	10 h	10 h

kDa: kilodalton; K_D: dissociation constant.
[a]Nonhuman studies.
Reprinted with permission from [26].

increments in temperature acting as a thermocouple (Figure 1) [21,25]. With rising temperatures, the intact RCL partially inserts into β-sheet A and helix D provisionally unwinds disrupting the cortisol-binding site resulting in cortisol release [21,24,25]. The CBG molecule remains intact, partially transitioning from the S to R state permitting a reversible temperature-dependent release of cortisol. Crystal structures and binding assays have supported the changes in CBG–cortisol binding affinity [20,24,25,34,35]. Using two novel monoclonal antibodies recognizing different moieties of the intact RCL spanning the NE cleavage site, Lewis et al. demonstrated that the RCL remains exposed and accessible to RCL-specific antibodies and NE in temperatures of up to 40°C [35].

2. Body

2.1. CBG, cortisol, and hypothalamic–pituitary–adrenal axis function in inflammation

Normal function of the hypothalamic–pituitary–adrenal (HPA) axis is critical for life. In acute physiological stress, cortisol has pleiotropic actions including increased hepatic glucose output, elevated blood pressure through synergy with catecholamines on peripheral vasculature and the heart, immunomodulation, increased cognitive focus, and arousal. Cortisol has initial pro-inflammatory actions, enhancing innate immune responses for short-term minor insults, followed by mechanistically better understood anti-inflammatory actions through glucocorticoid receptor-α-mediated suppression of intracellular pathways induced by the pro-inflammatory transcription factors nuclear factor kappa B and activator protein 1. Glucocorticoids target inflammatory cytokine production, neutrophil migration, T-cell apoptosis, and macrophage inhibition [36] (Figure 1). Potentially, initial local release of cortisol at inflammatory sites may involve the thermocouple function of CBG, with increased delivery of local cortisol due to increased CBG cleavage and a more general increase in free cortisol relying on HPA axis activation and lowered CBG levels, as the inflammatory state progresses [37].

Cortisol movement across cell membranes may involve free cortisol diffusion only, although CBG–cortisol interactions with a proposed CBG receptor have been partially characterized in rats [38].

2.2. In vivo study of CBG cleavage

The study of CBG cleavage *in vivo* requires measurement of CBG in its high-affinity intact and low-affinity cleaved forms. This can be done by electrophoretic methods or, in the case of our human studies, ELISA-based monoclonal assays. The development of monoclonal antibody 9G12 which recognizes the amino acid residues 341–348 within the RCL has allowed for the identification and quantification of intact haCBG [32]. A second monoclonal antibody directed against an epitope unaffected by NE cleavage allows direct measurement of total CBG, with laCBG inferred by subtraction.

Under basal conditions, 30–35% of circulating total CBG is laCBG in humans [27]. This suggests that laCBG is stable and not rapidly metabolized. Indeed, one study in rabbits showed equivalent circulating half-lives of human haCBG and laCBG [28].

2.3. CBG genetic variants

The discoveries of several human CBG genetic variants detected through clinical enquiry have revealed unexpected phenotypic implications. Nine CBG variants have been described in humans, seven of which effect either CBG synthesis or function [39]. The CBG Null/Adelaide G121A mutation resulting in a premature stop codon and complete loss of CBG synthesis was identified within an Italian–Australian kindred after the proband presented with hypotension and fatigue [40]. Where CBG is absent, as in these very rare CBG null individuals, circulating cortisol levels are commensurately reduced by approximately 80%. Five mutations affect CBG–cortisol binding affinity; the most severe seen in CBG Athens W371S [41] and CBG G237V [42] with a complete loss of binding affinity. CBG Leuven T433A [43–45] and CBG Lyon G1254A [40,41,46–49] result in a three- and fourfold reduction, respectively, and a partial loss is seen in CBG E102G [50]. CBG Santiago c.12delC [51] and CBG A51V

Table 2. Reactive center loop of native human corticosteroid-binding globulin (CBG) indicating known cleavage sites.

Human CBG	References	P17 E	P16 E	P15 G	P14 V	P13 D	P12 T	P11 A	P10 G	P9 S	P8 T	P7 G	P6 V	P5 T	P4 L	P3 N	P2 L	P1 T	P1′ S	P2′ K	P3′ P	P4′ I	P5′ I
Neutrophil elastase	[4]													^									
LasB	[27,30]														^	^	^*	^					
Chymotrypsin	[31]														^			^					
Epitope for monoclonal antibody-detecting total CBG	[32]									⟷	⟷	⟷	⟷	⟷	⟷	⟷	⟷						
Potential glycosylation site	[33]																^						
Human ATT		E	K	G	T	E	A	A	G	A	M	F	L	E	A	I	P	M	S	I	P	P	E
Neutrophil elastase																			^				

ATT: alpha-1 antitrypsin; LasB: *P. aeruginosa* elastase.
[a]Main cleavage site.
Adapted with permission from [26].

[50,52] mutations affect hepatic synthesis with in up to 50% reductions in plasma CBG concentrations. Another common variant CBG A224S found during candidate gene studies of patients with septic shock [44] is associated with increases in plasma CBG and lower plasma cortisol levels [53]; however, it does not appear to affect the function of CBG [44].

The characterization of CBG variants, detected in single-nucleotide polymorphism (SNP) databases and mutagenesis studies, has identified variants resistant to RCL cleavage (T342P and T342A), preserved cortisol-binding capacity after cleavage or both (I179V, I279F [with reduced CBG–cortisol binding affinity]), and increased sensitivity to cleavage (G335V). Altered cleavage has potential importance in the risk of developing inflammatory diseases [54,55]. The number of detected non-synonymous SNPs of the CBG gene has been increasing rapidly, now including over 400 SNPs, of which only 32 of which have been studied for their effects on CBG function [39]. CBG SNP variations have marked ethnic prevalence differences; for example, A51V is prevalent in Han Chinese and CBG Lyon is mostly found in Mediterranean people.

The CORNET study used a genome-wide association–SNP design to identify regions of the genome that influence elevated morning cortisol levels and found the strongest genetic variants in *SERPINA6*, the CBG gene. Hence CBG variants have a key role in determining cortisol levels and therefore risk of disease [56]. Physiologically, the major genetic influence on plasma cortisol is mediated by variations in the cortisol-binding capacity of CBG. This was determined by differences in the circulating concentration of CBG and on the NE cleavability of the RCL. The region encompassed by rs12589136 (4 kb upstream of *SERPINA6*) and rs11621961 (1 kb downstream of *SERPINA6*) with rs2749527 mid-region represented the genomic region associated with altered cortisol and CBG levels. All three T allele variants were associated with higher cortisol-binding affinities as assessed by [^{3}H] cortisol binding; higher total CBG levels as measured by radioimmunoassay were strongly associated with the T allele of rs2749527 and less strongly with rs11621961. Rs12589136 was not associated with total CBG but with immunoreactivity of the RCL of CBG using our 9G12 antibody [32,56]. The knowledge of polymorphic SNPs allows the opportunity to perform Mendelian randomization studies examining SERPINA6 genetic variants in diseases where cortisol and/or CBG are thought to have a causal effect on the development or severity of disease. Population-based studies of genes in the HPA axis and functional somatic syndromes found multiple SNPs in SERPINA6 to be associated with chronic widespread pain and maximal number of painful sites [53,57,58]. No association was seen with other HPA axis candidate genes despite the association between these disorders and lower serum cortisol. Of SERPINA6 variants, underlying the pathogenesis of pain and fatigue disorders is unknown but may involve cortisol transport generally or within the central nervous system [53].

The characterization of specific CBG mutants *in vitro* has allowed for a greater understanding of CBG secretion and function; however, the physiological and clinical implications of such variants, especially on the susceptibility, development, and perpetuation of various inflammatory conditions, are yet to be described. Overall, basic data suggest function-altering CBG SNPs have the potential to alter the prevalence and course of inflammatory conditions. *SERPINA6* SNPs also alter cortisol levels and may influence a range of inflammatory and metabolic diseases [59–63].

2.4. CBG glycosylation; potential implications for inflammation

CBG has six potential asparagine (N)-linked glycosylation sites, with five sites occupied by N-acetyllactosamine oligosaccharides, three are biantennary and two are triantennary, per molecule [64,65]. CBG glycosylation occurs posttranslation and prolongs half-life and influences binding affinity [21,28,66].

Position-specific glycosylation is important for CBG function. Glycosylation at Asn^{347} located within the RCL has been shown to influence cortisol-binding capacity by modulating protease cleavage and protein folding [30]. About 80% of circulating CBG has highly branched and voluminous glycans at the Asn^{347} site. Volume-enhancing glycation of Asn^{347} antagonizes NE cleavage of the RCL, slowing the rate of cortisol release. Glycosylation at Asn^{347} modulates the RCL cleavage susceptibility by *P. aeruginosa*-derived LasB (Figure 3) [30].

Glycosylation increases CBG thermosensitivity and is crucial for thermocouple functioning [21,67]. Glycosylation decreases CBG–cortisol binding affinity fourfold with a rise in temperature from 37°C to 42°C, where only a twofold decrease occurs with unglycosylated CBG [21,67].

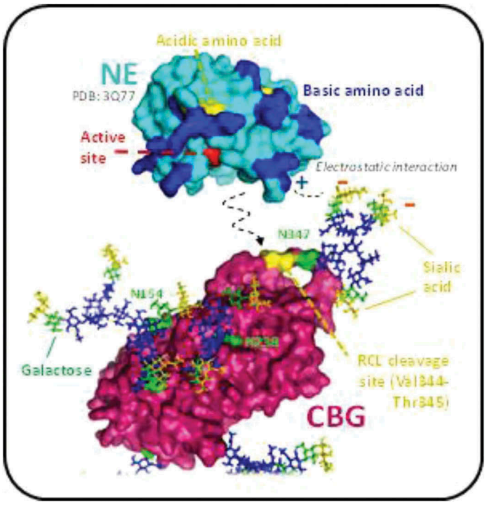


Figure 3. CBG Asn347 glycosylation. Carbohydrates (multi-coloured sticks) on Asn347 (N347, green) of CBG (magenta) modulate the RCL cleavage by NE (turquoise) (and in effect regulates the cortisol release) by, in part, sterically masking the RCL (yellow) and, in part, by electrostatically attracting the protease. RCL: reactive center loop, CBG: corticosteroid binding globulin, NE: neutrophil elastase. Reprinted with permission from [30].

2.5. Acute inflammation

2.5.1. CBG, sepsis, and septic shock

Total CBG and haCBG fall in sepsis and septic shock, due to CBG cleavage, as free cortisol [68] is released to fulfill its immunomodulatory roles and from cytokine-modulated inhibition of hepatic CBG synthesis [69]. In sepsis, circulating haCBG concentrations fall by 9%, in septic shock survivors by 51% and in septic shock non-survivors by 61% (Figure 4) [70]. The reduction in haCBG correlates more closely with sepsis severity than either total or free cortisol and thus could prove to be an important prognostic marker for sepsis. With the development of life-threatening systemic inflammation, haCBG continues to be cleaved, depleting the haCBG–cortisol reservoir and targeted delivery of cortisol to sites of inflammation perhaps perpetuating the inflammatory process.

In studies of CBG-deficient persons, it appears that free cortisol, not total cortisol, regulates HPA axis activity [71]. In inflammation, free cortisol levels are maintained by the release from CBG and reduction in cortisol metabolism [72]; however, in severe cases, free cortisol in turn may inhibit the HPA–glucocorticoid drive, causing a decrease in cortisol synthesis, reducing cortisol concentrations.

The use of exogenous hydrocortisone in septic shock remains controversial. While it improves blood pressure in some patients, mortality is not improved. Guidelines support the use of low-dose hydrocortisone (200 mg/day) in selected septic shock patients although outcomes cannot be predicted using basal or stimulated cortisol levels [73]. haCBG levels below a threshold identify those who may benefit from exogenous glucocorticoids, due to lack of appropriate cortisol–tissue targeting. Monoclonal antibodies directed against the RCL reduce CBG cleavage velocity by NE and Las B. Theoretically, such an approach could be used to lessen cortisol delivery, although a logical translational paradigm is not readily apparent [74].

CBG dynamics in inflammation has been replicated in animal models. In Charles River Sprague–Dawley rats, Freund's adjuvant used to incite inflammation resulted in a 30–50% reduction of total CBG within 24 h [75]. Rats that developed clinical features of inflammation had a more marked reduction in CBG of 40–80%. As seen in human sepsis studies, the degree of CBG decline correlated to inflammation severity. Hepatic CBG mRNA levels were lower; however, it did not correlate to cleaved CBG levels, suggesting cytokine-mediated inhibition of CBG synthesis during inflammation [75].

A rat model using two different Sprague–Dawley rat colonies, Harlen and Charles River, known to respond differently to inflammation, were administered Freund's adjuvant. Total CBG levels decreased in proportion to inflammation severity, with levels significantly lower, and a more severe inflammatory status seen within Harlen rat group [76]. In the absence of inflammation, Harlan rats had lower CBG levels compared to Charles River rats, portending a vulnerability to inflammation. While CBG genetic analyses revealed a synonymous single-nucleotide transition (C > T) in exon 2 for Phe 152 and two non-synonymous single-nucleotide transitions (A > G) in exon 4 that cause amino acid substitutions (Ile298Met and Met307Val) within Charles River rats, H^3-corticosterone-binding affinity studies were similar. While the significance of the differences in CBG coding sequences remains to be elucidated, it may underlie the differences in CBG levels between the two colonies and their susceptibility to inflammation [76].

Studies in CBG-knockout mice have provided the new insights into the potential physiologic roles of CBG [23,37,62]. Compared to wild-type mice, $Cbg^{-/-}$ mice have a complete loss of CBG, resulting in lower total corticosterone levels under basal conditions, with total corticosterone concentrations near halved in states of physiological stress. In $Cbg^{-/-}$ mice, free corticosterone levels were increased 10-fold during the light-morning sedentary phase compared to wild-type mice, but were similar in the dark-nocturnal active phase [37].

In a lipopolysaccharide (LPS) septic shock model, $Cbg^{-/-}$ mice had significantly reduced 48-h survival rates. With the administration of LPS, free corticosterone concentrations

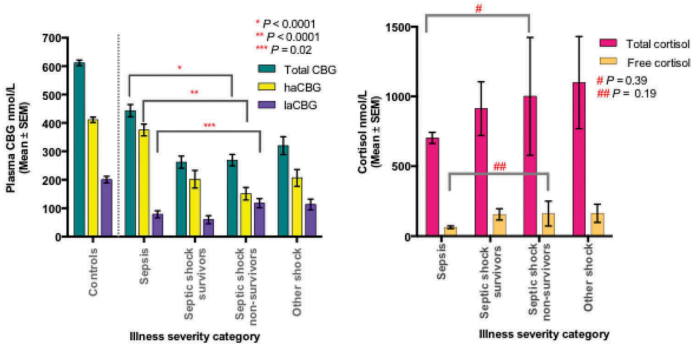


Figure 4. laCBG and haCBG profiles in sepsis and septic shock. Right panel: haCBG decreases as severity of illness increases *in vivo*, with a small, but significant increase in laCBG indicating CBG cleavage. Left panel: The association between free cortisol and illness severity, and total cortisol and illness severity, was not statistically significant. CBG: corticosteroid binding gobulin, haCBG: high affinity CBG, laCBG: low affinity CBG. Reprinted with permission from [70].

increased and were higher in $Cbg^{-/-}$ mice compared to wild type. This suggests that the susceptibility to septic shock was not due to reduced circulating free corticosterone [37]. Conversely, physical restraint stress resulted in an attenuated rise in free corticosterone despite higher adrenocorticotropic hormone (ACTH) levels in $Cbg^{-/-}$ mice compared to wild type, while in the non-stressed state, free corticosterone levels were normal but total corticosterone levels reduced [23]. $Cbg^{-/-}$ mice have a fourfold increase in corticosterone clearance compared to wild type. This may contribute to the attenuated rise in free corticosterone after physical restraint [37].

SERPINA6 variants have been associated with fatigue and pain disorders, perhaps due to altered cortisol access to the brain [59–63]. $Cbg^{-/-}$mice display altered behavior in response to intense and uncontrollable stress with markedly increased learned helplessness, higher rates of failed escape, and decreased number of avoiding foot shocks. Altered behavioral responses in $Cbg^{-/-}$ mice are associated with blunted free and total corticosterone responses to stress, an absence of hippocampal corticosterone rise, and a reduction of *Erg-1* gene expression within the hippocampus, a factor in mediating stress-related behavioral effects of glucocorticoids [23,63,77,78]. $Cbg^{-/-}$ mice display a lack of the normal impairment of memory retrieval that occurs with foot shock stress. This memory response to stress can be reversed in $Cbg^{-/-}$ mice with intrahippocampal infusions of glucocorticoid. This suggests that CBG has a role in glucocorticoid transport into the brain in stress conditions [78].

Inflammation and tissue damage stimulate the synthesis of pro-inflammatory and pyrogenic cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-6. TNF-α stimulates IL-6 production [79] and the HPA axis [80], increases NE activity [81], and reduces CBG levels [82]. A recent study analyzed CBG kinetics in 12 healthy human subjects receiving a TNF-α infusion versus saline control [83]. Total CBG, haCBG, and laCBG levels within the first 6 h did not differ, suggesting that the CBG cleavage is not immediate and hepatic synthesis of CBG remains unaffected within the initial hours of TNF-α-induced inflammation. Cortisol levels rose, including total, free, and ratio of free to total cortisol, in the setting of TNF-α infusion and pyrexia. The elevation of free cortisol proportionate to total cortisol while haCBG and laCBG remained unchanged may suggest weakening of the CBG–cortisol thermocouple, resulting in a TNF-α pyrexia-induced release of cortisol from CBG [83].

In infection, the balance of pro- and anti-inflammatory mediators along with an intact HPA axis is critical for immune system response and survival. An *in vivo* study of 100 patients with infection compared to 100 healthy controls found that those with an infection had 23% lower serum total CBG, 18% lower haCBG, and 31% lower laCBG with an 11% higher %haCBG:total CBG ratio. In infected patients, both free and total cortisol was higher, 2.6 and 4.0 times, respectively, with the ratio of free cortisol to total cortisol higher than in controls [84].

Many bacteria secrete proteases as a virulence factor to evade the host's immune defense mechanisms. *In vitro* studies of *P. aeruginosa* have shown the protease LasB to cleave CBG independent of NE releasing free cortisol [29]. When compared to other pathogenic bacterial infections, patients with a *P. aeruginosa* bacteremia had the lowest levels of total CBG, and laCBG and when compared to healthy controls, levels of total CBG were 1.5 times lower, haCBG 1.2 times lower, and laCBG 2.3 times lower. Low levels of total CBG and haCBG in patients with *P. aeruginosa* bacteremia occurred despite a milder degree of inflammation, supporting the specific enhanced CBG-cleaving effects of *P. aeruginosa* occurring independently of NE [84]. In the same study, haCBG was not further reduced in patients with *Escherichia coli*, *Klebsiella pneumonia, Candida spp*., or *Streptococcus spp*. bacteremia, suggesting protection of CBG from additional organism-specific cleavage in these infections. laCBG did not increase, and in the presence of *P. aeruginosa*, it decreased, in contrast to higher levels of laCBG seen in septic shock. This observation is not yet explained [70].

2.6. Chronic inflammation

2.6.1. CBG and rheumatoid arthritis

We examined CBG cleavage in rheumatoid arthritis (RA), a common chronic disease typified by synovitis, articular destruction, and systemic inflammation [54]. Paradoxically, despite the inflammation and pain, cortisol levels are normal [85]. RA generally responds to exogenous glucocorticoids. Patients with RA had lower total CBG levels, similar haCBG levels, and thus higher haCBG:total CBG ratio and lower laCBG levels compared to controls reflecting lower CBG cleavage rates in patients with RA. The lowest levels of total CBG, haCBG, and haCBG:total CBG ratios were seen in those in remission, while patients with the highest disease activity had higher total CBG, laCBG, and haCBG:totalCBG ratio. This suggests that higher rates of CBG cleavage occur in patients with disease remission, while lower CBG cleavage rates occur in those with active disease. Antibodies directed against the RCL have shown to decrease CBG cleavage by NE, protease LasB, and chymotrypsin [74]. Speculatively, RCL antibodies may be present in patients with inflammatory autoimmune disorders such as RA, inhibiting CBG cleavage. Total and free cortisol levels were similar in RA and controls. Within the RA cohort, there was no difference in total and free cortisol levels according to disease activity; however, there was a trend to lower total and free cortisol levels in patients with higher disease activity scores. Moreover, a lower ratio of free and total cortisol to IL-6 soluble receptor inversely correlated to RA disease activity, suggesting ineffective HPA activity proportionate to the degree of inflammation. Overall, this study suggested that impaired CBG cleavage, along with the known lack of HPA axis activation, may contribute to the pathogenesis of RA. Further, this lack of HPA axis activation potentially coupled with reduced cortisol delivery to inflammation may moderate the therapeutic response of RA to exogenous glucocorticoids such as prednisolone.

2.6.2. CBG and alpha-1 antitrypsin deficiency

We investigated CBG profiles in patients with α1-antitrypsin deficiency [86]. α1-Antitrypsin is a SERPIN protein produced by hepatocytes that inhibits NE. α1-Antitrypsin deficiency is a genetic condition with increased NE activity resulting in unregulated proteolysis and destruction of the lung parenchyma with resultant emphysema.

It was observed that CBG cleavage rates were lowest in patients with reduced α1-antitrypsin levels. Among patients with α1-antitrypsin deficiency, CBG levels were similar to controls, with higher haCBG levels and an increased haCBG:total CBG ratio. Together these findings suggest paradoxically impaired CBG cleavage in α1-antitrypsin deficiency.

Collectively these results, along with findings in sepsis, suggest that the role of CBG in chronic inflammation is different from that in acute inflammation, with reduced CBG cleavage and lower cortisol levels in chronic inflammatory disease states. This may reflect impairment of the HPA axis [87,88] with resultant initiation and propagation of chronic inflammatory disease. Posttranslational modifications of CBG, in particular glycosylation, or partial insertion of the RCL, may impair the cleavage of CBG by NE as seen in chronic inflammation. Genetic variations can impact CBG metabolism and binding affinity to cortisol that may predispose to inflammatory disease. Further research is required however to address these postulates.

2.6.3. CBG, pregnancy, and preeclampsia

Pregnancy is a physiological state of hypercortisolism with a two- to threefold increase in total and free cortisol by the third trimester stimulated by placental-derived corticotropin-releasing hormone [89,90]. CBG levels also increase two- to threefold until 36-weeks of gestation after which they fall, coupled with a rise in free cortisol at 38 weeks [89]. The increase in CBG during pregnancy is due in part to pregnancy-specific glycosylation, which affects metabolism, and in part to estrogen-stimulated hepatic synthesis [27,89]. Our recent study demonstrated that CBG levels in pregnancy were 1.9 times higher than that in nonpregnant controls and that this was comparable to a 3.2-fold rise in CBG in women taking the hormonal combined oral contraceptive pill (COCP), consistent with an estrogen-mediated increase in CBG synthesis. In pregnancy, the rise in CBG is attributed to haCBG; haCBG levels rise 2.8 times, while laCBG remains unchanged compared to controls. Women taking the COCP had elevations in both haCBG and laCBG; this supports the notion that the reduced CBG cleavage in pregnancy is due to pregnancy-induced CBG glycosylation altering its clearance [91]. The rise in haCBG during pregnancy theoretically provides for an increased anti-inflammatory cortisol reservoir, allowing for the possibility of a puerperal infection. In addition, higher haCBG levels in pregnancy may allow for the competition that high pregnancy levels of progesterone may produce for cortisol:CBG binding.

Preeclampsia occurs in 3–5% of all pregnancies and is a major cause of pregnancy and perinatal mortality and morbidity [92]. The etiology of preeclampsia is unclear; however, abnormal placental implantation, an increase in antiangiogenic factors and cytokines including IL-6 and TNF-α, and endothelial dysfunction are implicated [93,94].

Compared to normal pregnancy, CBG, total cortisol, and free cortisol levels are significantly lower during the third trimester in mothers with preeclampsia and gestational hypertension, and the fall in CBG and rise in free cortisol in pregnancy are less marked in preeclampsia [89]. The fetal HPA axis matures at midgestation and cortisol is synthesized from 20 weeks of gestation; however, up to a third of fetal cortisol variation occurs from maternal cortisol concentrations at term [95–97].

A recent study of 1182 adolescent offspring born to mothers with preeclampsia, gestational hypertension, and essential hypertension during pregnancy studied the HPA axis. Offspring whose mothers had had preeclampsia had higher total plasma cortisol (p = 0.024), calculated free cortisol (p = 0.052), and ACTH (p = 0.040) compared to controls. Systolic blood pressure was significantly higher in the gestational hypertension and preeclampsia group combined compared with controls (p = 0.006) [98].

The Barker hypothesis that the environment *in utero* can influence disease in adult life including diabetes, hypertension, heart disease, and stroke is well known. It is plausible that the fetal HPA axis is affected by maternal cortisol and that low maternal cortisol present in preeclampsia upregulates the fetal HPA axis resulting in elevated basal HPA activity in adolescents. While mothers with gestational hypertension have lower cortisol concentrations, elevated basal HPA axis activity was not found in the offspring, perhaps because the maternal changes are not as severe as that seen in preeclampsia [98].

2.6.4. Obesity and metabolic syndrome

Obesity, insulin resistance, and the metabolic syndrome are associated with a low-grade chronic inflammatory state with elevations of IL-6, C-reactive protein, and NE [99,100].

It has been well described that total CBG levels are reduced in obesity, insulin resistance, and polycystic ovarian syndrome [101–103]. CBG correlates negatively to several obesity parameters, including body mass index, fat mass index, waist circumference, and waist-to-hip ratios [101,102]. CBG levels also negatively correlate to glycated hemoglobin, insulin resistance, and hypertension, although not to fasting glucose [101,102].

A recent study revealed low total CBG levels in obese people consistent with previous studies; however, it further demonstrated laCBG to be low while haCBG was elevated. These perturbations of CBG suggesting impaired cleavage was more marked in those with central adiposity and those with metabolic syndrome [55]. It may be that impaired CBG cleavage may perpetuate the pro-inflammatory state of obesity, particularly in metabolic syndrome. The mechanism of impaired CBG cleavage in obesity is unknown. While formal glycosylation studies are yet to be performed in people with obesity and metabolic syndrome, a study using Western blot analysis has shown the molecular weight of CBG to be different in those with obesity compared to obese individuals with glucose intolerance, inferring CBG glycosylation is altered [99,100]. Reduced total CBG levels may result from cytokine-mediated suppression of hepatic synthesis [104,105].

3. Conclusions

CBG is the primary cortisol transport protein. While CBG allows for a circulating cortisol reservoir, it has a pivotal role in the targeted delivery of cortisol to inflamed tissues, modulating the immune response. CBG is capable of conformational change upon cleavage of the RCL by various proteases such as NE changing from a high-affinity form to a low-affinity form releasing bound cortisol [21,31,33].

In acute systemic inflammation, depletion of haCBG is associated with illness severity and mortality such as septic shock [70]. Paradoxically, in chronic inflammatory states such as RA, CBG cleavage is reduced, resulting in the diminished delivery of cortisol to inflamed tissues [54,86]. CBG is a thermosensitive protein, where cortisol release can be potentially effected by pyrexia through reduced CBG:cortisol affinity [21,25]. Inflammatory cytokines, such as IL-6, reduce hepatic CBG production [83]. Genetic polymorphisms of SERPINA6, the CBG molecule, or altered glycosylation profiles modify CBG function, for example, facilitating or inhibiting protease cleavage and thermocouple properties [30]. Further study of the CBG:cortisol delivery system in inflammatory diseases is warranted.

4. Expert commentary

The traditional view of CBG as a transport molecule for cortisol was broadened in 1990 with the knowledge that NE cleaves CBG, delivering cortisol at inflammatory sites. In the past 3 years, the capacity to measure uncleaved haCBG has led to important insights into divergent cleavage states of CBG, in relation to illness severity and in acute versus chronic inflammation. Further study in this area, coupled with study of genetic variants and posttranslational (glycan) differences in CBG, may shed light on an important aspect of inflammatory disease.

5. Five-year view

The intriguing data available to date suggest that CBG may modify the susceptibility to, and risk of progression of, inflammatory diseases. We now have the tools to measure cleavage function *in vivo* and *in vitro*, as well as CBG levels and cortisol-binding affinity. Initial pilot studies in inflammatory disease form an impetus to further study in this field. The next steps to elucidation of the importance of CBG in inflammation are to study larger groups of individuals with inflammatory disease and incorporate individual differences in CBG structure and function.

Key issues

- Corticosteroid binding globulin (CBG) is the primary binding molecule for cortisol in a 1:1 ratio and has a pivotal role in the targeted delivery of cortisol to inflamed tissues.
- CBG is a non-inhibitory serine protease inhibitor capable of conformational change upon cleavage of the reactive centre loop (RCL) by neutrophil elastase (NE), changing CBG from a high affinity form to a low affinity form reducing cortisol-binding affinity 10-fold and releasing bound cortisol.
- Recently *in vivo* monoclonal ELISAs have allowed the study of high and low affinity forms of CBG in the human circulation.
- In acute systemic inflammation, depletion of high affinity CBG (haCBG) is closely associated with illness severity and mortality in septic shock.
- In chronic inflammation, such as rheumatoid arthritis (RA), CBG cleavage is reduced perhaps implying impaired targeted delivery of cortisol.
- Nine CBG variants have been described in humans, seven of which effect either CBG synthesis or function.
- SERPINA6 polymorphisms and CBG protein glycosylation profiles alter CBG function and may modify the risk of developing, or progression of, inflammatory disease.

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Declaration of interest

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CHAPTER 2:

CBG CHEMISTRY IN RELATION TO INFLAMMATION.

CHAPTER OUTLINE

Chapter 2 provides additional introductory information necessary for the scope of this thesis and supports the background information summarised in the literature review in chapter 1. Chapter 2 provides a contextual background on septic shock, defines critical illness-related corticosteroid insufficiency (CIRCI) and discusses the contentious use of exogenous hydrocortisone. Here the evidence is provided to substantiate the hypothesis CBG deficiency may represent an independent risk factor for mortality in septic shock. Finally, the aims of this project and an outline of the research presented in this thesis are provided.

2.1 Sepsis and septic shock

Within Australia there are 15,700 cases of sepsis per year,[1] costing on average $39,000 AUD and contributing 612 million dollars to the annual health budget. Septic shock is a medical emergency, carries a 25–40% mortality rate and represents 20% of all global deaths (~100 deaths per week within Australia).[2] More notably, the incidence of severe sepsis and septic shock continue to rise.[2-4]

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to pathogenic infection, a syndrome identified by a constellation of clinical signs and symptoms, due to a multifaceted host response to an infection and activation of both pro- and anti-inflammatory responses, with significant effects on nonimmunologic pathways including cardiovascular, autonomic, hormonal, metabolic and coagulation. Organ dysfunction in sepsis is identified as an acute change in total Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score of 2 or more points consequence of infection (Table 2.1). This increase in SOFA score reflects an increase in overall mortality of 10%.[5]

Septic shock is subset of sepsis in which underlying circulatory and cellular and metabolic abnormalities are profound enough to cause a significant increase in mortality of 25–40%. It is hallmarked by persistent hypotension despite fluid resuscitation, necessitating vasopressors to maintain a mean arterial pressure >65 mmHg.[5] New treatments beyond fluids, antimicrobials, vasopressors, and support for organ failure, have not emerged in decades.

Table 1. Sequential [Sepsis-Related] Organ Failure Assessment Score[a]

System	Score 0	1	2	3	4
Respiration					
Pao_2/Fio_2, mm Hg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation					
Platelets, $\times 10^3/\mu L$	≥150	<150	<100	<50	<20
Liver					
Bilirubin, mg/dL (μmol/L)	<1.2 (20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (204)
Cardiovascular	MAP ≥70 mm Hg	MAP <70 mm Hg	Dopamine <5 or dobutamine (any dose)[b]	Dopamine 5.1-15 or epinephrine ≤0.1 or norepinephrine ≤0.1[b]	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1[b]
Central nervous system					
Glasgow Coma Scale score[c]	15	13-14	10-12	6-9	<6
Renal					
Creatinine, mg/dL (μmol/L)	<1.2 (110)	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440)	>5.0 (440)
Urine output, mL/d				<500	<200

Abbreviations: Fio_2, fraction of inspired oxygen; MAP, mean arterial pressure; Pao_2, partial pressure of oxygen.

[a] Adapted from Vincent et al.[27]

[b] Catecholamine doses are given as μg/kg/min for at least 1 hour.

[c] Glasgow Coma Scale scores range from 3-15; higher score indicates better neurological function.

TABLE 2.1 Sequential [Sepsis-Related] Organ Failure Assessment Score (SOFA).[5]

The SOFA scoring system using organ dysfunction parameters to identify patients with sepsis or septic shock in whom has either a known or suspected infection.[5]

2.2 Septic shock and the cortisol controversy

Septic shock and other critical illnesses are profound physiological stressors, activating the hypothalamic-pituitary-adrenal (HPA) axis. HPA axis stimulants include inflammatory cytokines (IL-1, IL-6 and TNFα), pain and psychological stress. Critical illness-related corticosteroid insufficiency (CIRCI) is conceptually defined as an impaired cortisol secretory response, blunting life sustaining effects on immunomodulation and vascular tone occurring in the face of critical illness.

In sepsis free cortisol associates more closely with illness severity than total cortisol.[6] However, total plasma cortisol increments or peak levels after cosyntropin (ACTH 1–24) injection, have not consistently predicted the clinical effectiveness of hydrocortisone.[7,8] CIRCI diagnosis was based on cortisol increment <248 nmol/L after 250 mcg ACTH 1–24, or random cortisol <276 nmol/L; however, recent consensus is that measuring cortisol does not assist in determining hydrocortisone administration.[9,10] The measurement of serum total cortisol does not allow for the assessment of free cortisol or fraction of cortisol bound to carrier proteins, namely CBG. Free cortisol and CBG measurements are not commercially available, hence routine measurements of total cortisol do not reflect the portion of cortisol most relevant to tissue action in the setting of sepsis.

Cortisol has important immunomodulatory properties, initially enhancing innate immune responses to short term minor insults, followed by anti-inflammatory actions. Cortisol acts through glucocorticoid receptor-α (GR-α), altering gene transcription, and activating secondary messenger pathways to suppress pro-inflammatory intracellular pathways induced by transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) (Figure 2.1). Through these pathways, cortisol targets inflammatory cytokine production, neutrophil migration, T cell apoptosis and macrophage inhibition.[11] The simultaneous activation of the central sympathetic nervous system in response to stress is augmented by cortisol. Cortisol increases the conversion of norepinephrine to epinephrine within the adrenal medulla and augments the pressor effects of norepinephrine on the vasculature to maintain blood pressure and redirect blood flow to vital organs. Septic shock represents a state of dysregulated excessive inflammation with consequent hypotension and damage to healthy tissues resulting in multi-organ failure and death.

Cortisol physiology is altered at both systemic and local levels in septic shock, altering cortisol secretion, metabolism, transport and delivery.

Known specific mechanisms include:

(i). Reductions in cortisol-binding proteins (CBG and albumin) within plasma. Reduced hepatic CBG synthesis due to IL-6 and TNFα, and the sequestration of albumin, a negative acute phase reaction altering the relationship between total and free cortisol.

(ii). Inhibition of cortisol steroidogenesis.

(iii). Variable HPA axis drive (increased or blunted relative to illness severity).

(iv). Reduced cortisol metabolism via alterations in 5α/5β reductases and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD).[12]

(v). Cellular glucocorticoid resistance due to NF-κB activation with subsequent antagonization of intracellular pathways used by the glucocorticoid receptor and reduced levels and transcription of GRα and increased levels and transcription of GRβ (dominant negative).[13,14]

(vi). Reductions in ACTH receptor activity and ACTH secretion.

(vii). Use of drugs inhibiting the HPA axis such as opioids, benzodiazepines and in some cases etomidate (a short acting anaesthetic agent which inhibits 11β-hydroxylase, a necessary enzyme required for cortisol steroidogenesis).

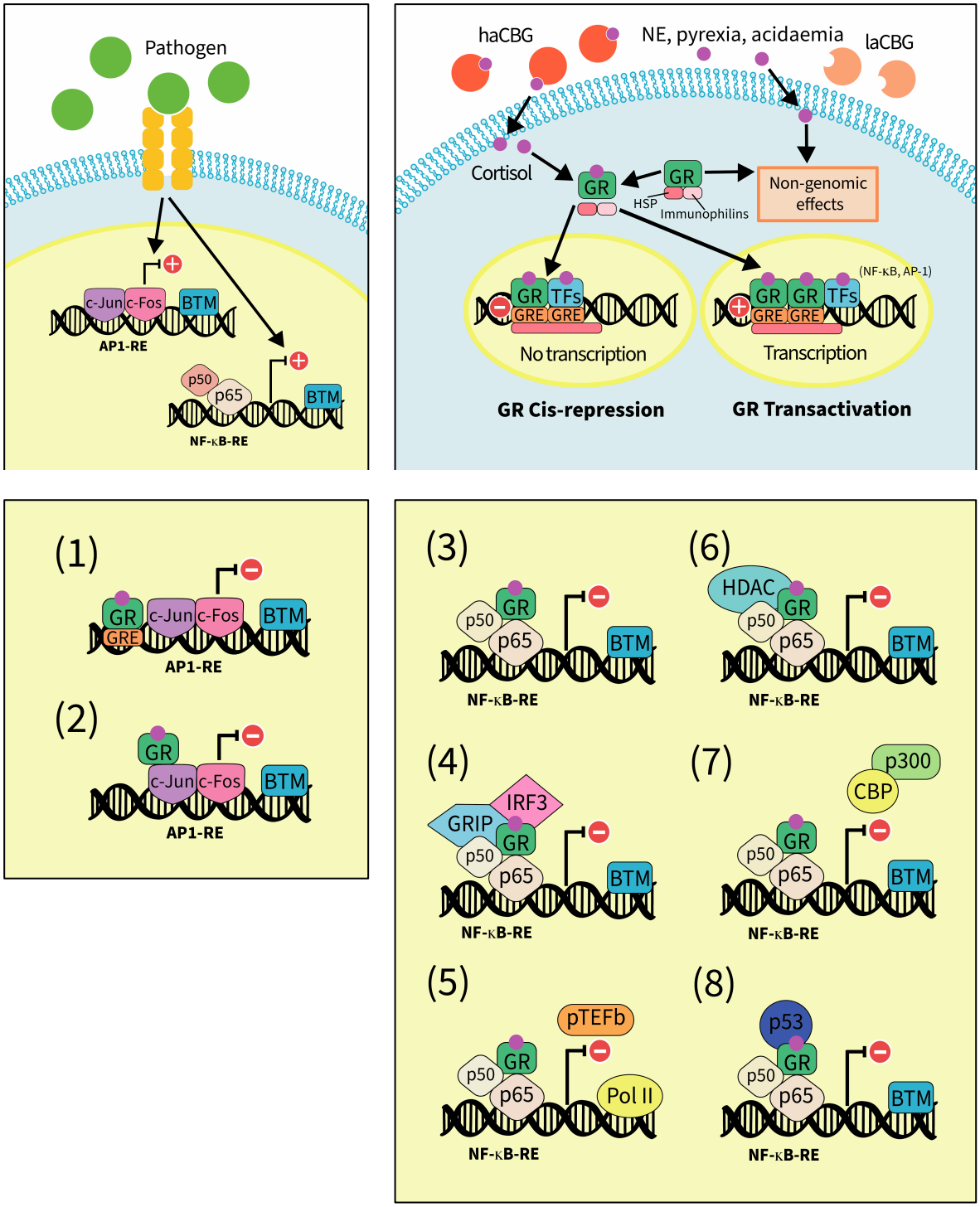


FIGURE 2.1 Glucocorticoid signalling in inflammation (Adapted from [11,15]).

At sites of inflammation, cortisol (purple) is released by haCBG (dark orange) through neutrophil elastase (NE) cleavage, and thermo-coupling mechanisms. Glucocorticoids diffuse through cell membranes and bind with glucocorticoid receptors (GR). GR reside in the cytoplasm in a multiprotein complex with chaperone proteins, heat shock proteins, and immunophilins. The classic GR (GR-α) is the major receptor involved in mediating the glucocorticoid responses to stress and inflammation. Upon

binding of cortisol, the GR undergoes a conformational change that allows it to dissociate from the chaperone proteins and translocation into the nucleus, where it binds to glucocorticoid response elements (GRE) to activate (transactivation) or repress (*cis*-repression) pro-inflammatory gene expression of various transcription factors such as nuclear factor-kappa B(NF-κB) and activator protein-1 (AP-1).

Pro-inflammatory stimuli trigger signalling cascades that result in the activation of the transcription factors AP-1 and NF-κB. Glucocorticoids bind their cytosolic receptor to repress activity of both these pro-inflammatory transcription factors through multiple mechanisms. Bottom left panel: Effect of glucocorticoid-GR complex on AP-1 activity: (1) at some promoters, GR is able to simultaneously bind to a GRE and tether to c-Jun to repress the transcriptional activity of AP-1; (2) at some promoters, GR physically interacts with c-Jun via tethering, which represses the activity of AP-1 and represses the transcription of pro-inflammatory genes including cytokines, cytokine receptors, chemotactic proteins, adhesion molecules, collagenases and matrix metalloproteinases. Bottom right panel: Effect of glucocorticoid-GR complex on NF-κB activity: (3) GR can physically interact with p65, which represses the activity of NF-κB; (4) GR can recruit GRIP (GR interacting protein) which blocks the formation of the NF-κB/IRF3 heterodimer; (5) GR is able to block the recruitment of the C-terminal tail kinase, pTEFb (positive transcription elongation factor), thus preventing RNA polymerase II (Pol II) phosphorylation and activation; (6) GR is able to repress NF-κB activity by recruiting a HDAC (histone deacetylase); (7) GR prevents NF-κB from interacting with p300 and CPB; and (8) p53 is able to interact with GR, altering its transcriptional activity, and thus preventing transcription of pro-inflammatory genes including cytokines and their receptors, chemotactic proteins, adhesion molecules, complement factors and cyclooxygenase 2.

2.3 Evidence for the use of hydrocortisone in the treatment of septic shock

Low dose hydrocortisone (200 mg daily administered 50 mg every 6 hourly, or by continuous infusion over 24 hours for 7 days without taper) is indicated in septic shock patients with hypotension, despite fluid resuscitation and vasopressor therapy, based on clinical judgement.[16]

Benefits of hydrocortisone in septic shock include pressor effect and reduced inflammatory cytokines.[17-19] Early studies of high-dose, short term synthetic glucocorticoids (equivalent to hydrocortisone 40,000 mg per day, often administered in a single dose) for septic shock showed an increased mortality.[20] Subsequent large randomised controlled trials of low dose hydrocortisone using 200 mg daily (approximating the normal adrenocortical cortisol output in response to severe stress) revealed a reduced time-to-withdrawal of vasopressor support (by 1–2 days) and a small mortality benefit in sicker patients.[9,10] Hydrocortisone in doses of 200 mg daily has full mineralocorticoid effects on fluid and electrolyte status. The addition of Fludrocortisone, 50 mcg daily administered via nasogastric tube, to hydrocortisone 200 mg has been associated with survival in studies.[8,10] The mineralocorticoid receptor (MCR type 1) has two physiological agonist ligands, aldosterone (fludrocortisone) and cortisol (hydrocortisone). Hydrocortisone at doses of 200 mg daily has full mineralocorticoid effects on fluid and electrolyte status. If fludrocortisone does indeed improve sepsis outcomes the mechanism remains uncertain, and dosing does not suggest it acts via the mineralocorticoid receptor.

Basal cortisol levels in sepsis and septic shock have not shown to be useful predictors of mortality except when levels are extremely high (>45 µg/dL [>1242 nmol/L]).[21] Initial trials in patients with CIRCI, defined as a reduced cortisol response to ACTH 1–24 250 mcg (<9 ug/dL [<248 nmol/L] at 60 minutes), suggested a predicted mortality benefit from hydrocortisone therapy;[8,22] however, this was not confirmed in larger trials.[23,24] At present, low dose hydrocortisone is indicated in septic shock patients with persistent hypotension despite fluid resuscitation and vasopressor therapy.[16] Deleterious side effects of systemic hydrocortisone use include secondary infections, muscle weakness, hyperglycaemia and neurocognitive disturbance.

2.4 Corticosteroid-binding globulin (CBG) in sepsis and septic shock

CBG is a 383 amino acid glycoprotein, binds cortisol in a 1:1 ratio and transports over 80% of circulating cortisol.[25] CBG has a cortisol tissue delivery role in sepsis, with pyrexia and acidosis reversibly reducing cortisol binding affinity and tissue NE cleaving high cortisol binding affinity CBG, irreversibly converting CBG to a low affinity state and thereby releasing cortisol at inflammatory sites (Figure 2.2).[26,27]

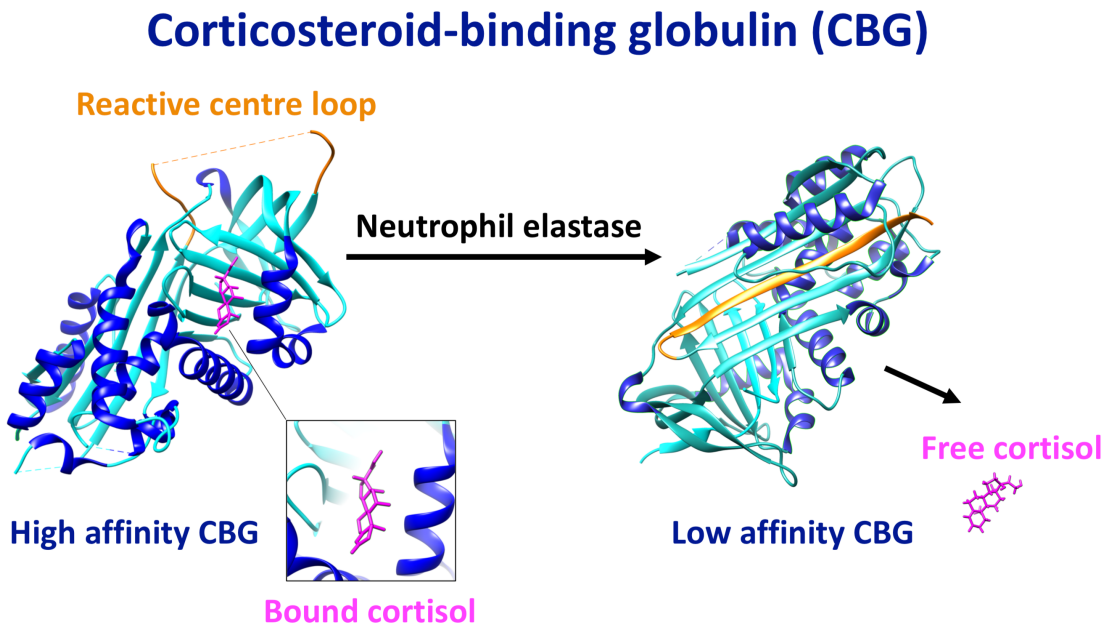


FIGURE 2.2 Cleavage of human corticosteroid-binding globulin by neutrophil elastase liberating free cortisol.

Crystal structure of human CBG (PBD ID 2VDY, 2.3 Å) undergoing conformational change from high affinity CBG to low affinity CBG after cleavage of the reactive centre loop (RCL; yellow) by neutrophil elastase and subsequent insertion of the RCL into the body of the molecule releasing bound cortisol.

Cortisol:CBG affinity is reduced 9-fold upon NE cleavage and cortisol is also liberated from CBG by increased temperature, and possibly also by acidaemia, conditions typical of sepsis.[26,28] NE is inhibited by alpha-1 antitrypsin (α1AT).[29] Inflammatory cytokines TNFα, IL-1 and IL-6 reduce CBG synthesis by inhibiting CBG mRNA production within hepatocytes[30-32] and

serum CBG has been observed to decline by up to 50% in humans within 24 hours following IL-6 administration.[30]

Our previous studies have shown CBG concentrations fall substantially in sepsis in proportion to illness severity, circulating haCBG fell by 9% in sepsis, 51% in septic shock survivors and 61% in septic shock non-survivors (Figure 2.3).[33] During the sepsis illness CBG concentrations continue to fall, often with mounting organ failures, so CBG nadir may be relevant to septic shock related organ failures and mortality. Upon sepsis recovery serum CBG levels return to normal within days (Figure 2.4).[34] These studies lacked the power to show that low CBG was an independent risk factor for mortality. If low plasma CBG is an independent mortality risk factor for septic shock death, its replacement is potentially feasible. Hence CBG administration could be subject to clinical trial in septic shock.

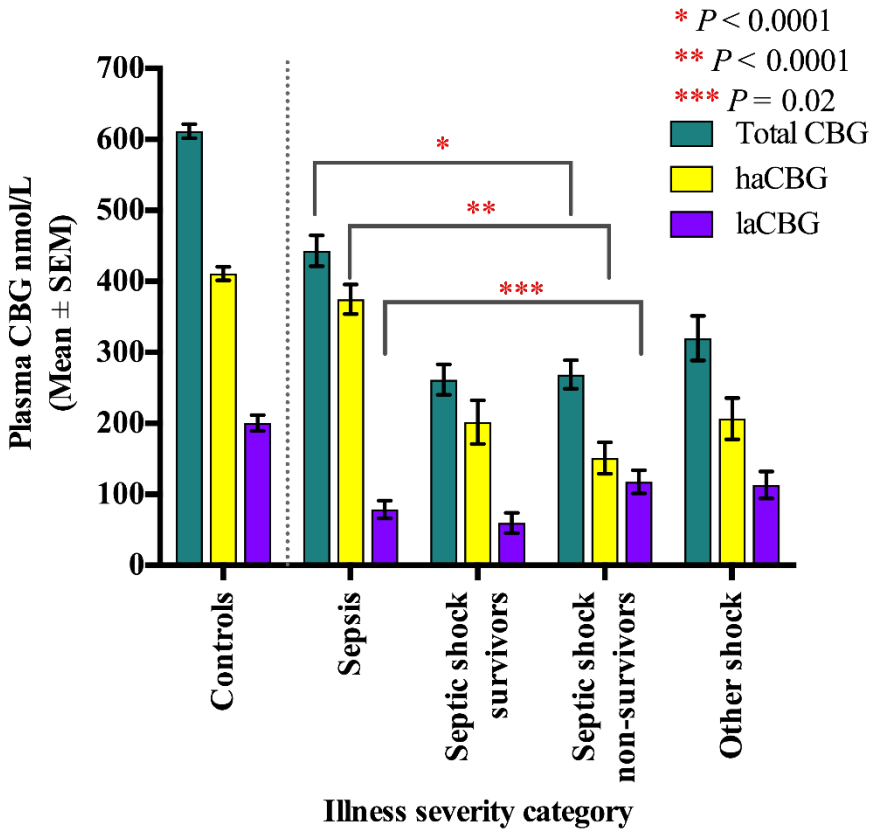


FIGURE 2.3 Total CBG, haCBG and laCBG profiles in sepsis and septic shock.[33]

Total CBG and haCBG decrease with sepsis illness severity. haCBG decreases as severity of illness increases in vivo, with a small but significant increase in laCBG, indicating CBG cleavage. * For linear trend r = –0.44, $P < 0.0001$; ** r = –0.48, $P < 0.0001$; *** r = 0.23, $P = 0.02$.

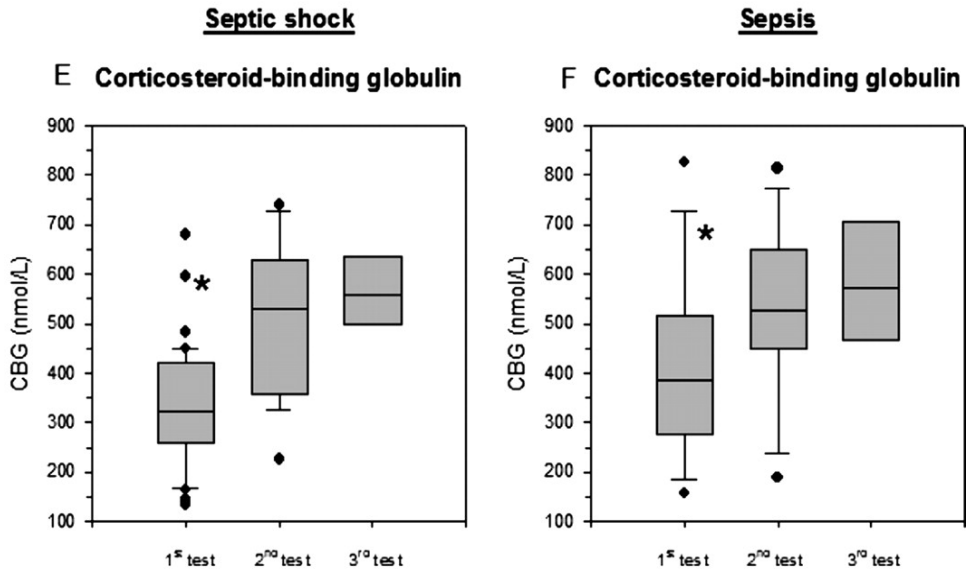


FIGURE 2.4 CBG levels during and upon recovery of septic shock and sepsis.[34]

Tests were performed at the following times: first test, at the time of illness; second test, immediately before hospital discharge; and third test, 6-12 weeks after discharge. Twenty septic shock (SS) and 10 sepsis (S) patients had the second test, and 14 SS and eight S patients had the third test.

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AIMS OF THE PROJECT

Septic shock is a maladaptive immune hyper-response with attendant injury to healthy tissues and consequent multi-organ failure. It is a state of persistent hypotension requiring vasopressors despite fluid resuscitation, and despite treatment with fluid resuscitation, antibiotics, vasopressors and often ventilation, mortality remains 25–40%. While glucocorticoids such as hydrocortisone increase blood pressure and attenuate shock, they often fail to control organ failure and avert death.

CBG acts to optimise the effect of the body's principal immunomodulatory hormone, cortisol. CBG deficiency is strongly associated with sepsis severity, limiting the targeted delivery of cortisol to the vasculature, immunocytes, inflammatory sites and tissues that require protection from overt inflammatory processes.

Our overall hypothesis is that CBG is crucial in protecting inflamed tissues from damage and the multi-organ failure that portends shock and death. The main objective of this project was to determine if low CBG concentrations in septic shock associate with mortality.

The specific aims for this project are:

1. To develop a novel ligand binding assay using surface plasmon resonance (SPR) technology to determine the equilibrium binding constants of a library of steroids to CBG under various conditions of temperature and pH, mimicking pathophysiological conditions relevant to sepsis.
2. To determine in patients admitted with septic shock if deficient CBG concentrations represents an independent risk factor for mortality.
3. To determine in patients admitted with septic shock if deficient CBG concentrations relate to disease progression events.
4. To determine in patients admitted with septic shock if deficient CBG concentrations relate to biochemical factors involved in inflammation and cortisol action.

CHAPTER 3:

PYREXIA AND ACIDOSIS ACT INDEPENDENTLY OF NEUTROPHIL ELASTASE REACTIVE CENTER LOOP CLEAVAGE TO EFFECT CORTISOL RELEASE FROM CORTICOSTEROID-BINDING GLOBULIN.

CHAPTER OUTLINE

The aim was to investigate the binding properties of CBG:cortisol in conditions reflecting normal physiology and sepsis to determine the specific binding characteristics which beget the targeted delivery of cortisol to sites of inflammation.

A novel direct ligand binding assay using surface plasmon resonance (SPR) technology was developed to investigate the affinities of steroid ligands to both haCBG and laCBG. Binding studies were performed at 25, 37 and 39 °C, and pH 7.4 and 7.0. Glucocorticoids and progestogens demonstrated binding affinity to both haCBG and laCBG, with cortisol displaying the highest binding affinity. An expected reduction in cortisol binding occurred with NE-mediated haCBG-to-laCBG conversion. CBG:cortisol bindng affinity was further reduced 3.5-fold at 39 °C relative to 37 °C, binding affinity was also reduced by acidosis for haCBG and laCBG.

The 9G12 monoclonal antibody's recognition of the reactive centre loop (RCL) was preserved for haCBG across the temperature ranges, confirming that the RCL remained intact and available for NE proteolysis even at elevated temperatures. Molecular modeling confirmed the critical role of Trp371, positioned within the steroid binding pocket, in ligand binding and the thermo-coupling mechanism of cortisol release.

This is the first study to investigate these binding interactions using such a systematic approach and has provided new insights into the mechanism of cortisol delivery by CBG. The synergy of NE-cleavage, pyrexia and acidosis on CBG:cortisol binding furthers our understanding in the targeted delivery of cortisol to sites of inflammation.

An “in house” monoclonal mouse antibody, 9G12 (RRID:AB_26632405) was used to quantify haCBG by ELISA[1-4] and have previously demonstrated a loss of 9G12 reactive CBG associates with sepsis severity.[4] 9G12 recognises a well-defined epitope of the RCL comprising the ^{342}STGVTLNL^{348} sequence of human CBG spanning the NE cleavage site.[2,3] NE-based cleavage of CBG disrupts of this epitope causing a loss of 9G12 binding. Concerns regarding

effectiveness of 9G12 in recognising haCBG due to the location of an N-glycan at N347 within the RCL have been raised.[5] (Table 3.1).

Human CBG	P17 E	P16 E	P15 G	P14 V	P13 D	P12 T	P11 A	P10 G	P9 S	P8 T	P7 G	P6 V	P5 T	P4 L	P3 N	P2 L	P1 T	P1' S	P2' K	P3' P	P4' I	P5' I
Amino acid sequence		Glu	Gly	Val	Asp	Thr	Ala	Gly	**Ser**	**Thr**	**Gly**	**Val 344**	**Thr 345**	**Leu**	**Asn 347**	**Leu**	Thr	Ser	Lys			
Neutrophil elastase cleavage site												↑										
Potential glycosylation site															↑							
9G12 epitope consensus sequence									↔													

TABLE 3.1. Reactive centre loop of native human CBG.

The 9G12 monoclonal antibody epitope consensus sequence includes the elastase cleavage site and a potential glycosylation site.

The SPR experiments provided further validation of the 9G12 antibody in the recognition of haCBG at the RCL epitope. I was able to demonstrate 9G12 reactivity to fully glycosylated recombinant human CBG and complete loss of 9G12 reactivity upon NE-mediated haCBG-to-laCBG conversion and subsequent reduction in CBG-cortisol binding affinity, thus supporting the use of 9G12 for the measurement of haCBG by ELISA in human clinical studies.

This work has been published and the manuscript is presented below.

1. Nenke, MA 2017, *Corticosteroid-Binding Globulin – a Targeted Delivery System for Cortisol. Physiology and Responses to Acute and Chronic Inflammation*.
2. Lewis JG, Elder PA. (2011). Corticosteroid-binding globulin reactive centre loop antibodies recognise only the intact natured protein: elastase cleaved and uncleaved CBG may coexist in circulation. *J Steroid Biochem Mol Biol* 127:289-294.
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CHALLENGES FACED DURING SPR BINDING ASSAY DESIGN

I was faced with many challenges in the development of the CBG:steroid binding assay using SPR technology. During development, the problems experienced, and solutions found, while at times frustrating, were a personal triumph, especially as I had no previous formal laboratory training.

The novel SPR binding assay protocol is described in detail within the "Methods" section of the published manuscript, Chapter 3. Detailed below were issues encountered through development:

The immobilisation technique used to couple CBG onto the sensor chip surface required careful consideration as to maintain RCL integrity, accessibility of the ligand binding pocket for steroid interaction and minimise steric hindrance. Three immobilisation and fixation techniques were considered: amine coupling; antibody coupling; and streptavidin-biotin coupling.

The first method tested was amine coupling, generally applicable to a wide range of proteins and a convenient method of attachment. This technique required the use of non-physiological pH conditions for CBG immobilisation and fixation onto a sensor chip surface. While CBG immobilisation was achieved, subsequent binding studies using hydrocortisone and progesterone did not yield expected results, confirming the denaturing of CBG during this process due to acidic coupling conditions. Furthermore, by virtue of this methodology the exposed amine groups on the protein surface determine the protein's position when coupled to the sensor chip surface, which may result in the obscuring of the RCL and steroid binding site.

An antibody methodology was subsequently tried. While antibody coupling resulted in successfully immobilising CBG onto the chip surface to a required concentration, subsequent steroid binding studies did not yield high response unit (RU) values as expected. Use of a coupling antibody positioned CBG:steroid interactions away from the sensor chip surface reducing sensitivity in the detection of refracted light.

Streptavidin-biotin coupling proved successful. Inspection of available X-ray structures of both haCBG and laCBG revealed that the C-terminus was optimal for the tags as it was distal to both the ligand binding pocket and the RCL containing the NE scissile site. CBG immobilisation was achieved to the required concentration under neutral pH such that protein integrity remained intact. Subsequent CBG:hydrocortisone and CBG:antibody binding studies confirmed protein integrity and RU sensitivity.

Binding studies are performed in SPR running buffer; however, due to the use of steroid analytes, dimethyl sulfoxide (DMSO) was required to maintain solubility. Organic solvents interfere with the detection of refractive index of samples; thus, a solvent correction was required to adjust measured RUs for the effects of varying concentrations of DMSO, adding to the complexity of the SPR studies.

The antibody binding SPR assay methodology used is detailed within the published manuscript, Chapter 3. The use of CBG antibodies (polyclonal and monoclonal 9G12) was integral to the experiment as it allowed for the 1) confirmation of successful fixation of CBG onto the chip surface, 2) conversion of haCBG-to-laCBG by NE-digestion prior to steroid binding studies, 3) provided insight into the CBG:cortisol thermocouple mechanism being independent of RCL action, and 4) allowed for the validation of 9G12 in detecting haCBG by ELISA in clinical studies. During development, regeneration was performed using 0.1 M glycine at pH 2.5 with a contact time of 60 s at 10 µL/min. The regeneration step, a process removing bound antibodies from CBG, was however observed to denature CBG with concomitant loss of ligand binding, similar to that experienced during exploration of amine coupling described above. The antibody binding step to confirm immobilised haCBG and laCBG was intended to occur prior to CBG:steroid binding studies. However, as the regeneration step was observed to denature CBG with concomitant loss of ligand binding and with no other suitable alternative available, this step was performed upon completion of the ligand binding studies.

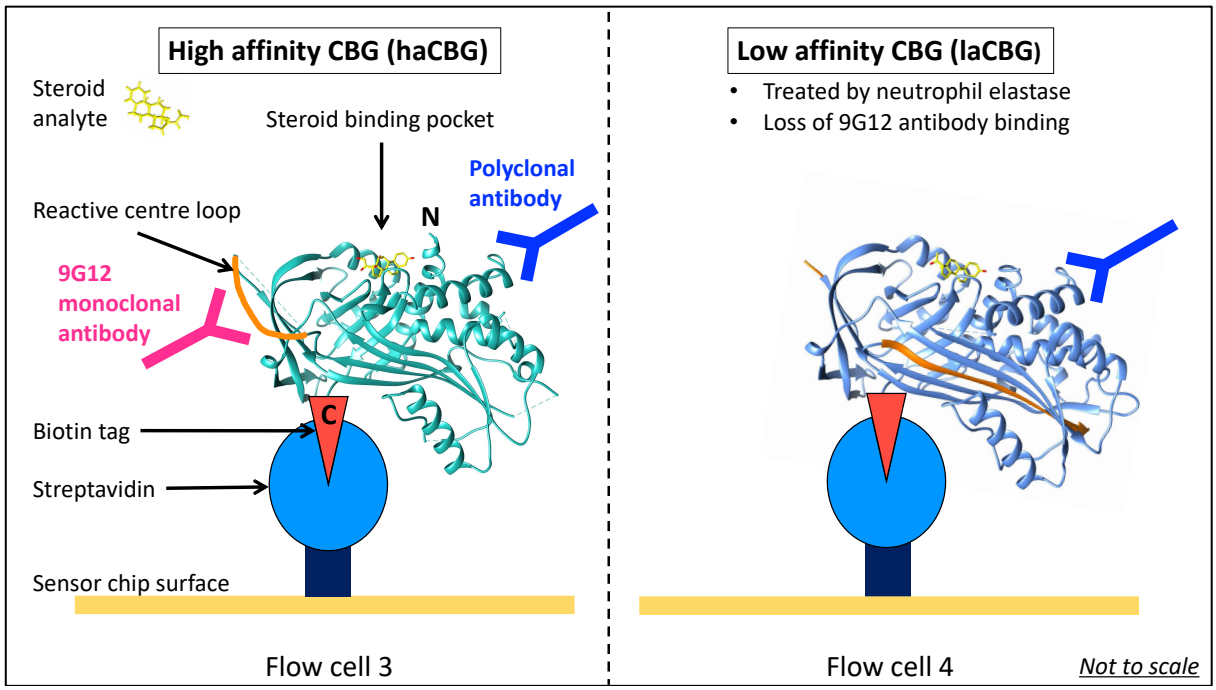


FIGURE 3.1 Surface plasmon resonance CBG:steroid binding assay schema.

Recombinant human CBG engineered with a biotin tag at the C-terminus were coupled to streptavidin-coated sensor chips flow cells 3 and 4. Flow cell 4 was treated with a neutrophil elastase to convert haCBG-to-laCBG. Flow cell 1 acted as a blank control and flow cell 2 acted as a streptavidin control. Steroid analyte binding studies were conducted under desired conditions (temperature and pH). Polyclonal CBG antibody (blue) and 9G12 monoclonal antibodies (blue) confirmed haCBG in flow cell 3 and laCBG in flow cell 4.

STATEMENT OF AUTHORSHIP

Statement of Authorship

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Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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ARTICLE

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Pyrexia and acidosis act independently of neutrophil elastase reactive center loop cleavage to effect cortisol release from corticosteroid-binding globulin

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Abstract

Corticosteroid-binding globulin (CBG) transports cortisol and other steroids. High-affinity CBG (haCBG) undergoes proteolysis of the reactive center loop (RCL) by neutrophil elastase (NE) altering conformation to low-affinity CBG (laCBG). Elevated temperature reduces CBG:cortisol binding affinity. Surface plasmon resonance was used to determine binding profiles of 19 steroids to haCBG and laCBG at 25, 37, and 39°C mimicking pyrexia and pH 7.4 and 7.0 mimicking acidosis, pathophysiological conditions relevant to sepsis. An expected 4–8-fold reduction in affinity for cortisol, cortisone, corticosterone, 11-deoxycortisol, progesterone, 17-hydroxyprogesterone, and prednisolone occurred with NE-mediated haCBG-to-laCBG conversion. CBG:cortisol binding affinity was further reduced 3.5-fold at 39°C relative to 37°C, binding affinity was also reduced by acidosis for both haCBG and laCBG. Using a conformational antibody generated against the RCL, we confirmed RCL antibody binding was eliminated by NE cleavage, but preserved in pyrexia and acidosis. Molecular modeling studies performed at 40°C confirmed a critical role for Trp371, positioned within the steroid-binding pocket, in ligand binding. These studies demonstrated CBG binding affinity to range of steroids is ligand specific and is reduced with NE-mediated haCBG-to-laCBG transition. Reduced CBG:cortisol binding occurs with increased temperature and in acidosis. Increased flexibility of the Trp371 side chain is proposed in the thermocoupling mechanism of cortisol release. The synergy of NE cleavage, pyrexia, and acidosis on CBG:cortisol binding may serve to enhance cortisol delivery to the interstitial space in inflammation.

KEYWORDS

acidemia, corticosteroid-binding globulin, cortisol, ligand binding affinity, pyrexia

Wayne Rankin and Steven W. Polyak senior co-authors.

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1 | INTRODUCTION

Corticosteroid-binding globulin (CBG), a serine protease inhibitor (serpin) family member,[1] is the principal transporter of circulating cortisol. Approximately 80% of blood cortisol is transported by CBG under basal conditions, while the remaining ∼15% is bound to albumin and ∼ 5% circulates as free hormone. CBG, a 383 amino acid glycoprotein, transports cortisol in a 1:1 stoichiometric ratio increasing the solubility of cortisol in serum, buffering fluctuating cortisol levels and, critically, allowing tissue-specific delivery.[2,3] One well characterized mechanism that facilitates the delivery of cortisol at target sites involves site-specific proteolysis of CBG by neutrophil elastase (NE). Infection and inflammation generates activated neutrophils that secrete the NE[4,5] that cleaves an exposed reactive center loop (RCL) of intact, high affinity CBG (haCBG). The liberated N-terminal fragment of the RCL then fully and irreversibly inserts itself into the central β-sheet of CBG as an additional β-strand.[6,7] Subtle conformational changes also accompany this large rearrangement, including unwinding of a connecting loop between α-helix D and β-strand 2 in the central β-sheet, which allosterically alters the structure of ligand pocket. Together these structural rearrangements generate low affinity CBG (laCBG), which has nine-fold reduced affinity for cortisol.[4] Structural studies have also revealed the plasticity of the ligand binding pocket governs CBG function. The binding pocket itself is dominated by the indole side chain of Trp371 which is essential for a stacking interaction with the hydrophobic rings present in the chemical structure of cortisol.[6,8] This side chain is mobile and can rotate to accommodate cortisol with optimal geometry observed when the indole moiety is in the same plane as cortisol.[6] CBG also binds other endogenous steroids of similar chemical structures including progesterone, 17-hydroxyprogesterone and cortisone, albeit with lower affinity, and is likely responsible for their transport and biodelivery to target tissues.[9–11] Thus NE-mediated cleavage of CBG leads to a three- to four-fold increase in interstitial fluid free cortisol concentrations[2,7,12] and represents a prereceptor mechanism for tissue physiologic-condition dependent steroid delivery, relevant to inflammation. Systemically, we have shown that falling serum concentrations of haCBG correlate to increasing illness severity in sepsis/septic shock and with the risk of mortality due to dysregulated delivery of sterols.[13,14]

In addition to this NE-mediated delivery mechanism cortisol is liberated from CBG by increased temperature and possibly also by acidemia, conditions typical of sepsis.[7,15] Hydrocortisone binding studies using glycosylated human CBG pooled from plasma revealed that the equilibrium binding constants (K_D) increased four-fold when the temperature was elevated from 37 to 42°C.[7,16] CBG:hydrocortisone binding affinity was not affected by pH but technical limitations were reported by the authors.[15] A molecular mechanism to explain the thermal effects on CBG:cortisol binding has been proposed based upon studies performed upon other serpin proteins. The uncleaved RCLs of structurally analogous antithrombin and thyroxine binding globulin partially and reversibly insert into the central β-sheet, inducing allosteric conformational changes that impact the ligand binding pocket.[6,17] This subtle "nudging" conformational change is sensitive to temperature, thereby providing the proteins with a thermocouple mechanism.[17] CBG is proposed to function in a similar manner, although direct evidence for the reversible insertion of the uncleaved RCL has not yet been verified by structural biology.[6,7,18] Thus, CBG represents a complex biodelivery system for maintaining circulating cortisol levels and mediating tissue specific delivery of steroid hormones.

In this study, surface plasmon resonance (SPR) technology was employed to determine the equilibrium binding constants of a library of steroids to haCBG and laCBG. Purified recombinant human CBG, shown to possess many of the same glycosylation modifications as native protein was utilized. CBG:steroid binding analyses were performed under physiological and pyrexial temperature and acidosis range pH conditions. The immunoreactivity of the RCL on the protein surface was also assessed by SPR using a conformational antibody generated against the RCL epitope. This is the first report to directly probe the proposed "in-and-out" submolecular switch movement of the RCL of CBG under various conditions. Finally, in silico molecular dynamic (MD) simulations were performed to delineate the effect of temperature on CBG structure and propose an additional mechanism of altered steroid binding. The in silico findings reinforce a critical role of Trp371 in the thermo-coupled release of steroid from CBG.

2 | MATERIALS

Glycosylated recombinant human CBG, expressed in HEK293 cells and tagged with biotin was purchased from Sino Biological Inc. (Beijing, China, Cat no. 10998-H27H-B). Human CBG (UniProtKB, P08185) purified from pooled sera of donors to greater than 90% purity, as assessed by SDS-PAGE was purchased from Affiland, Belgium. Pure steroid hormones at analytical grade or higher were purchased from Sigma Aldrich, with the exception of allopregnanolone (Med Chem Express, NJ).

3 | METHODS

3.1 | SDS-PAGE and western blot analysis of biotinylated recombinant human CBG

Gel electrophoresis of CBG was carried out using a 4–12% acrylamide gel. Following transfer to a nitrocellulose membrane, the membrane was blocked using 5% (w/v) skim milk powder in PBS-Tween for 48 hr. The membrane was then incubated with either 9G12 (RRID: AB_26632405) (1:50 dilution) or CBG polyclonal antibody (1:1,000 dilution) for 2 hr at room temperature, followed by anti-mouse IgG2a-HRP (1:500 dilution) then anti-rabbit IgG-HRP (1:2,500 dilution). All dilutions were made in 5% (w/v) skim milk powder PBS-Tween. Immunoconjugates were developed with BioRad Clarity ECL, containing a mixture of luminal and peroxide, and imaged using a ChemiDoc® Imager (Bio-Rad Laboratories, CA). A duplicate membrane was also prepared and probed with a Streptavidin-Alexia Fluor 488 conjugate (Thermo Fisher Scientific, Cat no. S11223) (1:100 dilution) to detect biotinylated protein. After incubating with streptavidin for 1 hr at room temperature, the membrane was washed three times for 5 min in PBS-Tween and then visualized using a ChemiDoc® Imager.

3.2 | N-glycan release and handling

The cysteine residues of human CBG isolated from donor blood (hereafter "Native CBG") and biotinylated recombinant human CBG (hereafter "Biotin-CBG") were reduced using 10 mM aqueous dithiothreitol (DTT) (Sigma-Aldrich, MO), 45 min at 56°C and carbamidomethylated using 40 mM aqueous iodoacetamide (Sigma-Aldrich, MO), 30 min in the dark at 20°C (all stated as final concentrations). Native and Biotin-CBG were each immobilized in three discrete spots (5 μg/spot) on a primed 0.45 μm polyvinylidene fluoride (PVDF) membrane (Merck-Millipore, MA) and processed essentially as described previously.[19] In brief, the dried protein spots were stained with Direct Blue (Sigma-Aldrich, MO), excised, transferred to separate wells in a flat bottom polypropylene 96-well plate (Corning Life Sciences, NY), blocked with 1% (w/v) polyvinylpyrrolidone in 50% (v/v) aqueous methanol and washed with water. The *N*-glycans were exhaustively released from the immobilised proteins using 10 U peptide: *N*-glycosidase F (PNGase F) (*Elizabethkingia miricola*, Promega, WI) in 10 μl water/well, 16 hr at 37°C. The released *N*-glycans were transferred into fresh tubes and deaminated by the addition of 10 μl 100 mM aqueous ammonium acetate, pH 5, 1 hr at 20°C. The *N*-glycans were then dried and subsequently reduced to alditols using 20 μl 1 M sodium borohydride in 50 mM aqueous potassium hydroxide, 3 hr at 50°C. The reactions were quenched by the addition of 2 μl glacial acetic acid to each sample. Dual desalting of the reduced *N*-glycans was performed using a strong cation exchange column (AG 50 W X8, Bio-Rad, Hercules, CA) and then porous graphitised carbon (PGC) packed as micro-columns on top of C18 discs in P10 solid-phase extraction (SPE) formats. The *N*-glycans were eluted from the PGC micro-columns using 0.05% trifluoroacetic acid:40% acetonitrile (ACN):59.95% water (v/v/v), dried and redissolved in 10 μl water for LC–MS/MS-based *N*-glycan profiling.

3.3 | N-glycan profiling

The liberated *N*-glycans were profiled using LC–MS/MS performed on LTQ Velos Pro ion trap mass spectrometer (Thermo Scientific, CA) coupled to a Dionex Ultimate-3000 HPLC (Dionex, CA). The *N*-glycans were separated on a PGC LC capillary column (Hypercarb KAPPA, 5 μm particle size, 250 Å pore size, 0.18 × 100 mm, Thermo Fisher Scientific, CA) using a linearly increasing concentration of solvent B (2.6–64% (v/v) 10 mM NH_4HCO_3 in ACN over 86 min) in solvent A (10 mM aqueous NH_4HCO_3) at a constant flow rate of 3 μl/min. The setup employed a postcolumn make-up flow configuration by supplying 100% isopropanol at a constant 4 μl/min flow rate throughout the LC–MS/MS run for enhanced sensitivity.[20] The acquisition MS1 scan range was set to m/z 570–2,000 with a zoom scan resolution of m/z 0.25 at full width at half maximum (FWHM) and a source voltage of +2.7 kV. Detection was performed in negative ion polarity mode with data-dependent MS/MS acquisition. The automatic gain control (AGC) for the MS1 scans was set to 5×10^4 with a maximum accumulation time of 50 ms. For the MS/MS events, the resolution was set to m/z 0.35 FWHM, the AGC was 2×10^4 and the maximum accumulation time was 300 ms. The top-five most abundant precursors in each MS1 scan were selected for MS/MS using resonance-activation (ion trap) based collision-induced dissociation (CID) at a fixed 35% normalized collision energy (NCE). Dynamic exclusion was disabled. The raw LC–MS/MS data was browsed and interrogated using Xcalibur v2.2 (Thermo Fisher Scientific, CA). The glycan structures were characterized using relative and absolute PGC-LC retention time, monoisotopic mass, and CID-MS/MS fragmentation pattern as previously described.[21]

3.4 | Preparation of sensor chips for SPR assays

SPR binding assays were conducted on a Biacore S200 (GE Healthcare, Uppsala, Sweden) using SPR running buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl and 0.05% (w/v) Tween 20. Streptavidin-coated CM5 sensor chips were prepared as previously described.[22] Briefly, all four flow cells (hereafter called Flow cell 1–4) of a CM5 sensor chip (GE Healthcare) were activated using NHS-EDC chemistry (GE Healthcare) for 900 s at a constant 10 µl/min flow rate. Flow cell 1, which served as a no-protein control, was then treated with blocking buffer (50 mM Tris pH 8.0) for 420 s at 10 µl/min to block unreacted sites. Streptavidin (100 µg/ml, pH 4.5 in sodium acetate) was immobilized onto the three remaining flow cells by injecting the protein solution at 2 µl/min until ~10,000 resonance units (RU) had been deposited. The activated surface was then washed with blocking buffer to any remove uncoupled streptavidin. Flow cell 2 remained a streptavidin only control and was not treated with biotinylated-CBG. Biotinylated-CBG (25 µg/ml in SPR running buffer) was then injected across Flow cells 3 and 4 until ~6,000 RU was captured. Human NE (Sigma Aldrich, Cat no. E8140) was reconstituted to 19.5 U/ml in a 50% glycerol solution (Sigma Aldrich Cat no. 56–81-5) then further diluted in SPR running buffer to a final concentration of 0.5 U/ml. NE was applied only to Flow cell 4, containing immobilized CBG, for 1,800 s at 25°C, at a flow rate of 10 µl/min to digest CBG in situ and generate laCBG, followed by equilibration in SPR running buffer for 900 s at 10 µl/min.

3.5 | Ligand binding assays using SPR

A library of 19 endogenous and exogenous steroid hormones was selected for binding analysis based on their known or predicted binding to CBG.[9–11] All steroid hormones were dissolved in dimethyl sulfoxide (DMSO), and diluted in SPR running buffer to a final concentration of 5% (v/v) DMSO. For each CBG-ligand interaction, a serial dilution of analyte covering a concentration range spanning 6,000–8.2 nM was prepared in SPR running buffer pH 7.4 containing 5% (v/v) DMSO. Binding curves were obtained by injecting varying hormone concentrations across the sensor chip for 40 s followed by a 100 s dissociation phase at a constant flow rate of 30 µl/min. Initial validation binding studies to both haCBG and laCBG were performed at room temperature 25°C. Ligands that demonstrated binding at 25°C were subsequently analyzed at 37 and 39°C, and pH 7.0 designed to mimic the pathophysiologic conditions of sepsis, while those which did not were not further analyzed at increased temperature as binding was not expected. Change in pH was achieved by adjusting the SPR running buffer from pH 7.4 to pH 7.0 by the addition of 0.1 M HCl. As all hormones completely dissociated from the surface during the acquisition period, no regeneration steps were required between samples. To correct for variations in DMSO concentration during the preparation of the analytes a solvent correction curve was included in the analysis by preparing a series of test solutions between 4.5 and 5.8% (v/v) DMSO. The increase in SPR signal observed with CBG:steroid binding interaction is expressed in response units (RU) and is calculated by subtracting the reference RU from the CBG:steroid RU. All RUs were referenced against the blank immobilised reference surface in Flow cell 1 and five buffer only injections to minimize system and injection artifacts. Each binding experiment was technically repeated 2–28 times. The double referenced data was globally fitted using a simple 1:1 L model using the Biacore T200 V3.0 evaluation software (GE Healthcare, Uppsala, Sweden).

3.6 | Antibody binding assays by SPR

Concentrations of total CBG and haCBG (and arithmetically inferred laCBG) were measured using an in-house polyclonal rabbit antibody and a monoclonal mouse antibody, 9G12 (RRID:AB_26632405), respectively. The 9G12 antibody recognizes the well-defined linear epitope of the RCL comprising the ^{341}STGVTLNL^{348} sequence of human CBG that encompasses the NE cleavage site at Val^{344}-Thr^{345}. NE-based cleavage of CBG reportedly disrupts of this epitope causing a loss of 9G12 binding.[23,24] Hence, 9G12, by implication recognizes only intact haCBG. The CBG polyclonal antibody recognizes multiple epitopes on CBG thus measuring total CBG regardless of conformation and structural integrity. Antibody binding was performed at the end of each binding series to confirm the presence of total CBG and laCBG on the respective flow cells. The polyclonal and 9G12 antibodies (1 mg/ml) were diluted in SPR running buffer to final concentrations of 6,000 and 4,000 ng/ml, respectively, run over the flow cells for a contact time of 30 s and dissociation time of 90 s at a flow rate of 10 µl/min. Regeneration was performed using 0.1 M glycine at pH 2.5 with a contact time of 60 s at 10 µl/min. The antibody binding experiments were performed upon completion of the ligand binding studies as the regeneration step was observed to denature CBG with concomitant loss of ligand binding.

3.7 | Equilibrium MD simulations

The structure of human cleaved CBG in complex with progesterone (PDB 4BB2, 2.48 Å)[25] was downloaded from the RCSB protein databank and missing residues between Ly96 and Ser100 were added using the molecular modeling program MODELLER.[26] The ligand-receptor complex was parameterized using the classical Chemistry at Harvard Macromolecular Mechanics 36 (CHARMM36)[27] force field with the aid of the CHARMM-GUI.[28] The system was solvated in a rectangular TIP3 water box fitted to the size of the protein with padding of 10.0 Å. KCl was added to the system for neutralizing, randomly replacing water molecules to simulate a concentration of 0.15 M.

All MD simulations were performed in GPU-accelerated nodes of the National Computing Infrastructure (NCI) Gadi supercomputer using the NAMD 2.13 package.[29] All simulations were performed with periodic boundary conditions in the NpT ensemble. The temperature of the simulation system was set to either 310 K or 313 K using the Langevin dynamics for temperature coupling. The system pressure was set to 1 atm using a Langevin piston. A distance cut-off of 11.0 Å was applied to short-range nonbonded interactions, whereas long-range electrostatic interactions were treated using the particle-mesh Ewald. Lennard-Jones interactions were updated at every step and electrostatic interactions at every two steps. The time step of integration was chosen to be 2 fs for all simulations performed. Before the production MD simulations, systems were minimized for 10,000 steps and equilibrated for 125,000 steps. Finally, production simulations were carried out for 60 ns with a 20 ps output timing.

3.8 | Simulation data analysis

Visualization was carried out using the VMD package.[30] The trajectories analysis, including root mean square deviation (RMSD) and root mean square fluctuation (RMSF) were carried out using TCL scripts developed in-house. Data plotting was carried out using the plotting tool Grace (https://plasma-gate.weizmann.ac.il/Grace/).

3.9 | Statistical analysis

SPR data was analyzed using GraphPad Prism version 8 for Mac OS X (GraphPad Software, Inc., CA). Two-way analysis of variance (ANOVA) was used to compare variables between the four temperature and pH conditions for both haCBG and laCBG. Results are expressed as mean ± standard error mean (SEM) unless otherwise stated. $p < 0.05$ was considered statistically significant.

4 | RESULTS

4.1 | Production and characterization of recombinant mammalian human CBG

To facilitate the development of a direct ligand binding assay using SPR, an engineered variant of recombinant human CBG was used for these studies. Specifically, two additional sequences were fused onto the C-terminus of the protein: a hexahistidine sequence to enable protein purification and a 14 amino acid avi-tag that allowed site-specific biotinylation of the protein for the SPR assay. Inspection of available X-ray structures of both haCBG and laCBG revealed that the C-terminus was optimal for the tags as it was distal to both the ligand binding pocket and the RCL containing the NE scissile site.[7] Therefore, we predicted that fusion of the additional amino acid residues onto the C-terminus was unlikely to disrupt ligand binding nor NE-proteolysis of the RCL. The attachment of biotin onto a single lysine residue present in the avi-tag permitted the immobilization of recombinant CBG onto streptavidin coated SPR sensor surfaces under mild binding conditions that maintained protein structure and biological activity. The commercially sourced CBG was expressed recombinantly in mammalian HEK293 cells to allow for human-type protein glycosylation and purified using immobilized metal ion chromatography. SDS-PAGE analysis revealed that, while CBG has a calculated molecular mass of 42 kDa, the apparent molecular weight was 50–60 kDa due to known *N*-glycosylation heterogeneity[31,32] (Figure 1). Western blot analysis showed that the purified protein was immunoreactive toward a polyclonal anti-CBG antibody as well as the conformational 9G12 monoclonal antibody that recognized a specific epitope within the intact RCL (Figure 1). A similar outcome was also observed on a Streptavidin blot, thus confirming the recombinant CBG was both biotinylated and uncleaved (Figure 1).

Glycemic analysis further confirmed that the recombinant human CBG contained many of the same *N*-glycosylation features as native human CBG isolated from healthy donor blood, albeit at different levels between the two variants of CBG obtained from different cellular sources (Figure 2). These included highly branched bi-, tri-, and possibly even higher branched complex sialoglycans variably modified with core fucosylation.[34] Our glycomics data revealed mostly quantitative differences between the *N*-glycans of HEK293-expressed biotinylated human CBG and native

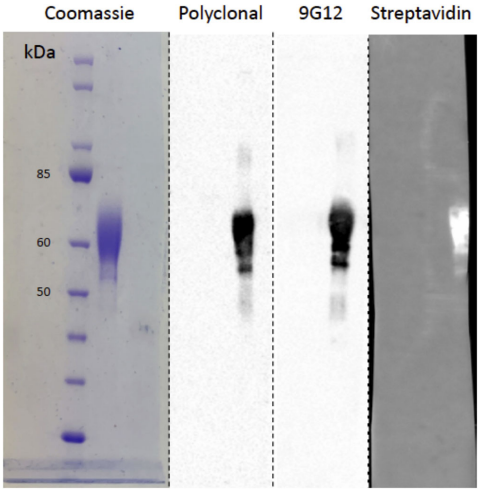


FIGURE 1 Analysis of recombinant human CBG. Coomassie-blue stained SDS-PAGE (left lane) and the corresponding Western blot analysis (three right lanes) of biotinylated recombinant human CBG probed with polyclonal anti-CBG antibody, anti-RCL monoclonal antibody 9G12 and streptavidin to detect biotinylated protein. This validation step demonstrates that biotinylated recombinant human CBG is uncleaved. Migration of molecular mass markers are also shown

CBG, but also a few structures uniquely carried by recombinant CBG[34] including LacdiNAc and sialyl-LacdiNAc and oligomannosidic-type *N*-glycans. Together these data suggested that the recombinant protein employed in this study possessed similar glycosylation properties as the native protein and was a suitable surrogate for *in vitro* ligand binding studies.

4.2 | Development and validation of SPR binding assay

Using the characterized recombinant protein we developed a direct ligand binding assay using SPR technology which allowed us to systematically investigate ligand binding to haCBG and laCBG under a variety of conditions that mimic those encountered under normal physiology, septic shock and other pathophysiological states. Streptavidin was first immobilized on to three SPR sensor surfaces then biotinylated human CBG was captured onto two of the SPR flow cells, while a third flow cell was left as a streptavidin only control. A polyclonal anti-CBG antibody was employed to demonstrate successful immobilization of CBG onto the sensor chip (Figure 3). One of the flow cells containing immobilised CBG then underwent treatment with NE for 30 min to facilitate the conversion of haCBG to laCBG in situ. Proteolysis was verified using the 9G12 monoclonal antibody with immunoreactivity specifically against the intact RCL (Figure 3). Complete conversion of haCBG-to-laCBG by NE treatment was confirmed by the loss of binding by 9G12 monoclonal antibody while maintaining polyclonal antibody binding. The known ligand hydrocortisone was then used to functionally validate the binding assay (Figure 4, for an example of the SPR sensograms for hydrocortisone binding to haCBG and laCBG, as well as the corresponding binding isotherms that underpinned the K_D determination). The measured equilibrium binding constants at 25°C for hydrocortisone (haCBG, K_D 42 nM; laCBG K_D 217 nM) were in excellent agreement with previous reports (haCBG, K_D 45 nM; laCBG K_D 422 nM).[25] Hydrocortisone is the name used for synthetic cortisol employed therapeutically.

4.3 | Steroid ligand binding studies

The validated SPR assay was then used to systematically determine the affinities of an extended library of 19 steroid ligands. Binding to both haCBG and laCBG were initially assayed at 25°C for validation with subsequent analysis at temperature and pH designed to mimic the pathophysiologic conditions of sepsis. Table 1 shows the equilibrium binding constants of all steroid hormones to haCBG and laCBG at both 25 and 37°C at pH 7.4. Affinities were measured for hydrocortisone, cortisone, 11-deoxycortisol, corticosterone, prednisolone, progesterone, 17-OH progesterone, aldosterone, and testosterone. The most potent binding was recorded for haCBG binding to hydrocortisone (K_D 42 nM) and corticosterone (K_D 45 nM). The data demonstrated the expected four- to five-fold decrease in affinity of the tested hormone upon haCBG-to-laCBG conversion. The greatest change, a seven-fold reduction in hormone binding affinity, was observed for cortisone (haCBG K_D 400 nM; la CBG K_D 2957 nM; p <.0001). Glucocorticoids and progestagens were the only hormones that displayed measurable binding to laCBG (i.e., K_D <6 μM). No binding to either ha-CBG or la-CBG (K_D >6,000 nM) was detected for 17-OH pregnenolone, β-estradiol, dexamethasone, dehydroepiandrosterone (DHEA), estrone, 5α-androstan-17β-ol-3-one, adrenosterone, dehydroisoandrosterone 3-sulphate, pregnenolone, or allopregnanolone.

The effects of temperature and pH on hydrocortisone (cortisol) binding to CBG were subsequently addressed (Table 2). Increasing temperature from 37 to 39°C, as observed in inflammation, caused a significant decrease in hydrocortisone binding to haCBG (37°C K_D 214 nM;

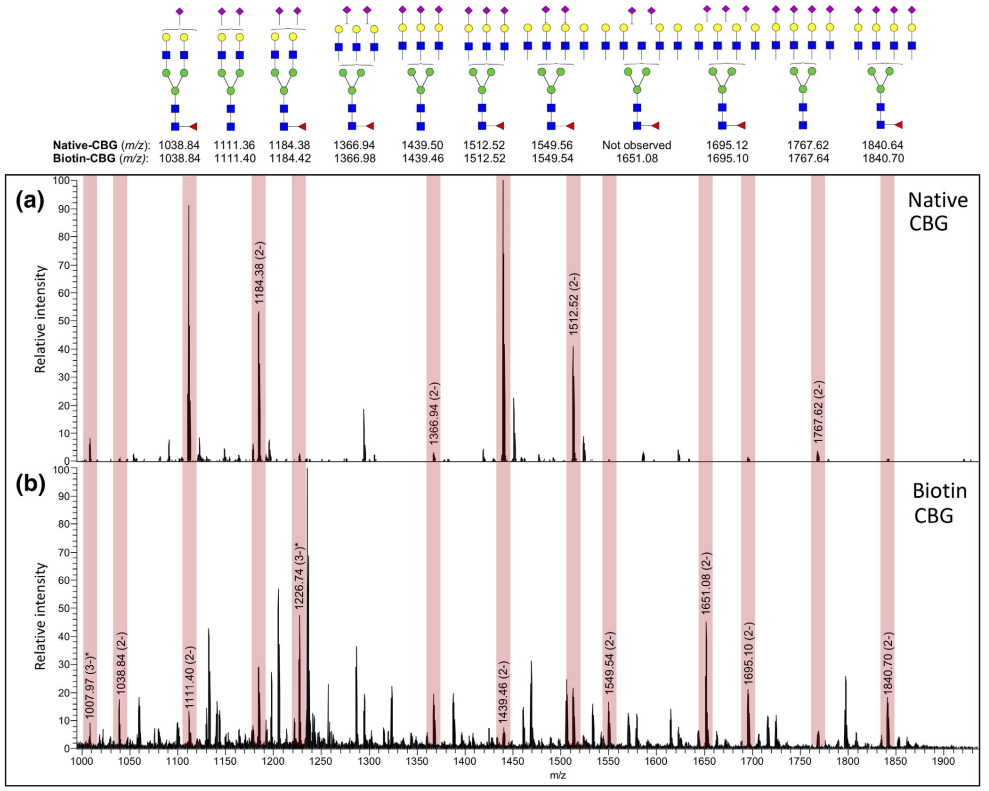


FIGURE 2 Comparative glycomic analysis of native and recombinant human CBG. Native human CBG isolated from donor blood (Native CBG, upper panel) and biotinylated recombinant human CBG expressed in HEK293 cells (Biotin-CBG, lower panel) are glycosylated with broadly similar *N*-glycosylation profiles, as documented using comparative PGC-LC–MS/MS *N*-glycome profiling. Vertical boxes in light red shading on the summed mass spectra highlight the *N*-glycans that are shared between the two CBG forms, albeit at different relative abundances. The *N*-glycan structures corresponding to the observed *m/z* values as supported by CID-MS/MS data are shown above using the conventional symbol nomenclature for glycans.[33] "*" denotes an already depicted *N*-glycan structure also present in another charge state

39°C K_D 507 nM; $p = .0042$). Likewise, acidification from the physiological pH 7.4 to pH 7.0 at 39°C caused further loss in binding activity (pH 7.4 K_D 507 nM; pH K_D 740 nM, $p = .0308$).

There was an additive effect of NE treatment, temperature elevation and acidification on CBG:hydrocortisone binding affinity. Hydrocortisone showed 3.5-fold reduced binding from haCBG 37°C, pH 7.4 (K_D 214 nM) to 39°C, pH 7.0 (K_D 740 nM; $p = <.0001$). NE-treatment further reduced binding with seven-fold reduced affinity observed between haCBG at 37°C, pH 7.4 (K_D 214 nM) and laCBG at 39°C at pH 7.0 (K_D 1,505 nM; $p = <.0001$). Similarly, a significant reduction in cortisol binding to laCBG was observed with increased temperature (Table 2). The data clearly demonstrate that temperature, pH, and NE-mediated proteolysis all contribute to the release of CBG bound hormone under the pathophysiological conditions that mimic septic shock.

4.4 | Antibody binding studies to investigate movement of the RCL

The effect of temperature and pH upon CBG structure was next investigated using SPR. Specifically, allosteric structural changes involving the reversible insertion of the RCL into the central β-sheet of haCBG were probed using the 9G12 conformational monoclonal antibody.

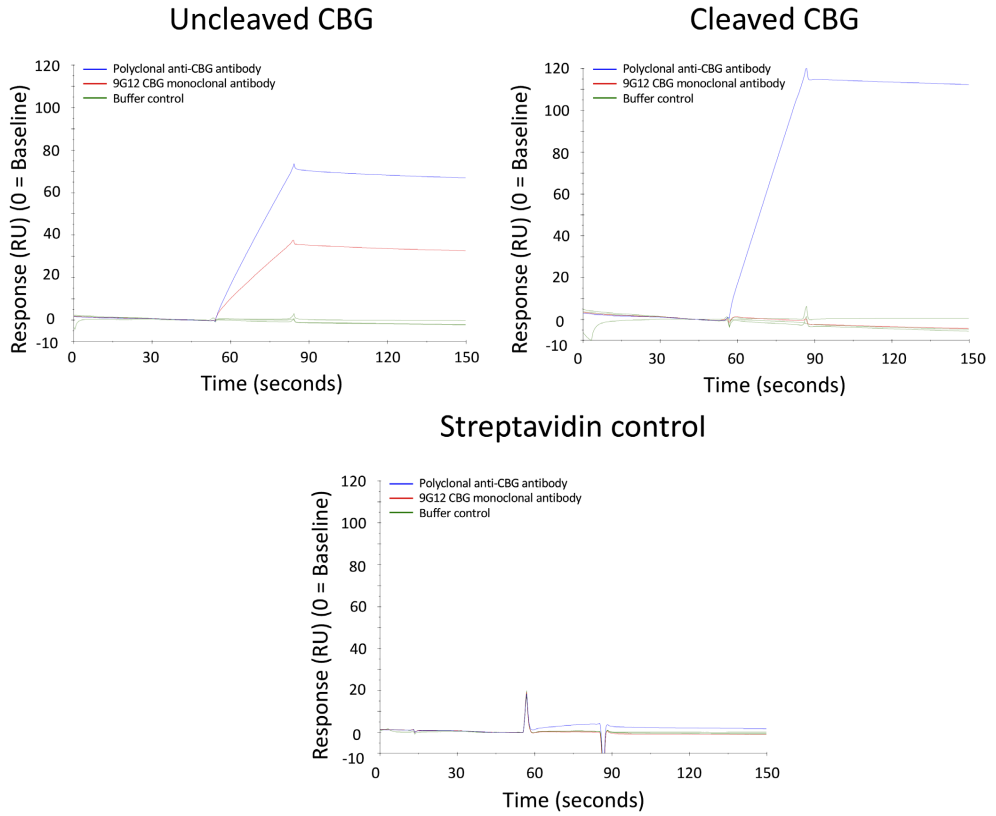


FIGURE 3 SPR analysis of immobilised CBG. Streptavidin was immobilized onto three separate flow cells on a CM5 sensorchip. The sensorchip was then treated with either biotinylated recombinant CBG (uncleaved CBG), biotinylated recombinant CBG followed by proteolysis with NE (cleaved CBG) or no protein (streptavidin control). Sensorgrams are shown for the binding of polyclonal anti-CBG antibody (blue trace), 9G12 monoclonal antibody recognizing only uncleaved haCBG (red trace) and a buffer control (green trace). The streptavidin only flow cell, devoid of CBG, served as a negative control to address nonspecific antibody binding

Binding studies were performed at 25 and 37°C at pH 7.4, and 39°C at pH 7.4 and 7.0 (Table 3). No changes in binding response were observed across the three temperatures tested, consistent with the RCL epitope being solvent exposed and immunoreactive despite the varying conditions. These results support the observation that the RCL remains intact and available for synergistic NE proteolysis, even at the elevated temperature of 39°C. Likewise, changes in pH did not alter 9G12 antibody binding at 25°C. However, reduced binding was observed at 39°C which may reflect synergistic effects of temperature and acidic pH on the solvent-accessibility of the RCL, as reflected by CBG:hydrocortisone binding studies performed under these conditions (Table 3).

4.5 | Equilibrium MD simulations

Finally, MD simulations were performed to further understand the impact of temperature upon CBG structure. Here the high-resolution crystal structure of human cleaved CBG in complex with progesterone (PDB ID 4BB2, 2.48 Å) was subjected to MD simulations after parameterization with the CHARMM36 all tom force field. This structure was selected as the crystal structure of human uncleaved CBG has not been reported, and the homologous rat CBG structures have regions unresolved including the RCL. During these initial equilibrium simulations at 310 and 313 K, no large changes in CBG structure were observed. The protein backbone reached

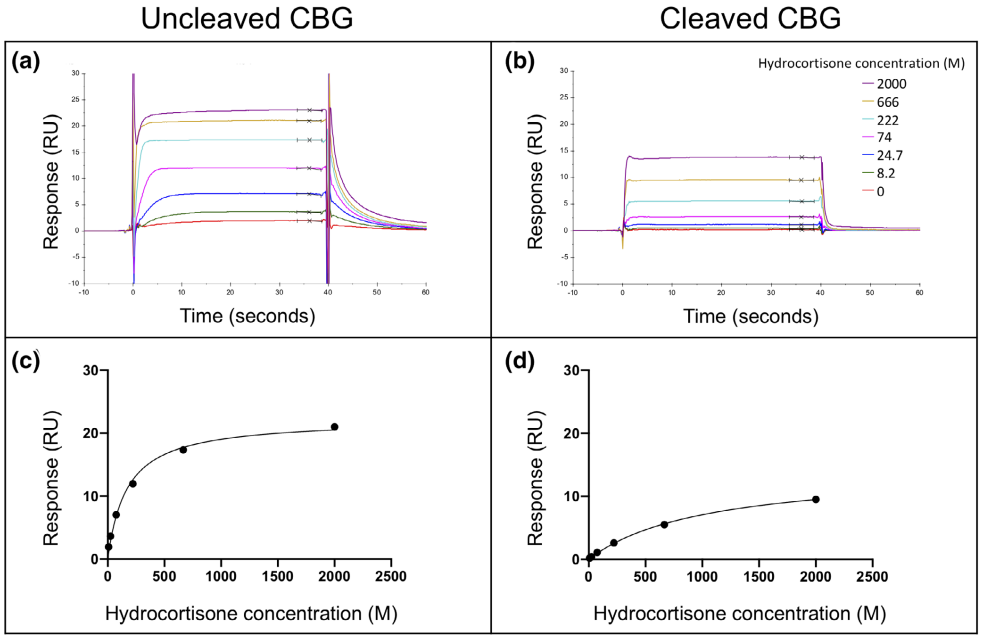


FIGURE 4 Hydrocortisone binding to CBG. Representative SPR sensorgrams are shown for hydrocortisone binding to (a) uncleaved CBG and (b) cleaved CBG at 37°C and pH 7.4. The corresponding binding isotherms used for determining equilibrium binding constants (K_D) are also shown (c) and (d). Each colored band indicates a different hydrocortisone concentration, ranging from 0 to 2000 nM

equilibrium dynamics of only ~1.6 Å deviated from the original conformation. Similarly, the ligand also reached an equilibrium structure ~0.3 Å from the original crystallographic position. Therefore, temperature-induced changes in CBG structure were most likely due to localized movement of amino acid side chains.

The relationship between ligand binding and temperature (37 and 40°C) was further probed by studying the dynamics of the CBG-ligand complex at 310 and 313 K for a period of 60 ns. Those amino acid residues located within 8 Å from the ligand were interrogated in further detail (Figure 5a). A comparison of the RMSF values between the two conditions showed that the indole side chain of Trp371 experienced the largest and most significant movement of any amino acid in the binding site (Figure 5b; Movies A https://figshare.com/s/5739e72874f70476ba2c and B https://figshare.com/s/fd5769ab24bbed371fe2). Visual inspection of the structure at 310 K revealed that the indole sidechain adopted the stable conformation required for a direct stacking interaction with the hydrophobic rings of the steroid. In sharp contrast, the indole heterocycle experienced much greater mobility at 313 K and was observed to rotate outside of the plane necessary for this binding interaction. Given the critical role of Trp371 in ligand binding, this increased rotation and mobility would conceivably impact upon a thermocoupled loss of ligand binding.

5 | DISCUSSION

This study confirms that the steroid binding to CBG is ligand specific and is reduced upon NE-mediated conversion of haCBG-to-laCBG. Importantly, this study has shown that CBG:cortisol binding is significantly reduced with increases in temperature and in acidosis for both haCBG and laCBG, representative of that which is experienced in systemic inflammatory states such as in sepsis and septic shock. Furthermore, the immunoreactivity of the RCL of haCBG remains largely preserved at 39°C and in acidosis. Molecular modeling studies of CBG performed at 40°C revealed the critical role of Trp371 in ligand binding. Taken together we propose the thermocoupling mechanism of cortisol release involves Trp371 and occurs while maintaining RCL integrity. Thus, the synergy of NE cleavage, pyrexia and acidosis on CBG

TABLE 1 Equilibrium binding constants (K_D, nM) of uncleaved and cleaved CBG determined at a constant condition of 25°C, 37°C, and pH 7.4

Steroid ligand	Uncleaved CBG (K_D, nM) 25°C	Cleaved CBG (K_D, nM) 25°C	Uncleaved CBG (K_D, nM) 37°C	Cleaved CBG (K_D, nM) 37°C
Hydrocortisone (cortisol)	42.0 (3.8)	217.5 (8.7)	214.4 (18.4)	1,064.5 (139.5)
Corticosterone	45.2 (6.4)	170.8 (6.6)	181.3 (2.3)	898.0 (29.3)
Prednisolone	77.9 (14.7)	458 (48.5)	329.8 (53.2)	2,265.5 (345.5)
11-deoxycortisol	85.3 (25.5)	348.7 (42.7)	249.8 (22.6)	1931 (201)
17-OH progesterone	118.4 (10.2)	516.4 (53.9)	889.1 (373.9)	2,837.5 (257.5)
Progesterone	318.2 (67.6)	1,570.3 (214.9)	1,680.5 (101.5)	6,143.0 (1266)
Cortisone	400.5 (97.8)	2,957.5 (243.5)	1,338 (281)	5,242
Testosterone	609.5 (22.7)	3,102.3 (564.5)	N/D	N/D
Aldosterone	1,008.8 (236.3)	4,634.5 (846.4)	N/D	N/D
17-OH pregnenolone	>6,000	>6,000	N/D	N/D
B-estradiol	>6,000	>6,000	N/D	N/D
Dexamethaxone	>6,000	>6,000	N/D	N/D
Dehydroepiandrosterone (DHEA)	>6,000	>6,000	N/D	N/D
Estrone	>6,000	>6,000	N/D	N/D
5a-Androstan-17B-ol-3-one	>6,000	>6,000	N/D	N/D
Adrenosterone	>6,000	>6,000	N/D	N/D
Dehydroisoandrosterone 3-sulphate	>6,000	>6,000	N/D	N/D
Pregnenolone	>6,000	>6,000	N/D	N/D
Allopregnanolone	>6,000	>6,000	N/D	N/D

Note: All data points are represented as the mean values and their variance indicated in brackets as SEM as determined from at least three technical replicates.
Abbreviation: N/D, not determined.

TABLE 2 Hydrocortisone (cortisol) equilibrium binding constants (K_D, nM) of uncleaved and cleaved CBG at different temperatures and pH

Hydrocortisone (cortisol)	Uncleaved CBG (K_D, nM)	Cleaved CBG (K_D, nM)	ΔK_D (fold change)
25°C, pH 7.4 N = 10	42.0 (3.8)	217.5 (8.7)	5.2
37°C, pH 7.4 N = 26	214.4[a,b] (18.4)	1,064.5[d,e] (139.5)	5.0
39°C, pH 7.4 N = 28	507[a, c] (19.0)	1,398.9[d] (37.7)	2.8
39°C, pH 7.0 N = 26	740.1[b, c] (24.8)	1,505.1[e] (44.1)	2.0

Note: All data points are represented as mean values and their variance indicated in brackets as SEM. Two-way ANOVA overall interaction $p = .0002$, row factor $p = <.0001$, column factor $p <.0001$.
Abbreviation: *N*, number of technical replicates.
[a]Comparison of haCBG:cortisol at 37°C pH 7.4 versus 39°C pH 7.4 $p = .0042$.
[b]Comparison of haCBG:cortisol at 37°C pH 7.4 versus 39°C pH 7.0 $p <.0001$.
[c]Comparison of haCBG:cortisol at 39°C pH 7.4 versus 39°C pH 7.0 $p = .0308$.
[d]Comparison of laCBG:cortisol at 37°C pH 7.4 versus 39°C pH 7.4 $p = .0010$.
[e]Comparison of laCBG:cortisol at 37°C pH 7.4 versus 39°C pH 7.0 $p <.0001$.

TABLE 3 RCL remains available over different temperature and pH ranges as demonstrated by antibody binding to uncleaved and cleaved CBG

	Uncleaved CBG		Cleaved CBG	
Temperature	**Polyclonal (RU)**	**9G12 (RU)**	**Polyclonal (RU)**	**9G12 (RU)**
25°C, pH 7.4	51.7 (3.5)	34.2 (7.2)	85.0 (3.9)	1.1 (0.4)
37°C, pH 7.4	63.9 (4.1)	32.2 (1.0)	87.6 (1.6)	−0.6 (0.3)
39°C, pH 7.4	84.5	31.9	126.8	0.7
39°C, pH 7.0	97.5	15.9	172.2	−2.5

Note: The binding response was measured in response units (RU). RU, increase in SPR signal observed with CBG:steroid binding interaction—reference RU. All data points are represented as mean values and their variance indicated in brackets as SEM.

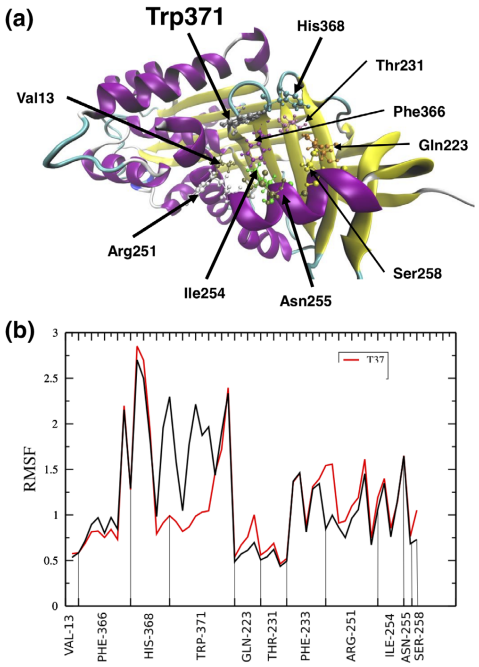


FIGURE 5 Molecular dynamic simulations. (a). The tertiary structure of the human CBG with key amino acids required for ligand binding highlighted. The human CBG (PBD ID 4BB2, 2.48 Å) structure was used. (b). The root mean squire fluctuation (RMSF) values for select amino acids in the ligand binding site during the simulation at 310 K (red curve) and 313 K (black curve). A comparison of curves show that the indole side chain of Trp371 experienced the largest and most significant movement of any amino acid within the ligand binding site. The naturally occurring Trp371 to Ser mutation (CBG Athens variant) resulting in the complete loss of CBG: cortisol binding activity in a Greek patient[35] further supports the critical role of Trp371 in the thermo-coupled release of steroid from CBG

structure may serve to enhance cortisol delivery to the interstitium at sites of inflammation.

The glucocorticoid cortisol is important in the regulation and modulation of the immune system in response to inflammation and essential for survival. In acute physiological stress, such as that experienced in sepsis, glucocorticoids work through secondary messenger pathways on pro-inflammatory transcription factors and at genomic sites to reduce inflammation.[36] Initially, minor insults elevate cortisol with pro-inflammatory actions enhancing the innate immune response,[37] after which cortisol exerts anti-inflammatory effects through glucocorticoid receptor (GR)-α mediated suppression of intracellular pathways induced by the pro-inflammatory transcription factors NF-$_{\kappa}$B and AP-1 and direct GR transcriptional actions at genomic glucocorticoid response elements.[38,39] Glucocorticoids target inflammatory cytokine synthesis, neutrophil migration, T cell apoptosis and macrophage inhibition. Cortisol has pleiotropic effects increasing hepatic glucose output and blood pressure through synergistic action with catecholamines on peripheral vasculature and the heart, immunomodulation, increased cognition and arousal.[36,40] Up to 80% of the body's cortisol is bound to CBG. CBG optimizes the human body's response to inflammation by facilitating cortisol delivery via four key mechanisms: pyrexia, reduced hepatic synthesis of CBG, NE cleavage, and tissue acidosis. These mechanisms feature in each pathogenic step in the cascade from localized inflammation to overwhelming septic shock. Our previous human studies have demonstrated that a lack of targeted delivery of natural cortisol and/or administered hydrocortisone to damaged tissue is related to deficient serum concentrations of CBG, which perpetuates organ failure and death in sepsis.[13,14]

To further probe the action of CBG in the finely tuned delivery of cortisol and other steroids, we developed a novel ligand binding assay. The chosen SPR technology provided a reproducible and readily applicable, direct ligand binding assay to quantitate the bimolecular

interaction between recombinant CBG and a library of 19 steroids. Our ligand panel consisted of both hormones with reported binding to CBG alongside previously untested hormones displaying similar chemical structures to the *bona fide* ligands that play possible neuromodulating roles in the brain.[41,42] Sex steroids were also profiled, in part due to the reported association of the CBG A51V polymorphism with higher female-to-male live births in Han Chinese.[43] Moreover, the assay was developed such that both uncleaved (haCBG) and cleaved (laCBG) isoforms could be tested in parallel under varying temperatures and pH. This is the first study to extensively investigate CBG binding interactions using SPR. Equilibrium binding constants for cortisol to haCBG (K_D 42 nM) and laCBG (K_D 217 nM) at 25°C pH 7.4 were in excellent agreement with those reported in the literature (i.e., Qi et al.[18]; K_D 26.9 nM for uncleaved recombinant CBG at 22°C, Gardill et al.[25] K_D 45 and 422 nM for uncleaved and cleaved recombinant CBG, respectively, at 25°C; and *Chan* et al[7] K_D 42.8 nM and 165 nM for uncleaved and cleaved recombinant CBG, respectively, at 25°C).

The SPR binding assay demonstrated a 3-to-8-fold decrease in binding affinity between haCBG and laCBG for cortisol, cortisone, corticosterone, 11-deoxycortisol, progesterone, 17-hydroxyprogesterone and prednisolone at physiological conditions (37°C, pH 7.4). Cortisol binding affinity also significantly decreased at higher temperatures and acidic pH for both haCBG and laCBG. With respect to other steroids, progesterone bound to haCBG with an eight-fold lower affinity than cortisol (K_D 1680.5 vs. 214.4 nM, respectively). During pregnancy progesterone concentrations at the intervillous space are 15-fold higher than in the peripheral maternal circulation, and CBG is largely progesterone-laden at this site.[44] The high circulating progesterone concentrations compete with cortisol for binding, contributing to the increase in free cortisol required for organ maturation neurodevelopment and neonatal transition.[45] 17-OH progesterone binds to CBG with four-fold lower affinity than cortisol (K_D 889.1 vs. 214.4 nM, respectively). In untreated and undertreated congenital adrenal hyperplasia, elevated 17-OH progesterone levels would preferentially bind CBG, increasing free cortisol.

Unique to our assay was the ability to directly study the RCL under temperature and pH conditions which would only be achievable using alternative methodologies such as mass spectrometry or X-ray crystallography. The 9G12 antibody recognition was preserved for haCBG across the tested temperature and pH range confirming that the RCL remains solvent exposed and available for NE proteolysis in these conditions. Presumably, CBG loses ligand affinity during pyrexia due to temperature-dependent flexibility of the hormone binding pocket independent of RCL modification (discussed below). Previous studies have proposed cortisol release in pyrexia may be driven by a reversible conformational change of haCBG due to a temperature-dependent insertion of the RCL into the CBG protein core,[6,7,18] described as the "flip-flop" or "nudging" submolecular switch mechanism as observed for other serpins.[17,18] Here, we demonstrated intact thermo-coupling occurs even after NE cleavage for laCBG, corroborating that the temperature-dependent mechanism of steroid release from CBG persists despite the full insertion of the RCL. Thus, these findings support a synergistic effect of both NE cleavage of haCBG and temperature-dependent mechanisms in the release of bound cortisol at sites of inflammation.

Serpins have the capacity to form a series of conformers due to RCL flexibility and A-sheet configurations resulting in graded ligand affinity. This equilibrium phenomenon is observed only for intact serpins which have not undergone proteolytic cleavage of the RCL.[6] CBG is proposed to exist within this dynamic landscape, but appears to have a more limited range of RCL flexibility than that observed for other serpins due to its higher ligand binding affinity and slightly shorter RCL by four residues.[6] With respect to the equilibrium of model of serpins, we acknowledge the potential limitation using antibody binding studies in this work to investigate RCL movement. Use of the 9G12 monoclonal antibody directed against the NE-cleavage site of the RCL will differentiate between a fully incorporated or exposed RCL, however, it may lack sensitivity to detect subtle micro movements of the RCL. Furthermore, the equilibrium of RCL movement under study conditions may be influenced by the virtue of antibody binding.

Our MD simulation experiments highlighted an important role for Trp371 in the thermo-coupled release of bound steroid. Previous studies have, similarly, implicated this amino acid residue in ligand binding. Substitution of Trp371 with threonine or lysine generated a mutant that was unable to bind cortisol in assays using radiolabelled ligand.[46] Likewise, a naturally occurring Trp371 to Ser mutation in a Greek patient with low morning cortisol levels (the CBG Athens variant) was characterized and shown to be devoid of cortisol binding activity.[35] Structural biology has shed further insights into the molecular basis of cortisol binding and mechanism of release. Comparative analysis of the available crystal structures of human and rat CBG in the unliganded state as well as both haCBG and laCBG in complex with steroids reveal that the ligand binding sites are all highly conserved.[6,8,25,47] The primary point of structural difference is the orientation of the indole sidechain of Trp371.[6] In co-complexes with cortisol or

progesterone, the indole ring of Trp371 is positioned parallel to ligand necessary for stacking interactions with the common A and B rings of these steroids. This is in stark contrast to the unliganded structure where the same side chain is rotated 90° and adopts a conformation suggestive that it is unreceptive to ligand binding.[6] In the current study, we measured significantly greater mobility of the same amino acid side chain essential for ligand binding when temperature was elevated above those expected under normal physiology. Our MD studies, in concert with biophysical binding assays and structural biology, provide an alternative mechanism for the thermo-coupled dissociation of ligand during pyrexia involving movement of the Trp371 side chain.

CBG glycosylation is known to be essential for cortisol binding through its influence on CBG protein folding and steroid binding at glycosylation site Asn238, one of five glycosylation sites positioned in proximity of the ligand binding site,[48] and influence RCL cleavability given there is glycosylation at Asn347, within the RCL, modulating the rate at which cortisol is released.[49] In the current study we used recombinant human CBG that was characterized for glycosylation modifications and demonstrated 9G12 immunoreactivity to untreated protein and the expected complete loss of 9G12 reactivity upon NE-mediated haCBG-to-laCBG conversion. Our previous research has revealed falling serum haCBG concentrations over time in proportion to septic shock severity and lower haCBG levels is associated with septic shock mortality.[13,14] In sepsis and septic shock with attendant multi-organ failure and hepatic dysfunction, protein glycosylation does not increase, thus it is unlikely that the reduction in 9G12 reactive CBG is due to an increase in CBG glycosylation of the RCL in these states. Furthermore, a loss in 9G12 reactive CBG correlates with rising free cortisol concentrations in sepsis and septic shock.[13,14] Data from the current study shows a clear temporal connection between *in vitro* NE cleavage under controlled conditions, reduction of CBG:cortisol binding and loss of 9G12 immunoreactivity. Thus, a reduction in 9G12 reactive CBG, as observed in human sepsis and septic shock, likely reflects a loss of circulating haCBG due to NE cleavage *in vivo*. The HEK293-expressed human CBG used in this study shared many glycosylation features with native human CBG. While some measurable differences may contribute to different 9G12 reactivity profiles, it is highly unlikely these minor changes would contribute to the results reported here further supporting the use of 9G12 antibodies to quantify haCBG in our human clinical studies.[14,45,50–54]

This study (1) systematically determines the binding affinity of glycosylated haCBG and laCBG to a large panel of steroid ligands, (2) demonstrates reduced CBG:cortisol binding affinity in response to acidosis, and (3) reveals CBG:cortisol binding affinity is reduced upon increases in temperature with the preservation of the thermo-coupling mechanism even after NE-cleavage. We demonstrate through molecular modeling the importance of Trp371 critically located within the binding pocket in the thermo-coupling mechanism of cortisol release and through RCL antibody binding studies that the NE-cleavage site remains largely intact at increased temperature. This has implications for the study of cortisol delivery in human sepsis and suggests that the conditions associated with sepsis separately contribute to tissue cortisol delivery via discrete structural effects on the CBG molecule. In sepsis, a sequential four-part process may operate to optimize free cortisol delivery, with pyrexia,[53] cytokine-based inhibition of hepatic CBG synthesis,[55] NE-mediated cleavage and acidosis acting in concert to produce immune activation, metabolic and neurocognitive effects, direct delivery of immunomodulatory cortisol at inflammatory sites and cortisol protection to healthy tissues affected by the inflammatory process. These findings demonstrate the modifiable hormone binding and delivery characteristics of CBG in both physiologic and pathologic conditions, supporting its significance in cortisol delivery to obviate systemic inflammation[4,5] and multi-organ failure in patients with septic shock and its association with mortality.[13,14]

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AUTHOR CONTRIBUTIONS

Emily Meyer: Conceptualization; data curation; formal analysis; investigation; methodology; writing-original draft. **David Torpy:** Funding acquisition; supervision; writing-review and editing. **Anastasia Chernykh:** Investigation; writing-review and editing. **Morten Thaysen-Andersen:** Investigation; supervision; writing-review and editing. **Marni Nenke:** Writing-review and editing. **John Lewis:** Writing-review and editing. **Harinda Rajapaksha:** Investigation; writing-review and editing. **Wayne Rankin:** Conceptualization; funding acquisition;

supervision; writing-review and editing. **Steven Polyak:** Conceptualization; methodology; supervision; writing-review and editing.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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SUPPLEMENTARY INFORMATION

Please access supplementary movies (A and B) published online using the Figshare database.

C) https://figshare.com/s/5739e72874f70476ba2c

D) https://figshare.com/s/fd5769ab24bbed371fe2

Supplementary movies of the tertiary structure of human CBG (PBD ID 4BB2, 2.48 Å) at 37 °C (**Movie A**) and 40 °C (**Movie B**) with key amino acids located within the steroid binding pocket required for ligand binding highlighted. Comparison shows spatial instability of Trp371 (depicted in silver), experiencing the most amount of movement of any amino acid within the binding site at 40 °C compared to 37 °C. The naturally occurring Trp371 to Ser mutation (CBG Athens variant) resulting in the complete loss of CBG:cortisol binding activity in a Greek patient supports the critical role of Trp371 in the thermo-coupled release of steroid from CBG.[1]

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CHAPTER 4:

TOTAL AND HIGH AFFINITY CORTICOSTEROID-BINDING GLOBULIN DEPLETION IN SEPTIC SHOCK IS ASSOCIATED WITH MORTALITY.

CHAPTER OUTLINE

In vivo study of CBG cleavage in human pathology became possible with the availability of a highly specific monoclonal antibodies directed to the RCL and a distant site, so that circulating CBG could be measured in both as total CBG and in its uncleaved (haCBG) form. Earlier studies showed that CBG concentrations fall substantially in sepsis in proportion to illness severity and in survivors return to normal.[1,2] These studies however failed to find an association between CBG and mortality due to small study numbers. The objective of this study was to determine whether low CBG concentrations in septic shock associated with mortality.

Here I present a retrospective pilot study showing an association between CBG (both total CBG and haCBG) depletion and mortality in septic shock patients. Patients with septic shock had CBG levels significantly lower than levels observed in health, with levels significantly lower in non-survivors when compared to survivors. Furthermore, there appeared to be a survival effect of CBG, with improvement in CBG levels over time in parallel with septic shock recovery.

CBG levels in non-septic critical illness did not portend mortality and were equivalent to that observed in health, suggesting CBG deficiency remained specific to sepsis associated critical illness.

Cortisol levels and albumin were not associated with mortality.

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Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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ORIGINAL ARTICLE

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Total and high-affinity corticosteroid-binding globulin depletion in septic shock is associated with mortality

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Abstract

Context: Corticosteroid-binding globulin (CBG) and albumin transport circulating cortisol. Cleavage of high-affinity CBG (haCBG) by neutrophil elastase at inflammatory sites causes cortisol release into tissues, facilitating immunomodulatory effects.

Objective: To determine whether depletion of haCBG is related to mortality in septic shock.

Design: A single-center prospective observational cohort study of patients recruited with critical illness or septic shock, using serum samples collected at 0, 8, 24, 48 and 72 hours. Serum total and haCBG, and total and free cortisol were assayed directly. Glucocorticoid treatment was an exclusion criterion. Mortality was assessed at 28 days from Intensive Care Unit admission.

Results: Thirty septic shock (SS) and 42 nonseptic critical illness (CI) patients provided 195 serum samples. SS/CI patients had lower total CBG, haCBG and low-affinity CBG (laCBG) than controls. Total CBG and haCBG were significantly lower in septic shock patients who died than in those that survived ($P < 0.009$, $P = 0.021$, respectively). Total and free cortisol were higher in septic than nonseptic individuals. Free/total cortisol fractions were higher in those with low haCBG as observed in septic shock. However, cortisol levels were not associated with mortality. Albumin levels fell in sepsis but were not related to mortality.

Conclusions: Low circulating haCBG concentrations are associated with mortality in septic shock. These results are consistent with an important physiological role for haCBG in cortisol tissue delivery in septic shock.

KEYWORDS
CBG, corticosteroid-binding globulin, cortisol, mortality, septic shock

1 | INTRODUCTION

Corticosteroid-binding globulin (CBG) binds 80% of cortisol under basal conditions in a 1:1 molar ratio, with ~15% bound to albumin and ~5% circulating free. CBG increases the solubility of cortisol in serum, provides circulatory transport and buffers fluctuating cortisol levels.[1,2] During states of physiological stress, cortisol concentrations above 300-500 nmol/L saturate CBG, resulting in an exponential rise in free cortisol.[3-6]

Corticosteroid-binding globulin, first isolated in 1958,[3] is a 383 amino acid protein, and a member of the serine protease inhibitor (SERPIN) family.[5] In 1988, Pemberton et al[7] found that cleavage by neutrophil elastase (NE) causes a 90% reduction in CBG:cortisol-binding affinity. Cortisol binds to the uncleaved high-affinity CBG

(haCBG) form inside a surface pocket. NE cleavage of the exposed protease bait domain, located within the reactive centre loop (RCL), between Valine[344] and Threonine[345] of haCBG, produces an irreversible conformational change, with full insertion of the now mobile RCL into the body of the molecule, disrupting the cortisol-binding site, reducing cortisol:CBG affinity and releasing cortisol.[8,9] This conversion of haCBG to low-affinity CBG (laCBG) by NE-mediated proteolysis facilitates the targeted delivery of cortisol to sites of inflammation[7,10] increasing local tissue cortisol concentrations 3-4-fold.[8,9] In addition, pyrexia induces a partial reversible insertion of the RCL into the body of the molecule, liberating cortisol from haCBG, so that CBG acts as a thermocouple.[8,11]

Septic shock, a common and severe inflammatory condition, carries a 30% mortality rate despite antibiotics and vasopressor use, and accounts for 5% of health expenditure.[12,13] Septic shock represents a failure of the host's stress response, where organ failure mediated by overwhelming inflammation develops, despite the actions of cortisol and catecholamines to improve cardiovascular stability and reduce tissue damage. Administered hydrocortisone has a pressor effect and may be life saving in some cases, but total cortisol levels do not predict this response.[14]

We have utilised a monoclonal antibody (9G12) which binds an epitope encompassing the NE cleavage site within the RCL of CBG, that is, 9G12 immunoreactive CBG (9G12-IR-CBG) in vivo. 9G12-IR-CBG represents uncleaved haCBG. Contemporaneous measures of total CBG using a second monoclonal antibody (12G2) to a non-RCL epitope allows direct measurement of total CBG. The difference between 12G2 and 9G12 IR-CBG, that is 9G12 nonreactive (NR) CBG indirectly represents the concentration of cleaved CBG.[15,16] Circulating haCBG concentrations fall in proportion to categorical sepsis severity (sepsis/septic shock), with the lowest levels seen in septic shock.[17]

Overall, a combination of haCBG cleavage, cytokine-mediated inhibition of CBG synthesis, reduced cortisol metabolism[18] and pyrexia-induced attenuation of CBG:cortisol binding may all act to elevate serum and tissue free cortisol levels beyond that achieved with hypothalamic-pituitary-adrenal axis stimulation. Moreover, CBG cleavage, through facilitating tissue-specific cortisol delivery, may be crucial in protecting inflamed tissues from damage and the multiorgan failure that portends shock and death. These mechanisms act synergistically to life-saving effect in acute inflammation, but may fail, in part due to depletion of the haCBG reservoir beyond the first few days of illness. It is possible that a lack of attention to cortisol delivery mechanisms may underlie the contradictory results of "low dose" (~200 mg/d) hydrocortisone administration in sepsis trials.[14,19] The objective of this study was to determine whether low haCBG concentrations in septic shock are associated with mortality.

2 | SUBJECTS AND METHODS

2.1 | Study patients

Inclusion criteria were age ≥18 years and blood lactate level >3.0 mmol/L on Intensive Care Unit (ICU) admission. Exclusion criteria were as follows: glucocorticoid treatment in ICU, liver failure (prothrombin time >15 second, International Normalized Ratio >1.5 or hepatic encephalopathy), liver surgery prior to ICU admission, contraindication to central venous catheterization, epileptic seizures (grand mal, shortly before or during admission), an anaerobic cause of hyperlactatemia (at the discretion of the treating physician), or do-not-resuscitate status. Septic shock was defined by Sequential Organ Failure Assessment (SOFA) III criteria.[20] Mortality was defined as death within 28 days of ICU admission. This was a substudy of a trial of lactate-directed fluid resuscitation.[21] One hundred healthy controls were recruited from outpatient clinics and community advertisements.[22]

2.2 | Study design

This was a prospective observational cohort study conducted in an adult tertiary level ICU. Patients were recruited from the ICU of the Erasmus University Medical Center (Rotterdam, Netherlands) between 2006 and 2008. The protocol was approved by the Dutch Central Committee on Research Involving Human Subjects. Delayed consent and data collected from enrolled patients who had died prior to consent were approved for use. Patients were enrolled at the time of ICU admission (time 0) and blood samples collected at 0, 8, 24, 48 and 72 hours. Biochemical and clinical variables required for calculation of the Acute Physiology and Chronic Health Evaluation (APACHE) II and SOFA III scores were collected at 0, 8, 24, 48 and 72 hours. Blood lactate levels, total CBG, haCBG, laCBG, free cortisol and total cortisol were measured at 0, 2, 4, 6, 8, 24, 48 and 72 hours. Samples were stored frozen until analysis. Glasgow Coma Scale, heart rate, mean arterial pressure and urine output were recorded at 0, 2, 4, 6, 8, 24, 48 and 72 hours. The use of fluids, inotropes, vasopressors, vasodilators, antibiotics, blood products and surgical procedures was recorded over the 72-hour study duration. Patients were followed to the earliest of 72 hours, ICU discharge or death. Mechanical ventilation, vasopressor use, and 28-day mortality were recorded.

2.3 | Laboratory measurements

Serum CBG assay: Total CBG, haCBG and laCBG levels were measured using an in-house parallel monoclonal noncompetitive direct enzyme-linked immunosorbent assays.[23] Monoclonal antibody, 9G12 (RRID:AB_26632405), recognizing the epitope sequence STGVTLNL, which corresponds to the amino acids 341-348 of human CBG, incorporating the elastase cleavage site on the exposed RCL. NE treatment of CBG, with subsequent cleavage of the exposed RCL, results in disruption of the epitope sequence and loss of 9G12 binding. Hence, 9G12, by implication, recognizes intact haCBG so that haCBG and 9G12-IR-CBG are synonymous. A second monoclonal antibody, 12G2 (RRID:AB_2632404), recognizing a separate epitope away from the RCL, not affected by NE cleaved CBG, is used to measure total CBG. Cleaved laCBG is inferred by subtraction of haCBG levels from total CBG levels. Intra-assay coefficients

of variation were 2.9% and 2.3% for total and haCBG, respectively. 9G12 binding is unaffected by CBG glycosylation, maintaining signal following peptide-N-glycosylation at a partially utilized consensus site within the 9G12 epitope sequence, thus accurately quantifying haCBG in vivo.[15,23] Control data for this study were derived from 100 healthy volunteers.[22]

The capability of the 9G12 antibody to bind the RCL epitope and thereby specifically detect haCBG has been validated with digestion of human CBG with NE, where loss of the haCBG molecule on Western blot was observed in association with a >95% reduction in haCBG concentration.[15,16] In addition, our in vivo human studies demonstrated a negative correlation between haCBG concentration and free cortisol fraction (free cortisol/total cortisol %).[17]

Duplicate microplates were coated with a CBG polyclonal antibody and incubated for 12 hours at room temperature. Nonspecific binding was blocked using assay buffer. The standard was prepared by serially diluting commercially available recombinant CBG (Sino Biological Inc. Beijing, Cat no. 10 998-H08H). After blocking, 100 μL/well of standard or diluted serum was added. After incubation at room temperature for 90 minutes, plates were washed and incubated with 100 μL/well of 12G2 or 9G12 monoclonal antibody (12G2 binding total CBG, and 9G12 binding haCBG) and incubated for 60 minutes. After washing the plates, 100 μL/well of diluted peroxidase conjugated anti-mouse antibodies was added to the respective plates, which were incubated for 30 minutes, washed and 100 μL/well of tetramethylbenzidine substrate was added. Colour development was terminated with 100 μL/well 0.9 mol/L hydrochloric acid. Absorbance read at 450 nm and concentrations determined by interpolation from standard curves. Assays were performed in parallel with two controls to ensure validity.[17,23]

Serum free and total cortisol: Free cortisol fraction estimation was performed using a temperature-controlled ultrafiltration/ligand-binding method that has been validated against equilibrium dialysis.[24] Free cortisol was calculated from total cortisol concentration and free cortisol fraction.[25] Serum (500 μL) was equilibrated with [^{3}H]-cortisol (0.1 Microcurie, NET396 Perkin Elmer, Boston, MA, USA) for 60 minutes at 37°C. A 50 μL aliquot was removed for measuring total counts; the remaining 450 μL was centrifuged for 15 minutes at 18 407 × *g* in a preconditioned ultrafiltration device (VS0102, Sartorium Stedium Biotech, Goettingen, Germany). A 50 μL portion of the filtration as removed for measurement of free counts. Comparison of the radioactive counts of the free and total portions determined the percentage free cortisol. Radioactivity was counted in 6 mL of xylene-based scintillant (Irgasafe Plus, Perkin Elmer, Glen Waverly, Vic, Australia). Total cortisol was measured using a commercial electrochemiluminescence immunoassay on a Roche e601 analyzer (Roche Diagnostics, Castle Hill, NSW, Australia).

2.4 | Statistical analysis

Data was analysed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc., San Diego, CA). Unpaired Student's *t* tests were used to compare septic shock to critical illness groups. Pearson correlation coefficients with line of best fit were used to relate two continuous variables. Categorical variables were analysed using Chi-squared test. One-way analysis of variance (ANOVA) was used to compare continuous variables between the four illness categories. Repeated-measure ANOVA was used to compare variables over serial time points. Results are expressed as mean ± standard error mean unless otherwise stated. $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Patients

Seventy-two patients were studied; their characteristics are outlined in Table 1. Patients were categorized into nonsepsis critical illness survivors (CI-S), nonsepsis critical illness nonsurvivors (CI-D), septic shock survivors (SS-S) and septic shock nonsurvivors (SS-D). Thirty patients had septic shock (SS-S and SS-D) with a 50% 28-day mortality rate (SS-D, n = 15), of whom, the majority (n = 9, 60%), died within the 72-hour study. Forty-two had nonsepsis critical illness (CI-S and CI-D) such as multitrauma, postsurgical care and complications, neurological emergencies, hypovolemic shock, cardiogenic shock including cardiopulmonary arrest and hematologic malignancy. The 28-day mortality rate within the CI group was 38% (n = 16), of whom 62.5% (n = 10) died within the 72-hour study. One hundred and ninety-five blood samples were collected, the median number of samples per patient was two (range 1-5, mean 2.7). There was no difference in age or BMI ($P = 0.219$ and $P = 0.364$, respectively) across the four groups. Most patients were male 78% (M:F 56:16); however, the ratio of males to females did not differ between groups ($P = 0.213$) (Table 1). Eighteen CI patients were admitted to ICU postsurgery (elective n = 9, nonelective n = 9), with ten surgical procedures occurring during ICU admission. Three SS-S patients had preceding surgery (elective n = 1, nonelective n = 2) with six surgical procedures during ICU admission. Five SS-D patients had prior surgery, all nonelective, with a further five surgical procedures during ICU admission. The mean length of ICU stay was 8.4 days (range <1-39). There was no significant difference in APACHE II and SOFA III scores across the four groups ($P = 0.310$ and $P = 0.785$, respectively).

Mean albumin levels were lower in both sepsis groups (SS-S 19.0 ± 1.3 g/L and SS-D 18.3 ± 1.6 g/L) than in nonsepsis critical illness groups (CI-S 28 g/L and CI-D 25 g/L; $P < 0.001$); however, there was no significant difference in albumin concentrations between septic shock survivors and nonsurvivors (SS-D vs CI-S $P = 0.008$, vs CI-D $P = 0.020$) (SS-S vs CI-S $P = 0.002$, vs CI-D $P < 0.001$) ($F = 50.53$, $df = 3$, $P < 0.001$) (Table 1).

3.2 | Serum corticosteroid-binding globulin affinity forms

Mean total CBG, haCBG and laCBG concentrations were all significantly lower in the four acute severe illness groups than in

TABLE 1 Patient baseline characteristics

Group	Nonsepsis critical illness survivors	Nonsepsis critical illness non-survivors	Septic shock survivors	Septic shock nonsurvivors	*P* value
Participants (n)	26	16	15	15	-
Age, years mean (range)	56.6 (17-80)	59.7 (23-82)	57 (37-70)	66 (37-80)	0.219
Gender (M:F)	22:4	14:2	9:6	11:4	0.213
BMI (kg/m^2) mean (range)	26.5 (17.8-48-4)	26.9 (21.2-51.1)	24.5	24.0	0.364
APACHE II mean (range)	17.5 (5-31)	29.9 (23-42)	26.6 (10-45)	28.6 (21-39)	0.310
SOFA-III mean (range)	7.1 (2-14)	11.4 (4-17)	10.5 (7-14)	11.5 (6-16)	0.785
ICU length of stay (days) mean (range)	7 (<24 h-38)	6 (<24 h-24)	17 (1-34)	6.4 (< 1-26)	<0.001
Albumin at admission to ICU g/L mean (range)[a]	28 (11-47)	25 (13-46)	19 (8-27)	18 (7-32)	<0.001[a]
Surgery (n)					
Pre-ICU admission	15	3	3	5	
During ICU admission	7	3	6	5	
Main source of infection (n)					
Pulmonary	3	1	6	5	
Intra-abdominal	0	3	6	6	
Genitourinary	0	0	1	1	
Other	0	1	1	1	
Unknown	1	1	1	1	
Microbiology (n)					
Gram positive	1	2	4	6	
Gram negative	1	7	8	11	
Pseudomonas sp.	2	0	0	1	
Fungi	0	1	1	2	
Multiple	1	3	3	7	
Not identified	0	0	6	3	

APACHE II, Acute Physiology and Chronic Health Evaluation II; BMI, Body Mass Index; F, female; ICU, intensive care unit; M, male; N, number; SOFA III, Sequential Organ Failure Assessment Scoring III.
[a]$P < 0.001$.

healthy controls (SS-S $P < 0.0001$, SS-D $P < 0.001$, CI-S $P < 0.001$, CI-D $P < 0.001$) (Figure 1). Mean total CBG, haCBG and laCBG concentrations were lower in septic shock than critical illness (SS 230.7 ± 9.0, 171.4 ± 8.8, 59.5 ± 3.1; CI 294.1 ± 8.9, 195.2 ± 5.5, 100.0 ± 5.8 nmol/L; $P < 0.001$, $P = 0.018$, $P < 0.001$, respectively).

Total CBG levels were significantly lower in septic shock nonsurvivors compared to septic shock survivors ($P = 0.009$) (Time x group interaction $F = 44.02$, $df = 3$, $P < 0.001$) (Figure 1A). There was no significant difference in total CBG comparing survivors and nonsurvivors with nonseptic critical illness.

haCBG levels were lower in nonsurvivors than survivors of septic shock ($P = 0.009$) (Time x group interaction $F = 44.2$, $df = 3$, $P = 0.0003$) (Figure 1B). Within septic shock non-survivors total and haCBG levels appeared to reach a nadir at 24 hour, due to deaths (n = 9) among those with the lowest total and haCBG concentrations. In those who died within 72 hours of ICU admission, the last 9G12-IR-CBG measurement prior to death was significantly lower in septic shock nonsurvivors (mean + SEM, 121 + 22, n = 9) compared to critical illness nonsurvivors (mean + SEM, 213 + 23, n = 10) unpaired two-tailed *t* test, $P = 0.011$). Compared to septic shock survivors, nonsurvivors had a 34% lower haCBG overall, reflected in a 29% lower total CBG. LaCBG levels were not significantly different between survivors and nonsurvivors of septic shock ($P = 0.122$) (Figure 1C). Total and haCBG levels were highly correlated ($R = 0.83$, $P < 0.001$), as expected.

3.3 | Serum total and free cortisol concentrations

Serum total cortisol concentrations were significantly higher in septic shock compared to nonseptic critical illness individuals and cortisol rose as illness progressed. However, there was no difference in total cortisol between survivors and nonsurvivors (Figure 2A). Serum free cortisol was also significantly higher in septic shock compared to critical illness (Figure 2B). Free:total cortisol ratios (%) were higher in septic shock patients who died compared to those who survived, also those with the

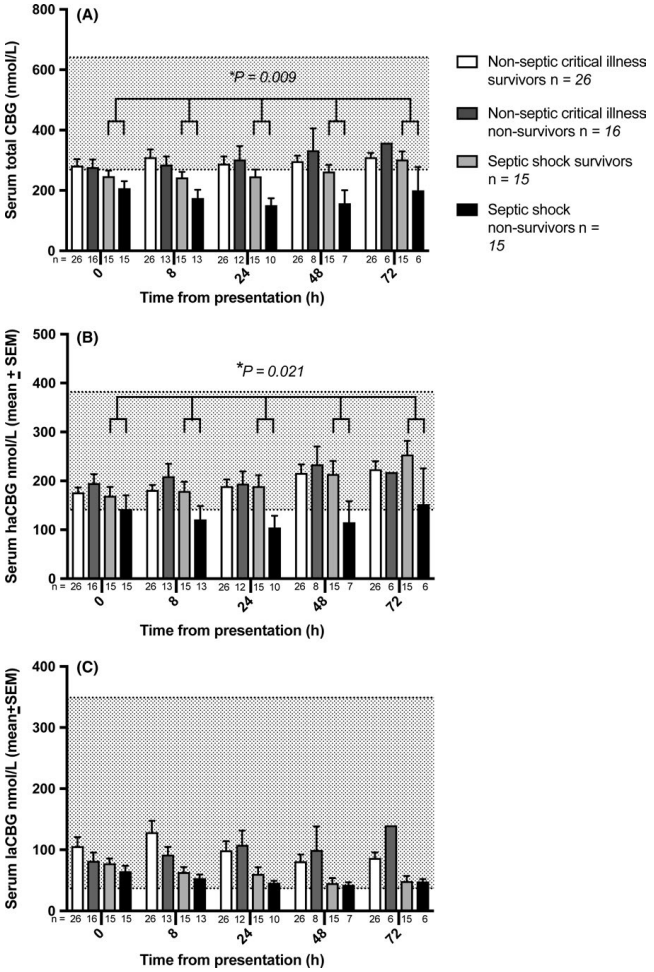


FIGURE 1 A, Sequential serum total corticosteroid-binding globulin (CBG) in nonseptic critical illness and septic shock survivors and nonsurvivors over 72 h. Total CBG was significantly lower in septic shock nonsurvivors (black bars) compared to septic shock survivors* (light grey bars, $P = 0.009$), critical illness survivors (white bars, $P = 0.002$) and critical illness nonsurvivors (dark grey bars, $P = 0.008$). ($F = 44.02$, $df = 3$, $P < 0.001$). B, Sequential serum haCBG in nonseptic critical illness and septic shock survivors and nonsurvivors over 72 h. HaCBG was lower in critical illness patients than in controls (shaded area). HaCBG was significantly lower in septic shock nonsurvivors (black bars) than in survivors* (light grey bars, $P = 0.021$), nonseptic critical illness survivors (white bars, $P = 0.012$) and nonseptic critical illness nonsurvivors (dark grey bars, $P = 0.006$). A survival relationship was not seen in nonseptic individuals. ($F = 26.11$, $df = 3$, $P < 0.001$). C, Sequential serum laCBG in nonseptic critical illness and septic shock in survivors and non-survivors over 72 h. LaCBG was significantly decreased in septic individuals, but laCBG levels were not different between septic shock survivors (light grey bars) and nonsurvivors (black bars). ($F = 12.57$, $df = 3$, $P = 0.012$). Data represented as mean ± standard error of the mean. Shaded background depicts normal serum total CBG, haCBG and laCBG, respectively, taken from 100 healthy controls.[22] The number (n) of patients alive at the various time points is indicated underneath *x*-axis

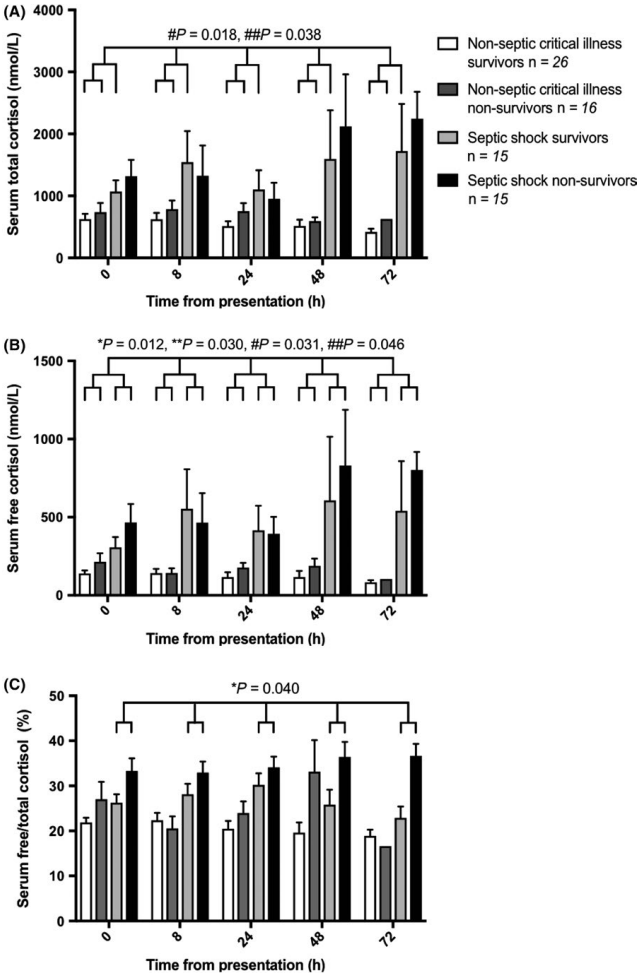


FIGURE 2 A, Sequential serum total cortisol concentrations in nonseptic critical illness and septic shock in survivors and nonsurvivors over 72 h. Higher total cortisol levels were seen in septic vs nonseptic critical illness (SS-S vs CI-S $^{\#}P$ = 0.018, vs CI-D $^{\#\#}P$ = 0.038), patients but cortisol levels were not associated with mortality in the critical illness or septic groups. (F = 13.68, df = 3, P = 0.015). B, Sequential serum free cortisol concentrations in nonseptic critical illness and septic shock in survivors and nonsurvivors over 72 h. Free cortisol was higher in septic compared to nonseptic groups (SS-S vs CI-S $^{*}P$ = 0.012, vs CI-D $^{**}P$ = 0.030) (SS-D vs CI-S $^{\#}P$ = 0.031, vs $^{\#\#}P$ = 0.046). Free cortisol did not associate with mortality in either the critical illness or septic shock groups. (F = 19.53, df = 3, P = 0.004). C, Sequential serum free cortisol as a percent (%) of total cortisol in nonseptic critical illness and septic shock in survivors and nonsurvivors over 72 h. Free/total cortisol % was significantly higher in SS-D* (black bars) compared to SS-S (light grey bars), reflecting the lower total and ha CBG (P = 0.040). (F = 13.71, df = 3, P = 0.005). Data represented as mean ± standard error of the mean

lowest haCBG (P = 0.04) (Figure 2C). There was a significant correlation between total CBG and free cortisol (R = −0.220, P = 0.003). Free cortisol negatively correlated with total CBG (P = 0.003) and a trend was seen for haCBG (P = 0.077) (Figure 3). Serum albumin did not correlate with free cortisol (R = −0.118, P = 0.126).

4 | DISCUSSION

This is the first study showing that total CBG and haCBG depletions are associated with mortality in human septic shock. Serum total CBG and haCBG levels were significantly lower in septic shock nonsurvivors compared to septic shock survivors (by 29% and 34%, respectively). haCBG, represented by 9G12-IR-CBG, is of particular interest due to its role in tissue cortisol delivery. Among nonsepsis critical illness patients, haCBG levels were lower than controls but not as low as those with septic shock. From this observation, we propose that depletion of haCBG may impair cortisol transport to inflammatory sites, contributing to organ failure and death seen in septic shock.

The results are consistent with in vitro evidence of haCBG cleavage by NE and cytokine-modulated inhibition of hepatic CBG synthesis.[26] Lower laCBG concentrations in septic shock compared to nonseptic critical illness may reflect a relative lack of haCBG as substrate for cleavage. LaCBG concentrations for all four groups fall within the normal reference range, albeit the lower reference range for septic shock groups, this may reflect altered clearance in the

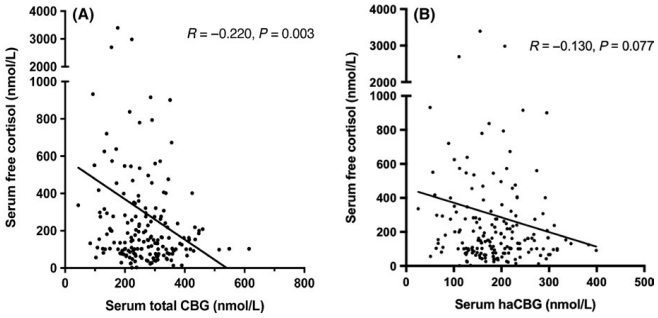


FIGURE 3 A, Correlation between total corticosteroid-binding globulin (CBG) and free cortisol concentration with line of best fit. For correlation $R = -0.220$, $P = 0.003$. B, Correlation between haCBG and free cortisol with line of best fit. For correlation $R = 0.130$, $P = 0.077$, n = 187

setting of end-organ dysfunction; however, the mechanism of CBG metabolism is unknown.

HaCBG reached a nadir around 24-48 hours in septic shock nonsurvivors, following the early deaths, within 72 hours of ICU admission (n = 9), in those with the lowest haCBG. In contrast, among septic shock survivors, 9G12-IR-CBG progressively increased over 72 hours, suggesting an abatement of CBG cleavage due to falling tissue NE levels in those in recovery.

Serum albumin was reduced in septic shock compared to nonsepsis critical illness; however, albumin was not associated with septic shock mortality. Albumin remained unchanged over the 72-hour study duration in septic shock groups. In septic shock, there is a loss of serum albumin from an increase in transcapillary loss of up to 300% due to cytokine and vasoactive peptide-mediated endothelial dysfunction and capillary leakage[27] Albumin synthesis is also inhibited by proinflammatory cytokines.[28,29]

Total and free cortisol was significantly higher in septic shock than in nonseptic critical illness. However, neither total nor free cortisol concentrations were higher in the groups that died. In septic shock, the free:total cortisol ratio was significantly increased in those who died, reflecting the lower, 9G12-IR-CBG, haCBG. Basal cortisol values in sepsis and septic shock are not useful predictors of mortality except at extremely high cortisol levels (>1242 nmol/L)[30] and free cortisol correlates more closely with sepsis severity than total cortisol,[24] consistent with our findings.

The use of exogenous glucocorticoids to treat septic shock remains in contention, with guidelines leaving administration to the discretion of the treating intensivist.[31] Septic shock patients may exhibit a reduced time-to-vasopressor withdrawal (shock reversal) with hydrocortisone, possibly indicating relative insufficiency of the hypothalamic-pituitary-adrenal axis.[19,32] High-dose, short-course glucocorticoids, equivalent to 40 000 mg of hydrocortisone per day, in septic shock increased mortality,[33] while lower glucocorticoid doses, equivalent to 200-300 mg of hydrocortisone per day, over a longer duration of up to 7 days, reduced time-to-vasopressor withdrawal and in some cases mortality, with a tendency for those with lower cortisol response to cosyntropin to show the most benefit[32]. In 2018, two large randomized control studies of hydrocortisone in septic shock were published.[34,35] In one study, hydrocortisone 200 mg per day did not reduce 90-day mortality; however, there was improved time to shock resolution, and reduced length of time to discharge from both intensive care and hospital.[35] Another study did find reduced mortality and reduced vasopressor use in patients who received hydrocortisone and fludrocortisone.[34] The second study involved a patient group with a higher mortality rate: 43% hydrocortisone/fludrocortisone and 49% placebo vs 28% hydrocortisone and 29% placebo in the first study. Hence, the risk:benefit ratio of hydrocortisone use in septic shock remains contentious and it may be that determination of individuals with altered cortisol physiology, particularly cortisol tissue delivery as represented by CBG cleavage could inform future trials of hydrocortisone administration.

Limitations of this study include a relatively low number of positive blood cultures to allow cross-organism comparisons and a design that did not allow analysis of pyrexia effect on free cortisol concentrations, although we have examined this phenomenon in early pyrexia after TNF-α administration.[36,37] Important mechanistic questions about the CBG cleavage phenomenon remain including the influence of CBG genetics, known to influence cleavage and a potential host factor in septic shock, as well as the effect of glycosylation on RCL function.[38,39] We have not related our measures of CBG cleavage to organism in the sepsis patients, but our previous study of patients referred for blood culture found that organism or blood culture positivity (generally around 50% or less in septic shock) did not relate to extent of cleavage.[37] Multiple factors contribute to mortality in the critically ill, including comorbid conditions and primary diagnoses; those with nonseptic critical illness were a heterogenous group. A multivariate analysis has not been undertaken, and this is subject to further research. 9G12 immunoreactive CBG was designed to represent intact CBG based on its ability to recognize the epitope within the RCL, 9G12 nonreactive CBG is not directly measured by our assay with in vitro evidence that NE treated CBG lose 9G12 reactivity implying cleavage.[23] Further in vivo studies are underway to determine the nature of 9G12 nonreactive CBG to elucidate whether this does represent laCBG or another biological variant.

In summary, this is the first study showing that haCBG depletion is associated with mortality in septic shock in humans. Septic

shock and nonseptic critical illness patients have lower haCBG than controls. In septic shock, serum total CBG and haCBG levels were profoundly low in nonsurvivors compared to survivors. These findings raise the possibility that low haCBG, as represented by 9G12-IR-CBG, leads to ineffective targeted cortisol delivery which may be crucial for protecting tissues and averting multiorgan failure, the *sine qua non* of septic shock. Total CBG and/or haCBG may prove a useful predictor of mortality in septic shock and also identify a subset of patients who would potentially benefit from adjunctive hydrocortisone therapy. haCBG replacement therapy in human septic shock potentially may restore normal cortisol transport, potentially obviating multiorgan failure and mortality.

CONFLICT OF INTEREST

The authors report no conflict of interest in this work.

AUTHORS' CONTRIBUTIONS

EM performed data and statistical analysis, and prepared the manuscript. MN undertook laboratory measurements of CBG and cortisol, and reviewed the manuscript. WR and JL reviewed the manuscript. LK, MS, TJ, JB, JH, RF undertook sample and data collection, and reviewed the manuscript. DT contributed to and reviewed the manuscript.

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CHAPTER 5:

CORTICOSTEROID-BINDING GLOBULIN DEFICIENCY INDEPENDENTLY PREDICTS MORTALITY IN SEPTIC SHOCK.

CHAPTER OUTLINE

Septic shock represents a state of dysregulated excessive inflammation in response to infection, with consequent hypotension and damage to healthy tissues resulting in multi-organ failure and death. Sepsis and septic shock are the leading causes of death worldwide and there have been no therapeutic advances in over 20 years.[1]

Cortisol has important immunomodulatory properties, acting through glucocorticoid receptor-α (GRα), altering gene transcription, and activating secondary messenger pathways to suppress pro-inflammatory intracellular pathways induced by transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) **(Chapter 2, Figure 2.1)**. Corticosteroid-binding globulin (CBG) has a highly specialised cortisol tissue delivery role in sepsis, with pyrexia and acidosis reversibly reducing cortisol binding affinity and tissue neutrophil elastase cleaving high cortisol binding affinity CBG, releasing cortisol at inflammatory sites **(Chapter 3, Meyer *et al*., 2020)**. Our previous small studies have shown serum CBG concentrations fall substantially in proportion to sepsis severity[2] with association between CBG and mortality **(Chapter 4, Meyer *et al.,* 2019)**.

Our primary hypothesis was that plasma CBG concentrations may represent an independent risk factor for Intensive Care Unit mortality in septic shock. Our aim was to assess a large cohort of septic shock patients to determine if CBG represents a risk factor for mortality and disease progression events.

This manuscript represents a 3-year single centre study aimed at addressing the potentially deleterious role of CBG deficiency in septic shock, involving 135 consecutive patients.

The findings are that CBG concentrations below the 30th percentile (<200 nmol/L) for septic shock patients is independently associated with mortality and represent the only directly remediable risk factor for death among a range of otherwise known mortality risks. Low CBG was also associated with a range of other indicators of disease severity, norepinephrine requirements and cardiac dysfunction. CBG was here associated with disease severity markers biochemical factors known to influence CBG and cortisol action (α-1 antitrypsin and

inflammatory cytokines). This accords with CBG's role in targeted delivery of immunomodulatory cortisol to tissues to quell systemic inflammatory processes. Furthermore, the lack of association between serum cortisol levels and outcomes are in keeping with previous studies[3,4] and support the notion that CBG is not simply a proxy for cortisol and are consistent with CBG's role in targeted cortisol-tissue delivery.

The findings are entirely novel and are relevant in view of the difficulty in finding new therapies for septic shock after several decades, the largely equivocal studies published in NEJM in 2018[5,6] of hydrocortisone therapy and the ongoing equipoise regarding this treatment in Intensive Care Units internationally.

Given the 3.2-fold increase in mortality, replenishing CBG to levels >200 nmol/L may have a large therapeutic effect in the treatment of septic shock avoiding the multiorgan failure cascade that accompanies severe illness and death.

The manuscript opens the path for therapeutic trials of CBG therapy in septic shock.

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STATEMENT OF AUTHORSHIP

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Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	2/2/22

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By signing the Statement of Authorship, each author certifies that:

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Corticosteroid-Binding Globulin Deficiency Independently Predicts Mortality in Septic Shock

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Abstract

Context: Hydrocortisone administration in septic shock remains controversial. Corticosteroid-binding globulin (CBG) transports cortisol to inflammatory sites and is depleted in septic shock.

Objective: To determine whether severely deficient serum CBG < 200 nmol/L (reference range 269-641 nmol/L) independently predicts septic shock mortality.

Methods: A prospective observational study in patients with septic shock. Patients were categorized into 2 groups: mean plasma CBG concentrations <200 nmol/L and ≥200 nmol/L (day 1/2), with additional categorization by nadir CBG. Primary outcome was intensive care unit (ICU) mortality. Secondary outcomes were 28- and 90-day mortality, norepinephrine requirements, renal replacement therapy, and clinician-instituted hydrocortisone.

Results: 135 patients were included. Mortality rates in ICU were higher in the CBG < 200 nmol/L vs the CBG ≥ 200 nmol/L group: 32.4% vs 13.9% [odds ratio (OR) 2.97 (95% CI 1.19, 7.41); $P = 0.02$] with 28-day mortality OR 2.25 (95% CI 0.99, 5.11) and 90-day mortality OR 2.21 (95% CI 0.99, 4.91). Multivariate analysis revealed 4 factors independently associated with ICU mortality: CBG < 200 nmol/L (adjusted OR 3.23, 95% CI 1.06, 9.88), Acute Physiology and Chronic Health Evaluation II > 25 (adjusted OR 3.58, 95% CI 1.20, 10.68), Sequential Organ Failure Assessment (SOFA) liver score (adjusted OR 1.98, 95% CI 1.04, 3.72), and renal replacement therapy (adjusted OR 6.59, 95% CI 2.17, 20.01). Nadir CBG levels were associated with higher SOFA cardiovascular scores and norepinephrine total dose (μg; $P < 0.01$) and duration (days; $P < 0.01$). Plasma cortisol concentrations and hydrocortisone administration did not relate to ICU mortality.

Conclusion: Septic shock patients with CBG < 200 nmol/L had higher norepinephrine requirements and 3.2-fold higher ICU mortality. CBG concentration was the only directly reversible independent mortality risk factor.

Key Words: corticosteroid-binding globulin, CBG, cortisol, septic shock, sepsis, mortality

Septic shock is characterized by life-threatening organ dysfunction due to a dysregulated host response to systemic infection, defined empirically by persistent hypotension requiring vasopressors to maintain a mean arterial pressure > 65 mmHg despite fluid resuscitation (1). Septic shock is common, mortality is 30% to 40%, and new treatments beyond fluids, antimicrobials, vasopressors, and support for organ failure have not emerged in decades. Septic shock and other critical illnesses are profound physiological stressors, activating the hypothalamic-pituitary-adrenal (HPA) axis. Critical illness-related corticosteroid insufficiency is conceptually defined as an impaired cortisol secretory response, blunting life-sustaining effects on immunomodulation and vascular tone. Critical illness-related corticosteroid insufficiency diagnosis was based on cortisol increment < 248 nmol/L after 250 mcg cosyntropin (adrenocorticotropic hormone 1-24) or random cortisol < 276 nmol/L; however, recent consensus is that measuring cortisol does not assist in determining hydrocortisone administration (2, 3).

Hydrocortisone has a pressor effect in septic shock, and large randomized controlled trials of low dose hydrocortisone (200mg daily) revealed a reduced time-to-withdrawal of vasopressor support (by 1-2 days) and a small mortality benefit in sicker patients (2, 3). At present, low-dose hydrocortisone is indicated in septic shock patients with hypotension, despite fluid resuscitation and vasopressor therapy, based on clinical judgment (4).

Septic shock represents a state of uncontrolled inflammation with consequent hypotension and damage to healthy tissues resulting in multiorgan failure and death. Cortisol has important immunomodulatory properties, initially enhancing the innate immune responses to short-term minor insults, followed by anti-inflammatory actions. Cortisol acts through glucocorticoid receptor-α, altering gene transcription

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and activating secondary messenger pathways to suppress proinflammatory intracellular pathways induced by transcription factors nuclear factor kappa B and activator protein 1. Hence, cortisol targets inflammatory cytokine production, neutrophil migration, T-cell apoptosis, and macrophage inhibition (5).

Approximately 80% of blood cortisol is transported by corticosteroid-binding globulin (CBG) under basal conditions, while the remaining 10% to 15% is bound to albumin and 5% circulates as free cortisol. CBG, a 383 amino acid glycoprotein, binds cortisol in a 1:1 ratio (6). CBG has a cortisol tissue delivery role in sepsis, with pyrexia and acidosis reversibly reducing cortisol binding affinity and tissue neutrophil elastase cleaving high cortisol binding affinity CBG, irreversibly converting CBG to a low affinity state thereby releasing cortisol at inflammatory sites (Fig. 1) (7-10). Cortisol:CBG affinity is reduced 9-fold upon neutrophil elastase cleavage, by 3.5-fold at 39°C relative to 37°C with further reductions occurring due to acidosis (7, 9). Thermo-coupling mechanism of cortisol release is mediated through the destabilization of Trp371, a tryptophan residue located within the cortisol-binding pocket (9). Neutrophil elastase is inhibited by alpha-1 antitrypsin (α1AT) (11). Inflammatory cytokines tumor necrosis factor alpha (TNFα), interleukin 1l, and interleukin 6 (IL-6) reduce CBG synthesis by inhibiting hepatocyte CBG messenger RNA production (12-14), and serum CBG has been observed to decline by up to 50% in humans within 24 hours following IL-6 administration (12) (Fig. 2).

Our previous studies showed that serum CBG concentrations fall substantially in proportion to sepsis severity (15), and a small retrospective pilot study showed an association between CBG and critical illness mortality (16). CBG concentrations fall progressively during sepsis, so CBG nadir may be relevant to septic shock–related organ failures and mortality. Approximately one third of septic shock patients have admission CBG concentrations below 200 nmol/L (refence range 269.1-641.3 nmol/L) (15-17). In septic shock survivors, CBG concentrations recover within days (15). These studies lacked the power to show that CBG was an independent risk factor for mortality. If low plasma CBG is an independent mortality risk factor for septic shock death, its replacement is potentially feasible.

Our primary hypothesis was that plasma CBG concentrations may represent an independent risk factor for intensive care unit (ICU) mortality in septic shock.

Research Design and Methods

Study Design and Oversight

The study was an investigator-initiated and led, single center, observational study investigating the relationship between plasma CBG concentrations and clinical outcomes in septic shock. The study was conducted at the Royal Adelaide Hospital, Australia, within the ICU with study participants being recruited consecutively. Data were collected by the lead investigator and trained research staff and recorded in the web-based Research Electronic Data Capture (REDCap) software (version 11.0.3, Nashville, TN, USA). All aspects of patients' care were conducted at the discretion of the treating clinicians.

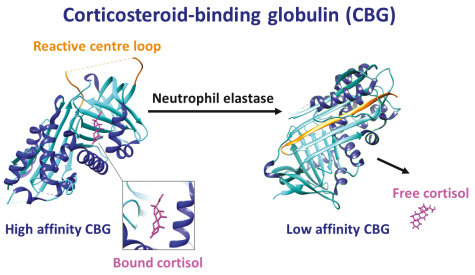


Figure 1. Cleavage of human corticosteroid-binding globulin (CBG) by neutrophil elastase liberating free cortisol. Crystal structure of human CBG (PBD ID 2VDY, 2.3 Å) undergoing conformational change from high affinity CBG to low affinity CBG after cleavage of the reactive center loop (RCL; yellow) by neutrophil elastase and subsequent insertion of the RCL into the body of the molecule releasing bound cortisol.

A convenience sample size of 140 participants was chosen, 2 years of local single-center recruitment. This was based on evidence from 2 previous pilot studies (1) indicating that plasma CBG is reduced in sepsis in relation to categorical illness severity (controls, sepsis, septic shock) (15, 18) and (2) suggesting an association between low CBG and septic shock mortality in 30 patients (16).

Patients

Eligible patients were ≥18 years and were receiving intravenous (IV) norepinephrine for septic shock in the ICU. Septic shock was defined as the presence of a clinically or microbiologically documented infection; ≥2 points on the Sequential [sepsis-related] Organ Failure Assessment (SOFA) score; receiving norepinephrine for at least 6 hours to maintain a mean arterial pressure > 65 mmHg (1). Exclusion criteria included pregnancy; expectation of death within 24 hours; receipt of norepinephrine infusions for >48 hours; known conditions that alter cortisol secretion, including the use of systemic glucocorticoids for ≥3 months and disorders of the HPA axis; or the employment of systemic glucocorticoids within the ICU for indications other than septic shock. Etomidate was not used.

Study Procedures

Patients were consecutively recruited. Blood samples were drawn at enrollment and on subsequent mornings until septic shock recovery (defined as the cessation of norepinephrine for 6 consecutive hours) or death, up to a maximum of 7 days. The management of norepinephrine infusions was protocolized [Supplemental Table 1 (19)]. IV hydrocortisone was administered at a dose of 50 mg every 6 hours in patients with hypotension unresponsive to fluids and vasopressors at the discretion of the treating clinicians. Demographic, clinical, and mortality data out to 90 days were collected.

Measurements

Plasma CBG concentrations were measured by enzyme-linked immunosorbent assay using a polyclonal rabbit and monoclonal mouse antibodies 12G2 (RRID:AB_2632404; https://scicrunch.org/resources/Antibodies/search?l=&q=RRID%3AAB_2632404) (20). C-reactive protein (CRP), albumin, lactate, and adrenocorticotrophic

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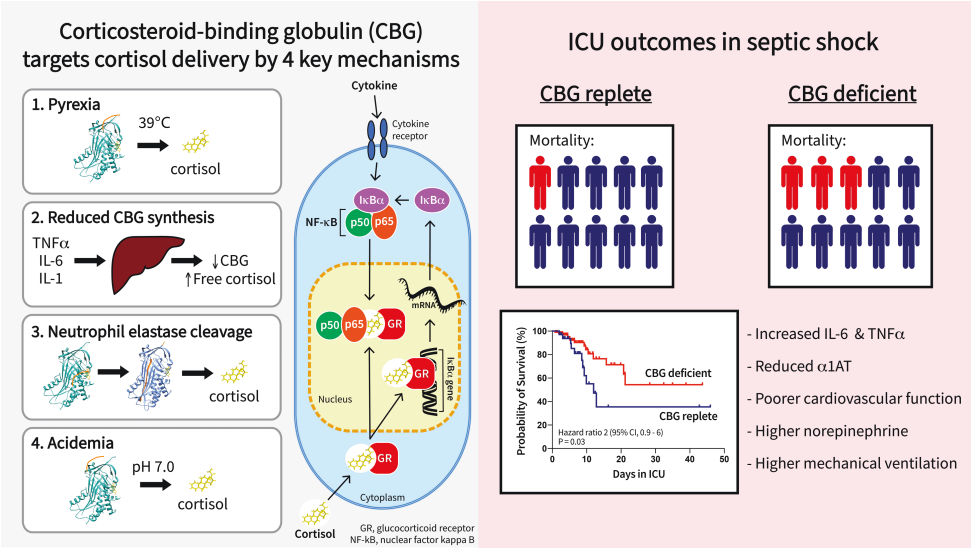


Figure 2. Graphical abstract—4-phase actions of corticosteroid-binding globulin (CBG) in delivering cortisol to sites of inflammation and outcomes of CBG deficiency in septic shock. Left panel: CBG's role in targeted delivery of immunomodulatory cortisol to tissues to quell inflammatory processes. CBG targets cortisol delivery via 4 key mechanisms: (1) CBG releases cortisol in pyrexia independent of cleavage; (2) inflammatory cytokines inhibit CBG messenger RNA production in hepatocytes, and reduction in CBG availability leads to a rise in serum free cortisol; (3) neutrophil elastase cleaves CBG releasing cortisol at inflamed sites; and (4) CBG releases cortisol in acidosis independent of cleavage. Right panel: Septic shock patients with a deficiency of serum CBG < 200 nmol/L had cardiac dysfunction, higher norepinephrine and mechanical ventilation requirements, and 3.2-fold higher intensive care unit mortality. Biochemical factors known to influence CBG levels, alpha-1-antitrypsin, and inflammatory cytokines (interleukin 6 and tumor necrosis factor alpha) were shown to directly correlate with serum CBG concentrations in septic shock.

hormone (ACTH) concentrations were assayed on Roche Cobas autoanalyzers, and total white cell, monocyte, and lymphocyte counts were assayed on Sysmex XN analyzers. Total serum cortisol was assayed by competitive enzyme-linked immunosorbent assay (IBL America, Minneapolis, MN, USA) with free cortisol fraction determined after ultracentrifugation, as previously described (18). Mean day 1/2 total and free cortisol values were used given low diurnal variation in serum secretion of cortisol in septic shock (21).

Outcomes

The primary outcome was ICU mortality in relation to admission (day 1/2) plasma CBG concentration. Secondary outcomes were all cause-mortality at 28 and 90 days; ICU admission duration in survivors; norepinephrine total dose (μg) and duration (days) (in all participants and ICU survivors); mechanical ventilation; and use of renal replacement therapy. Physiological agents known to effect CBG concentrations were assessed: IL-6, TNFα, and α1AT.

Plasma CBG Concentrations

The use of day 1/day 2 mean CBG was to reduce any effect of fluid resuscitation; however, there was no significant difference between these days in CBG (day 1: 276 ± 9.7 vs day 2: 269 ± 9.3, P = ns). Where day 1's value was missing (n = 7) the other day's concentration was used as the mean value.

Mean CBG in health is 455.3 nmol/L (95% CI 436.7, 473.6 nmol/L; reference range is 269.1-641.3 nmol/L, mean ± 2 SD) (Fig. 3) (17). Previous pilot studies showed higher mortality in patients with CBG < 200 nmol/L (15, 16). A CBG level of 200 nmol/L falls approximately 3 SDs below the healthy range median. For analysis, baseline CBG was dichotomized into values ≥200 and <200 nmol/L. An accompanying pathologically low CBG during illness was defined as nadir < 150 nmol/l, in accord with a mean decrement in CBG (day 1/day 2 mean minus lowest value) of 53 ± 5.5 nmol/L.

Statistics

The primary outcome is presented as the odds ratio (OR) for ICU mortality in patients with low CBG, with corresponding 95% CIs derived from a logistic-regression model, and as a hazard ratio for ICU survival, with corresponding 95% CI and P-values. Odds for ICU mortality were further examined by including covariates (age, sex, glucocorticoid administration, cortisol category; baseline SOFA score; Acute Physiology and Chronic Health Evaluation II (APACHE II) score, renal replacement therapy, ventilation, total dose of norepinephrine (μg), average dose of norepinephrine per day (μg/24 hours), CBG category, free cortisol, SOFA scores (liver [recoded into 3 categories (0, 1, 2) due to sparse data in the higher categories], coagulation, respiratory, and central nervous system), and surgical admission category (defined as patients being admitted to ICU from the operating room or from recovery) into the logistic regression model. Odds for ventilation were analyzed using the same covariates, excepting SOFA scores, in a logistic regression model.

Statistical significance of the primary outcome was determined by a 2-tailed P-value < 0.05. Effect size estimates and

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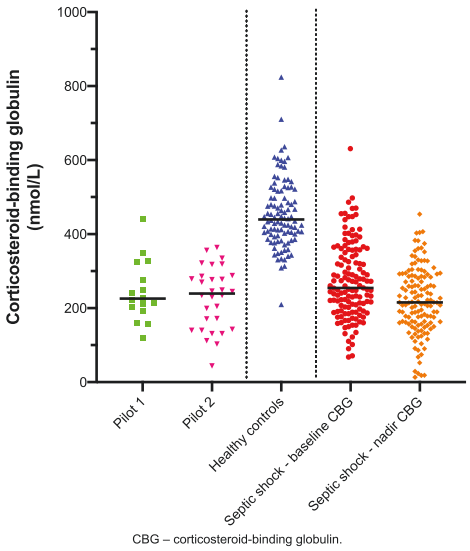


Figure 3. Distribution of total corticosteroid-binding globulin (CBG) in septic shock and healthy controls. Pilot 1 comprises 16 septic shock patients (15); Pilot 2, 30 septic shock patients (16); and healthy controls, 100 . Mean 455.3 (95% CI 436.7, 473.6) nmol/L, reference interval (269.1-641.3 nmol/L) ± 2 SDs (17). For septic shock cohort (n = 135), both CBG mean of days 1 and 2 and nadir during septic shock are represented. Scatter dot plots with median represented by horizontal line.

95% CI for the secondary outcomes are reported without *P*-values. These intervals are not adjusted for multiple comparisons and should be interpreted accordingly. Analysis of physiological associations with CBG have significance set at a *P*-value < 0.05, with no adjustment for multiple testing. Categorical variables were compared using the Chi-squared test, and continuous variables were assessed using the Pearson correlation coefficients and linear regression analysis. Group comparisons for continuous variables were undertaken using *t* tests or analysis of variance, with appropriate adjustments for inequality of variances.

Data are reported as percentages, mean ± SE, or median (interquartile range). Log_{10} transformation of continuous variables was used to normalize the distribution where appropriate. Analyses were conducted using IBM SPSS Statistics for Macintosh, version 27.0 (IBM, Armonk, NY, USA) and GraphPad Prism, version 9.0.0 for Macintosh (GraphPad Software, San Diego, CA, USA).

Study Approval

The Central Adelaide Local Health Network Human Research Ethics Committee approved the study protocol and amendments (Protocol no. R20160208 HREC/16/RAH/29). Participants or their legally authorized next-of-kin were provided with written informed consent. When death occurred after enrollment and prior to consent, participants were included due to the study's low-risk nature. As the study did not involve therapeutic intervention, it did not require registration with the Australian New Zealand Clinical Trials Registry.

Results

Patients

From November 2016 through March 2020, we identified 364 eligible patients, of whom 224 met an exclusion criterion and 140 met the inclusion criteria and were enrolled from the Royal Adelaide Hospital ICU [Supplemental Figure 1 (19)]. A major exclusion criterion was physician-determined likelihood of death within 24 hours (n = 22). Of 140 patients enrolled, 135 were included in the analysis as 4 were voluntarily withdrawn from the study by next-of-kin and 1 by investigators through identification of exclusion criteria. Plasma CBG concentrations of the remaining 135 patients were low relative to healthy controls (Fig. 3) with 89/135 (65.9%) below the reference range. Baseline CBG < 200 nmol/L was found in 34 (25.2%) patients. A nadir CBG < 150 nmol/L was identified in 27 (20.0%) patients.

Patient characteristics were similar at baseline for the CBG groups < 200 nmol/L and ≥ 200 nmol/L (Table 1). Key characteristics in these respective CBG groups were age (mean + SD) of 62.5 + 15.4 years and 63.4 + 13.9 years; male sex 44.1% and 36.6%; body mass index 27.5 + 7.2 and 31.6 + 10.4 kg/m^2; prevalent diabetes 20.6% and 37.6%; APACHE II scores 22.2 + 6.6 and 21.8 + 7.4; and admission SOFA scores 11.7 + 3.2 and 10.1 + 2.8 ($P = 0.01$). Median time to septic shock onset was 5 hours from hospital admission and 0 hours from ICU admission for the cohort.

Physiological variables were similar at baseline for the 2 CBG groups for ACTH, CRP, lactate, and white cell count (Table 1). The median lactate levels were 4.0 (IQR 2.3-5.1) and 3.9 (IQR 1.7-5.2) mmol/L, respectively and median white cell count 19.2 (IQR 7.5-24.3) and 17.8 (IQR 10.9-22.8) cells × 10^{-9}/L, respectively.

The mean number of infections per patient was 2, including nosocomial infections. Primary sites of infection were pulmonary in 28.1%, abdominal in 20%, bacteremia in 17%, soft tissue in 19%, urosepsis in 9%, sepsis of unknown origin in 8%, and septic arthritis or osteomyelitis in 6%. Infective organisms were bacterial (gram-negative n = 110, gram positive n = 77), viral (n = 20), and fungal (n = 20) (Table 2). There was no significant difference in septic shock therapy between the CBG groups. Hydrocortisone therapy were similar for the CBG groups, prescribed in 55.9% and 41.2%, respectively (Table 1). Additional inotropes or vasopressors included vasopressin (16.3%), epinephrine (8.9%), dobutamine (3.0%), and milrinone (1.5%). Surgical procedures during admission between the high and low CBG groups were 61.8% and 40.6%, respectively. A total of 6 patents had liver cirrhosis (low CBG group n = 3, high CBG group n = 3), of whom 3 had associated liver failure (low CBG group n = 2, high CBG group n = 1).

Outcomes

Primary Outcome—ICU Mortality

Among patients with a baseline CBG level < 200 nmol/L, 32.4% (11/34) died in ICU, compared to 13.9% (14/101) with a CBG ≥ 200 nmol/L (OR 2.97, 95% CI 1.19, 7.41; $P = 0.02$) (Table 1). Time-to-event analysis for ICU admission and baseline CBG was significant [log-rank (Mantel-Cox) $P = 0.03$, hazard ratio 2.0; 95% CI 0.9, 6.0] (Fig. 4). Median survival of patients with baseline CBG < 200 nmol/L category was 12.0 (95% CI 8.4, 15.6) days. Time-to-event analysis of CBG nadir was also significant [log-rank

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Table 1. Baseline characteristics and outcomes of the patients

	CBG < 200[a] (n = 34)	CBG ≥ 200[a] (n = 101)	Odds ratio[b] or mean difference (95% CI)	*P*-value
Patient characteristic				
Age, years	62.5 ± 15.4	63.4 ± 13.9	–	0.77
Male sex	15/34 (44.1)	37/101 (36.6)	–	0.54
BMI (n = 101)	27.5 ± 7.2	31.6 ± 10.4	–	0.06
Diabetes	7 (20.6)	38 (37.6)	–	0.09
Immunocompromised	2/33 (6.1)	16/94 (17.0)	–	0.15
APACHE II score[c]	22.2 ± 6.6	21.8 ± 7.4	–	0.78
Total SOFA score[d]	11.7 ± 3.2	10.1 ± 2.8	–	0.01
Admission type[e]	10/24 (29.4)	16/85 (15.8)	–	0.13
Hydrocortisone use[f]	19/34 (55.9)	42/101 (41.2)	–	0.17
Physiological variables[g]				
ACTH, pmol/L (n = 124)	4.3 ± 6.3	6.7 ± 13.6	–	0.20
CRP, mg/L (n = 125)	215.0 ± 116.2	218.2 ± 108.3	–	0.89
Lactate, mmol/L (n = 131)	4.0 (2.3-5.1)	3.9 (1.7-5.2)	–	0.67
White cell count, cells × 10^{-9}/L	19.2 (7.5-24.3)	17.8 (10.9-22.8)	–	0.85
Primary outcome				
ICU mortality	11/34 (32.4)	14/101 (13.9)	2.97 (1.19, 7.41)	0.02
Secondary outcome				
28-day mortality	14/34 (41.2)	24/101 (23.8)	2.25 (0.99, 5.11)	0.05
90-day mortality	16/34 (47.1)	29/101 (28.7)	2.21 (0.99, 4.91)	0.05
ICU mortality associated in patients receiving hydrocortisone[h]	8/19 (42.1)	11/42 (26.2)	2.05 (0.66, 6.42)	0.22
ICU-free days at day 28	12.2 ± 12.7	16.6 ± 10.4	−4.41 (−9.26, 0.43)	0.07
Mechanical ventilation	27 (79.4)	51 (50.5)	3.78 (1.51, 9.47)	<0.01
Renal replacement therapy	12 (35.3)	27 (26.7)	1.50 (0.65, 3.43)	0.34
Norepinephrine[i]				
Total dose, µg	96 391.1 ± 176 499.7	51 320.9 ± 60 924.7	–	–
(Log_{10}) total dose, µg	4.6 ± 0.5	4.5 ± 0.5	0.27 (0.09, 0.44)	0.08
Average daily use, µg/24 hours	33 874.1 ± 88 444.6	17 751.0 ± 18 951.5	–	–
Duration, days	3.2 ± 1.6	2.7 ± 2.0	0.95 (0.28, 1.61)	0.11
Norepinephrine[j]				
Total dose, µg	94 034.5 ± 208 211	45 637.1 ± 60 491.7	–	–
(Log_{10}) total dose, µg	4.6 ± 0.5	4.3 ± 0.5	0.30 (0.11, 0.49)	0.04
Average daily use, µg/24 hours	39 143.9 ± 107 252.9	14 701.6 ± 9442.2	–	–
Duration, days	2.8 ± 1.4	2.6 ± 2.0	0.75 (0.03, 1.46)	0.48
Physiological correlates[g]				
IL-6, pg/mL (n = 122)	699.9 (144-1610.9)	149.0 (56.5-1039.3)	–	0.02
TNFα, pg/mL (n = 122)	125.9 (69.9-1664.1)	81.9 (48.6-169.7)	–	0.03
α1AT, g/L (n = 124)	1.8 ± 0.6	2.2 ± 0.7	–	<0.01
SOFA-CV scores[k]	3.9 ± 0.3	3.8 ± 0.4	–	0.20

Data are given as mean ± SD, median (interquartile range), and n or n/total n (%).
Abbreviations: α1AT, alpha-1-antitrypsin; ACTH, adrenocorticotropic hormone; APACHE II, Acute Physiology and Chronic Health Evaluation II; BMI, body mass index; CBG, corticosteroid-binding globulin; CRP, C-reactive protein; ICU, intensive care unit; IL-6, interleukin-6; TNFα, tumour necrosis factor alpha; SOFA-CV scores, Sequential Organ Failure Assessment cardiovascular scores.
[a]Baseline CBG values represent the mean result from the first 2 days during septic shock admission. The 2 days' values were comparable (day 1: 276 ± 9.7 vs day 2: 269 ± 9.3 nmol/L, *P* = ns).
[b]Derived from a logistic regression model, unadjusted for other variables.
[c]Scores on the APACHE II are assessed on a scale from 0 to 71, with higher scores indicating a higher risk of death.
[d]Scores on the SOFA range from 0 to 24, with higher scores indicating greater severity of illness. The baseline SOFA score was calculated from admission data.
[e]Data are given as surgical n/medical n (% surgical). Surgical admissions were defined as patients being admitted to ICU from the operating room or the recovery room. Medical admissions were defined as patients being admitted to ICU from the emergency department, hospital ward, or interhospital transfer.
[f]Four patients who received hydrocortisone-equivalent doses of glucocorticoids are included intravenous dexamethasone in 1, intravenous methylprednisolone in 1, and oral prednisolone in 2.
[g]The values represent the most recent results when admitted with septic shock.
[h]Only those patients receiving hydrocortisone.
[i]All patients (n = 135, survivors and non survivors) included in analysis of norepinephrine.
[j]Patients discharged from the ICU alive included in analysis (n = 110).
[k]SOFA-CV score of 0 to 2 represents no norepinephrine requirement; scores of 3 and 4 represent low-medium and high norepinephrine requirements, respectively.

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(Mantel-Cox) $P = 0.04$, hazard ratio 2.0; 95% CI 0.9, 6.0] (Fig. 5).

Multivariate logistic regression analysis identified 4 factors that independently predicted ICU mortality: APACHE II > 25 (adjusted OR 3.58, 95% CI 1.20, 10.68), baseline CBG < 200 nmol/L (adjusted OR 3.23, 95% CI 1.06, 9.99), SOFA liver score (adjusted OR 1.98, 95% CI 1.04, 3.72), and the need for renal replacement therapy (adjusted OR 6.59, 95% CI 2.17, 20.01) (Fig. 4C).

Secondary Outcomes

Mortality at both 28 and 90 days was higher in the low CBG group [CBG < 200 nmol/L vs CBG > 200 nmol/L; 28 days: 41.2% (14/34) vs 23.8% (24/101), OR 2.25 (95% CI 0.99, 5.11); 90 days: 47.1% (16/34) vs 28.7% (29/101), OR 2.21 (95% CI 0.99, 4.91)]. The patients who received hydrocortisone (n = 61) had comparable ICU mortality to those who did not receive hydrocortisone (n = 74) (Table 1). Renal replacement use did not differ between the CBG groups. A higher proportion of patients in the CBG < 200 nmol/L group received mechanical ventilation during ICU admission [79.4% (27/34) vs 50.5% (51/101), OR 3.78 (95% CI 1.51, 9.47)]. After inclusion of relevant covariates in the logistic regression model, the following factors were predictors of the use of mechanical ventilation [OR (95% CI)]: (Log_{10}) total norepinephrine dose, 6.12 (2.50, 15.00); surgery category type, 4.67 (1.47, 14.88) among operated patients; and baseline CBG category 2.96 (1.06, 8.10) for low CBG relative to high CBG. Patients receiving mechanical ventilation had lower mean CBG levels [mean difference −39 (95% CI−73, −4.4) nmol/L] and lower nadir CBG levels [mean difference −30 (95% CI −61, −0.2)] (Fig. 6C).

Table 2. Infection and pathogen characteristics

Pathogen characteristic	All patients (n = 135)
Total number of infections per patient, mean	2
Primary source of septic shock, n (%)	
Sepsis of unknown origin	8 (5.9)
Bacteremia	23 (17.0)
Pulmonary sepsis	38 (28.1)
Abdominal sepsis	27 (20.0)
Urosepsis	9 (6.7)
Soft tissue sepsis	19 (14.1)
Septic arthritis/osteomyelitis	6 (4.4)
Missing data	5 (3.7)
Confirmed pathogens per patient, n (%)	
1	58 (43.0)
2	38 (28.1)
3+	34 (25.2)
Missing data	5 (3.7)
Documented pathogen, n	
Positive blood culture	65
Positive culture other[a]	142
Viral PCR or serology	13
Culture negative sepsis	36
Organism type, n	
Gram-positive bacteria	77
Gram-negative bacteria	110
Viral	20
Fungal	20

Abbreviation: PCR, polymerase chain reaction.
[a]Urine, sputum, stool, surgical site, tracheal aspirate, and central venous catheter tip.

Norepinephrine dose, although not strongly associated with baseline CBG (Table 1), was associated with nadir CBG (Fig. 6A and 6B). Nadir CBG < 200 nmol/L was associated with higher total norepinephrine doses Log_{10} (μg) and longer infusion durations (days) in the entire patient cohort [mean difference 0.27 (95% CI 0.09, 0.44) and 0.95 (95% CI 0.28, 1.61), respectively] and in patients who survived ICU [mean difference 0.30 (95% CI 0.11, 0.49) and 0.75 (95% CI 0.03, 1.46), respectively] (Fig. 6A).

Serum cortisol (free or total) in patients not receiving exogenous hydrocortisone was not associated with ICU mortality (Fig. 4B). Time-to-event analysis for ICU survival was not affected significantly by hydrocortisone administration [log-rank (Mantel-Cox) $P = 0.05$] (Fig. 7).

Physiologic Correlates of Altered CBG Concentration

Among patients not receiving exogenous hydrocortisone (n = 74), concentrations of total plasma cortisol were similar between high and low CBG groups (669.1 ± 413.7 and 900.7 ± 455.3 nmol/L, respectively). Free cortisol concentrations were also similar between the CBG <200 and > 200 nmol/L groups (168.7 ± 37.0 and 190.8 ± 125.4 nmol/L, respectively, P = ns), as were serum albumin levels (23.7 ± 6.3 and 24.5 ± 6.2 g/L, respectively, P = ns). CBG negatively correlates with percentage free cortisol (proportion of free to total cortisol; Pearson −0.36, 2-tailed $P < 0.01$) in those who did not receive hydrocortisone treatment (n = 74).

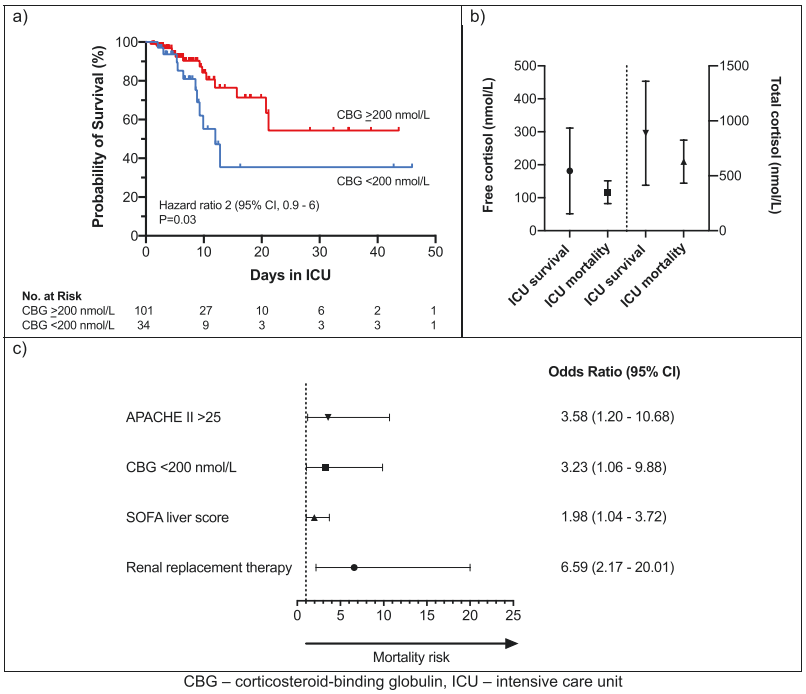
IL-6 and TNFα concentrations (Log_{10}) were both negatively correlated to CBG levels ($r = -0.29$, $P < 0.01$, and $r = -0.27$, $P < 0.01$, respectively) (Fig. 8C and 8D) whereas (Log_{10}) α1AT, an inhibitor of neutrophil elastase, positively correlated to plasma CBG concentrations ($r = 0.36$, $P < 0.01$) (Fig. 8E). The illness severity marker lactate negatively correlated to CBG ($r = -0.23$, $P < 0.01$) while monocyte count positively correlated with CBG ($r = 0.24$, $P < 0.01$). There was no correlation between baseline CBG and serum albumin, CRP, or lymphocyte count. Albumin was not an independent predictor of mortality in septic shock.

Baseline total SOFA scores negatively correlated with CBG ($r = -0.21$, $P = 0.02$), but there was no correlation between CBG and APACHE II ($r = 0.02$, $P = 0.81$). Lower nadir CBG concentrations were significantly associated with greater levels of cardiovascular dysfunction as measured by SOFA-CV (Fig 9A-9D).

Discussion

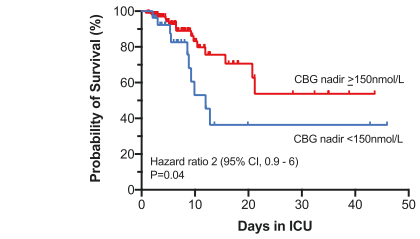
This prospective observational study of 135 patients with septic shock revealed the novel finding that CBG deficiency on ICU admission, defined as a plasma CBG concentration < 200 nmol/L, independently predicted ICU mortality (3.2-fold risk ratio) and was associated with the need for ventilator and vasopressor support, both markers of illness severity.

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CBG – corticosteroid-binding globulin, ICU – intensive care unit

Figure 4. Septic shock survival within intensive care. (A) Kaplan-Meier curve for septic shock survival in intensive care by mean corticosteroid-binding globulin (CBG), <200 and ≥ 200 nmol/L (n = 135). The survival rate was significantly higher in the baseline CBG > 200 nmol/L group, $P = 0.03$ by log-rank. (B) Free and total cortisol by septic shock survival in intensive care in patients who did not receive hydrocortisone treatment (n = 74). Results were nonsignificant. (C) Multivariate analysis revealed 4 factors independently associated with survival. Odds ratio of death within the intensive care unit due to septic shock is shown for these 4 independent factors. Scores on the Acute Physiology and Chronic Health Evaluation (APACHE) II are assessed on a scale from 0 to 71, with higher scores indicating a higher risk of death (a score of >25 has been used as a cutoff point to identify patients of a higher risk of death) (22-24).



CBG – corticosteroid-binding globulin, ICU – intensive care unit.

Figure 5. Septic shock survival by corticosteroid-binding globulin nadir. Kaplan-Meier curve for septic shock survival in intensive care by corticosteroid-binding globulin (CBG) nadir <150 and ≥ 150 nmol/L. The survival rate was significantly higher in the CBG nadir ≥ 150 nmol/L group, $P = 0.04$ by log-rank.

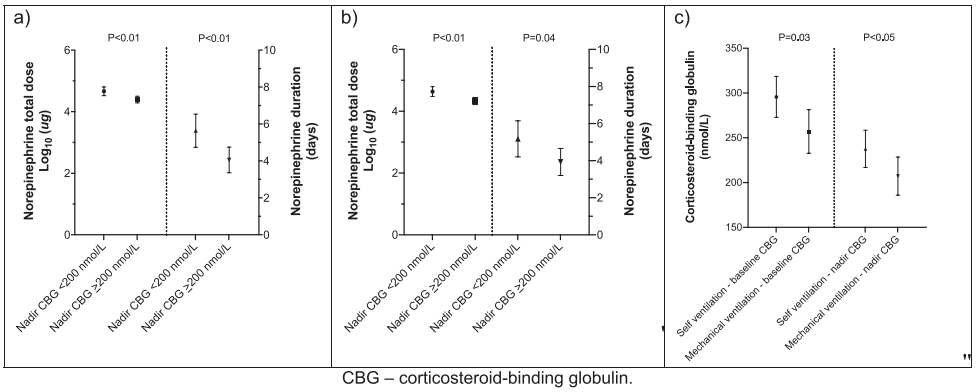
The cohort had a mortality rates similar to current best practice and the current definition of septic shock. In addition, the data set exhibited high integrity across all clinical, physiological, and biochemical measures facilitating the capacity to develop statistical analyses relating the study question, low CBG, to the septic shock state.

CBG levels were comparable to our previous exploratory pilot studies; however, this much larger study allowed a broader assessment of the importance of low CBG in terms of its importance as an independent mortality risk factor and its association with both disease severity markers and biochemical factors involved in inflammation and cortisol action. The interactions between low CBG in these domains have a logical basis given CBG's cortisol delivery action to inflamed tissues.

Independent mortality risk factors were plasma CBG, APACHE II score, renal replacement therapy, and SOFA liver score. Of these factors, plasma CBG < 200 nmol/L is the only potentially reversible risk factor.

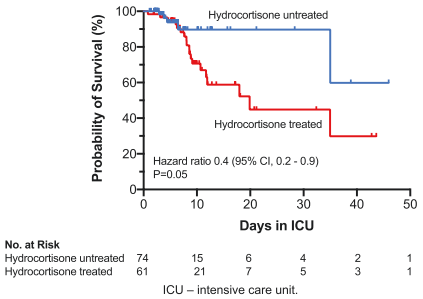
Nadir CBG was also associated with SOFA-CV, a composite measure of mean arterial pressure and norepinephrine requirement, suggesting that CBG may be particularly important in cardiovascular maintenance in sepsis (25, 26). Low circulating CBG may have a direct adverse effect on cortisol delivery to cardiovascular structures or its presence along with CBG messenger RNA in the cytoplasm and cell membrane of cardiomyocytes, Purkinje fibers, endocardial

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CBG – corticosteroid-binding globulin.

Figure 6. Serum corticosteroid-binding globulin (CBG), norepinephrine, and mechanical ventilation use. Lower levels CBG were associated with higher norepinephrine and mechanical ventilation requirements in septic shock. (A) Norepinephrine administration measured in total dose Log_{10} (μg) and duration (days) for septic shock by nadir CBG <200 and ≥ 200 nmol/L. *P*-values < 0.01 and < 0.01, respectively, unpaired *t*-test. All patients (n = 135, survivors and nonsurvivors) included in analysis. (B) Norepinephrine administration measured in total dose Log_{10} (μg) and duration (days) for septic shock by nadir CBG <200 and ≥ 200 nmol/L. $P < 0.01$ and $P = 0.04$, respectively, unpaired *t*-test. ICU survivors (n = 110) included in analysis. c) Ventilation status for septic shock by baseline and nadir corticosteroid-binding globulin. *P* values 0.03 and < 0.05 respectively, unpaired *t*-test. Mean baseline corticosteroid-binding globulin is calculated over days 1 and 2 of septic shock admission.



ICU – intensive care unit.

Figure 7. Septic shock survival by hydrocortisone treatment. Kaplan-Meier curve for septic shock survival in intensive care by hydrocortisone treated and hydrocortisone untreated. Survival rate was significantly higher in the hydrocortisone untreated group, $P = 0.05$ by log-rank. This cohort was not randomized for the use of hydrocortisone, which was given according to clinician preference. Those who received hydrocortisone treatment had more severe septic shock and were hence more likely to die.

cells, and arterial smooth muscle cells may also be reduced in sepsis, further altering cortisol delivery and action (25, 26).

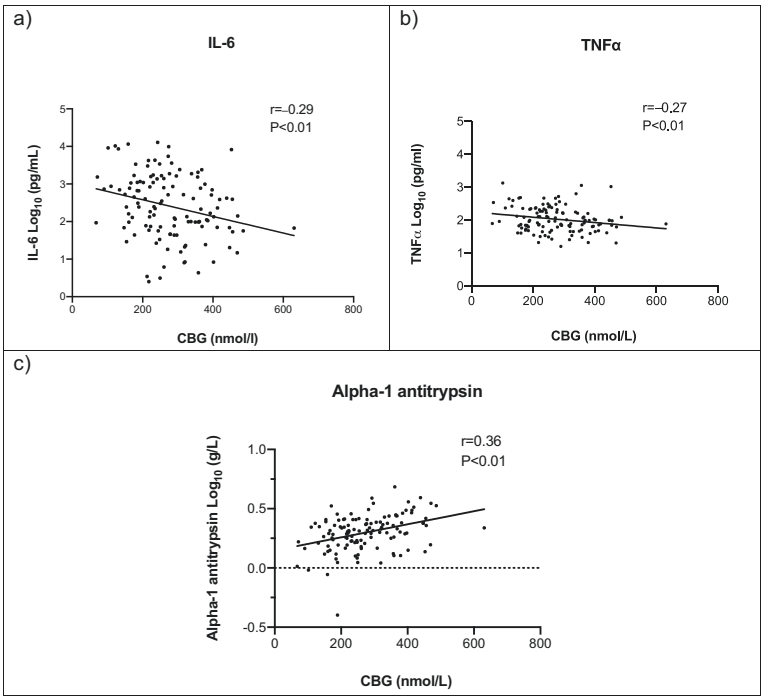
While CBG had a strong relationship with mortality and organ failures, particularly cardiovascular, cortisol levels did not. Our study was not intended to address the value of cortisol concentrations in septic shock; we did not measure stimulated cortisol to standardize the concentrations, and the size of our study was insufficient for this purpose, particularly as hydrocortisone was delivered on intensivist judgment to approximately half of the patients, and the cortisol concentrations in that setting would be highly variable and subject to pharmacokinetic considerations. Cortisol concentrations have been shown to be associated with illness severity and pressor sensitivity but are not recommended for use in determining hydrocortisone therapy (15, 27-31).

Cortisol levels were elevated, although not markedly so, and ACTH levels remained within the normal range. During sepsis and septic shock, ACTH synthesis and secretion is decreased and the HPA axis is mainly activated by corticotrophin-releasing hormone independent pathways involving immune mediators (32). Clinical studies have found ACTH levels to be lower in septic shock patients compared to healthy controls (33).

The lack of association between serum cortisol levels and outcomes are in keeping with previous studies and support the notion that CBG is not simply a proxy for cortisol and are consistent with CBG's role in targeted cortisol tissue delivery. Cortisol concentrations do not reliably relate to cortisol tissue effect since cellular glucocorticoid resistance, which varies between tissues and individuals, occurs in sepsis (5). This is consistent with previous reports where cortisol levels are highly variable in sepsis (34) and are not of value in clinical decisions regarding hydrocortisone therapy (4). Furthermore, large trials of hydrocortisone administration in the treatment of septic shock have not shown consistent mortality benefit (2, 3). In contrast, CBG is a cortisol transport glycoprotein uniquely adapted to targeted cortisol tissue delivery in sepsis, with reduced CBG-cortisol binding in pyrexia (9, 35), acidosis (9), and cytokine-suppressed hepatic CBG synthesis (12-14) and, finally, by irreversible neutrophil elastase cleavage of CBG at inflammatory sites (7, 9, 36).

Serum albumin concentrations are reduced in septic shock, consistent with the acute phase response, but serum albumin was not associated with mortality. Albumin binds 10% to 15% of cortisol with low affinity, acting as a buffer within the circulation, and does not have a biochemical role in specific cortisol transport to tissues or inflammatory sites, as far as is known. Evidence is conflicting regarding benefit of albumin administration in critical illness, and albumin was rarely administered to the 135 patients within the study; thus, we cannot comment on the relationship between albumin administration and outcome.

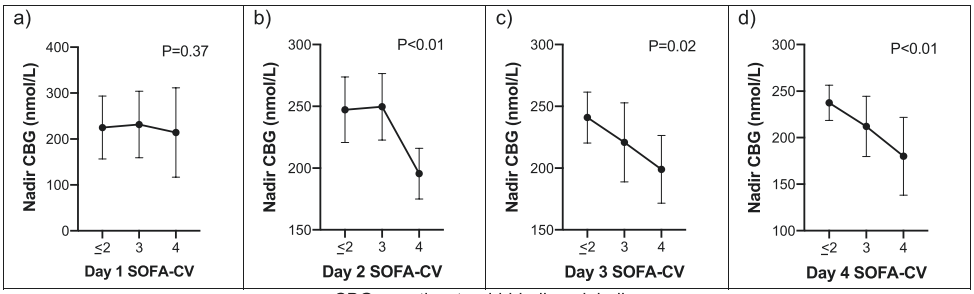
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CBG – corticosteroid-binding globulin; IL-6 – interleukin-6; TNFα – tumour necrosis factor alpha.

Figure 8. Biochemical factors that reduce circulation of corticosteroid-binding globulin (CBG). (A) Correlation between CBG (nmol/L) and interleukin-6 Log_{10} (pg/mL) with line of best fit. For correlation r = –0.29, $P < 0.01$. (B) Correlation between CBG (nmol/L) and tumour necrosis factor alpha Log_{10} (pg/mL) with line of best fit. For correlation r = –0.27, $P < 0.01$. (C) Correlation between CBG (nmol/L) and alpha-1 antitrypsin Log_{10} (g/L) with line of best fit. For correlation r = 0.36, $P < 0.01$. Alpha-1 antitrypsin inactivates neutrophil elastase, which causes proteolytic degradation of corticosteroid binding globulin.

CBG knockout mice have an increased mortality in sepsis (37). The risk of septic shock and its outcomes are thought to be largely host dependent. In humans, CBG polymorphisms exist that alter CBG concentrations and cortisol binding affinity (11). CBG polymorphisms are also predicted to alter neutrophil elastase cleavability (11). Hence, genetically determined variations in CBG may alter the risk of low circulating CBG concentrations and disease outcomes.



CBG – corticosteroid-binding globulin.

Figure 9. Nadir corticosteroid-binding globulin associations with cardiovascular outcomes. Lower nadir CBG categorical concentrations significantly associates with cardiovascular dysfunction as measured by Sequential Organ Failure Assessment (SOFA) cardiovascular (SOFA-CV) scores, test for linear trend (A-D). SOFA-CV scores of 0 to 2 represent no norepinephrine requirement; scores of 3 and 4 represent low-medium and high norepinephrine requirements, respectively.

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Although low CBG concentration was strongly related to early mortality (within ICU), the strength of this association appeared to reduce over time (28- and 90-day mortality). This could reflect a type II error through patient mortality. However, declining effect of CBG on mortality may become evident as other nonsepsis contributors to mortality emerge. CBG, lowered by neutrophil elastase cleavage and suppression of synthesis by inflammatory cytokines, recovers rapidly in convalescence; our previous study showed plasma CBG concentrations normalized 24 to 48 hours after recovery from septic shock (18). Baseline CBG was low, reflecting prodromal illness pre-ICU. CBG declines within 24 to 48 hours in healthy controls after administration of IL-6 (12). As inferred by our pilot studies, dichotomizing baseline CBG at 200 nmol/L (40th percentile in septic shock and 1st percentile in healthy controls) in this study showed a clear contrast between low or high mortality.

Underlying the mechanisms of CBG deficiency, the inflammatory cytokines IL-6, and TNFα, are greatly elevated in septic shock and were strongly negatively associated with plasma CBG concentration. Circulating α1AT concentrations were strongly associated with CBG concentrations, reflecting the role of α1AT in inhibiting neutrophil elastase within tissues.

This study showed an independent effect of low plasma CBG on worsening mortality and an association with norepinephrine requirements (both dose and duration) as well as ventilation. This finding is compelling because it suggests that CBG supplementation in sepsis could improve cardiovascular failure and survival. It could be argued that CBG is a negative acute phase reactant and the associations noted may represent illness severity. However, the finding that CBG is a predictor of mortality independent of acute phase reactant CRP argues against this. Moreover, CBG's function with the release of bound cortisol in conditions of pyrexia and acidemia and with neutrophil elastase cleavage provides a mechanistic rationale for understanding CBG's role in sepsis and inflammation.

We were not able to determine the duration of CBG deficiency as the duration of illness prodrome before ICU admission was unknown; many patients had low CBG upon ICU admission, and CBG concentrations fall up to 50% within 24 hours of systemic illness (15, 18) and, in this study, fell little during septic shock. Hence, CBG concentrations may not be entirely host specific but may be influenced by the length and severity of the sepsis prodrome. Validation cohorts in septic shock with different characteristics such as a high mortality group, other critical illness–related conditions, or simple replication studies would be of interest. Similarly, ventilatory requirement was based on clinical judgment, required in those with a surgical admission category, and was not an independent predictor of ICU mortality. In contrast, norepinephrine use was protocolized, hence reducing the variability in this secondary outcome.

Septic shock is a medical emergency and represents 20% of all global deaths (38), and new treatments are sorely needed. Our data demonstrate a strong relationship between CBG deficiency and disease severity, cardiac dysfunction, and a 3.2-fold increase in mortality in septic shock. We hypothesize that CBG supplementation in septic shock may be of practical benefit and warrants investigation.

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Disclosures

The authors have declared that no conflict of interest exists.

Data Availability

Some or all data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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SUPPLEMENTARY INFORMATION

FIGURE 5.1. Study screening record.

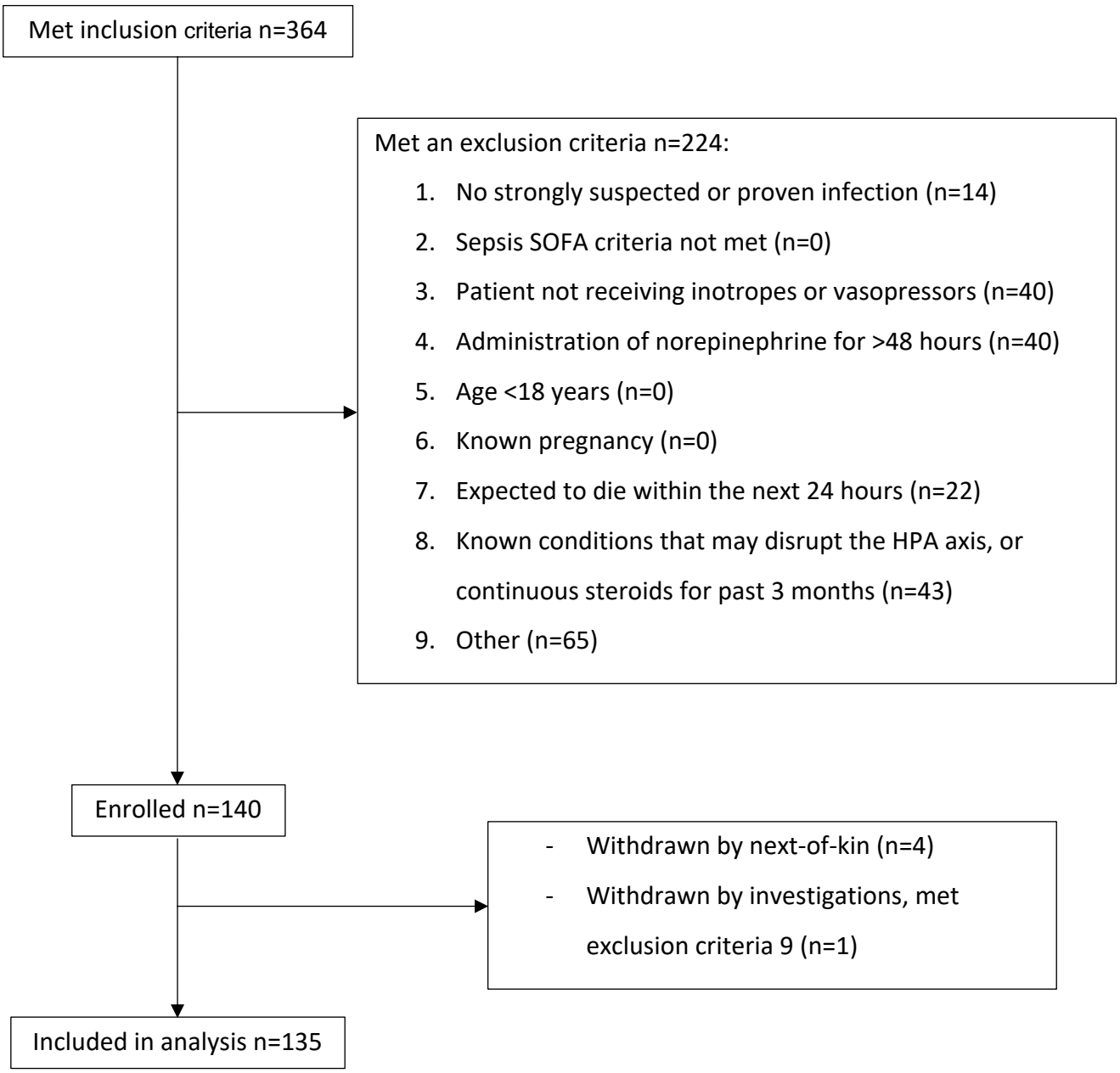


TABLE 5.1. Norepinephrine infusion protocol:

1. Invasive monitoring of relevant parameters (always including mean arterial pressure [MAP] by continuous intra-arterial measures and hourly urine output.
2. Fluid replacement “cardiovascular filling” will be given.
3. A desirable MAP for each patient will be determined by the treating physician, between 60-100 mmHg. Norepinephrine infusion will be commenced, starting at 2−5 mcg/min and titrated to that the patient’s target MAP at a rate determined by the patient’s treating physician.
4. Urine output will be assessed hourly while on norepinephrine.
5. Once MAP ≥5 mmHg above goal for 30 mins, norepinephrine dose reduction will begin as follows;

If infusion rate >50 mcg/min	Reduce rate by 5 mcg/min every 15 min
If infusion rate 20−50 mcg/min	Reduce rate by 2 mcg/min every 15 min
If infusion rate <20 mcg/min	Reduce rate by 1 mcg/min every 15 min

6. If MAP >20 mmHg above goal reduce rate by 50%. If MAP falls below target >5 mins increase rate to target MAP.
7. Cessation of vasopressors will be defined as being off norepinephrine for 6 or more consecutive hours.
8. Required deviations from this protocol are at the discretion of the treating intensivist.

CHAPTER 6:

FINAL DISCUSSION AND FUTURE DIRECTIONS.

CHAPTER OUTLINE

This chapter will review my main findings regarding CBG's role in the targeted delivery of cortisol in inflammation. These findings encompass: 1. Basic research into CBG's three intrinsically mediated and one extrinsically mediated functional alterations in sepsis, 2. A clinical study demonstrating CBG's interaction with key players in the inflammatory cascade and demonstrating the relation between acquired CBG deficiency and mortality in septic shock. The position of these results in juxtaposition with the extant literature and the logical steps for research and development will be discussed.

6.1. Summary of key findings

Through basic and clinical inquiry, the studies presented in this thesis examined the role of CBG in cortisol delivery in sepsis and septic shock.

The major findings in this thesis include:

Basic research:

(i). The development of a novel ligand binding assay using surface plasmon resonance (SPR) technology, which provided a reproducible and readily applicable, direct ligand binding assay to quantitate the bimolecular interaction between recombinant CBG and a library of 19 steroids, including cortisol. **(Chapter 3)**

(ii). SPR binding assays demonstrated a 3-to 8-fold decrease in binding affinity between high affinity (ha) CBG and low affinity (la) CBG for cortisol, cortisone, corticosterone, 11-deoxycortisol, progesterone, 17-hydroxyprogesterone and prednisolone at physiological conditions (37°C, pH 7.4). **(Chapter 3)**

(iii). CBG:cortisol binding affinity significantly decreases at higher temperatures (39°C) and acidic pH (pH 7.0) for both haCBG and laCBG, representative of conditions experienced in systemic inflammatory states, such as sepsis and septic shock. **(Chapter 3)**

(iv). 9G12 antibody immunoreactivity of the reactive centre loop (RCL) of haCBG is preserved at 39°C and in acidosis, with preservation of the thermo-coupling mechanism even after neutrophil elastase (NE)-cleavage. Molecular modelling studies of CBG at 40°C revealed the critical role of Trp371, located within the binding pocket, in ligand binding. Taken together, we propose that the thermo-coupling mechanism of cortisol release involves Trp371 and occurs while maintaining RCL integrity. Thus, the effects of NE-cleavage, pyrexia and acidosis on CBG structure may serve synergistically to intrinsically enhance cortisol delivery to the interstitium at sites of inflammation. **(Chapter 3)**

Clinical research:

(i). Total and haCBG depletions are associated with septic shock mortality, due in part to factors extrinsic to the CBG molecule, whereas total and haCBG does not associate

with non-sepsis related critical illness mortality. **(Chapter 4)**

(ii). **CBG deficiency in septic shock, defined as a plasma CBG concentration < 200 nmol/L (reference interval 269–641 nmol/L), independently predicted ICU mortality (3.2-fold risk ratio**). **(Chapter 5)**

(iii). CBG levels <200 nmol/L are associated with **key disease events** such as increased mechanical ventilation and increased noradrenaline use. **(Chapter 5)**

(iv). Multivariate analysis revealed **four factors independently predicted mortality**; APACHE II >25, CBG <200 nmol/L, SOFA liver score and renal replacement therapy. **(Chapter 5)**

(v). **Nadir CBG associated with SOFA-CV**, a composite measure of BP and norepinephrine requirement. **(Chapter 5)**

(vi). The **pro-inflammatory cytokines**, IL-6, and TNFα were predictably elevated in septic shock and displayed a strong negative association with plasma CBG concentration.

(vii). **Circulating α1AT concentrations** strongly associated with CBG concentrations, reflecting the role of α1AT in inhibiting neutrophil elastase, consistent with the known cleavage mechanism of CBG, which yields cortisol to inflamed tissues. **(Chapter 5)**

(viii). **Low CBG concentrations** were associated with: increased serum lactate, a marker of impaired tissue metabolic function; and associated with increased circulating monocytes concentrations, a marker of hypercortisolism. **(Chapter 5)**

6.2 Implications and future directions

In sepsis, our studies support the concept of a sequential 4-part process optimising free cortisol delivery through CBG-specific mechanisms. These include with pyrexia, acidosis, cytokine-based inhibition of hepatic CBG synthesis and NE-mediated cleavage, acting in concert to deliver immunomodulatory cortisol directly to inflammatory sites, obviating systemic inflammation and multi-organ failure in patients with septic shock, and its association with mortality. Note that pyrexia and acidosis do not deplete CBG, but loosen CBG:cortisol binding reversibly, whereas inhibition of CBG synthesis and irreversible cleavage deplete the pool of CBG capable of binding cortisol.

In 135 patients with septic shock, statistically independent mortality risk factors on multivariate analysis were four in number: plasma CBG < 200 nmol/L, APACHE score, renal-

replacement therapy, and SOFA liver score. Of these factors' plasma CBG < 200 nmol/L is the only risk factor that has the potential for direct intervention through administration of this natural protein, if it were available. The cardiovascular component of SOFA associated with low CBG, pointing to a novel mechanistic role for CBG in cortisol delivery to the heart and vasculature in septic shock.

CBG deficiency also associated with disease progression and mortality in septic shock. This accords with CBG's role in targeted delivery of immunomodulatory cortisol to tissues to quell systemic inflammatory processes.

Given the 3.2-fold increase in mortality, replenishing CBG may have a large therapeutic effect in those with CBG < 200 nmol/L in the treatment of septic shock. Sepsis and septic shock are the leading causes of death worldwide and there have been no therapeutic advances in over 20 years.[1] Our group hypothesize that CBG supplementation in septic shock may reduce illness severity and mortality and warrants an interventional clinical trial.

The potential role of CBG as a therapeutic is of interest, however, lies outside the scope of this thesis. While recombinant human CBG products are commercially available, they are not produced to World Health Organisation good manufacturing practice (GMP) requirements for use in humans. There is no data on the safety or efficacy of CBG administration in animals or humans. Future work in the development and translation of CBG as a therapeutic for the treatment of septic shock is required and is a logical progression of the research presented in this thesis.

6.3 Disputing the notion of reverse causality

It could be argued that low concentrations of CBG in patients critically ill with septic shock represent illness severity reflecting reverse causality.

There are several arguments against this position: 1. CBG has a known role in cortisol delivery to the interstitial compartment of threatened tissues, after cleavage by neutrophil elastase, 2. Numerous other biochemical and most clinical factors that change with sepsis do not

independently associate with mortality, 3. Animal models of low CBG are associated with sepsis induced mortality.

CBG's function in release of bound cortisol in conditions of pyrexia, acidaemia and with NE cleavage provides a mechanistic rationale for understanding CBG's role in sepsis and inflammation. We postulate that Moreover, circulating α1AT concentrations were strongly associated with CBG concentrations, reflecting the role of α1AT in inhibiting NE within tissues.

CBG is not simply a negative acute phase reactant in septic shock, as it has functional importance in CBG transport to inflammation through specific cortisol delivery functions at inflammatory sites through its proven interaction with NE. In addition, many other measurable markers that are altered in accordance with septic shock severity do not independently predict mortality. Furthermore, the finding that CBG is a predictor of mortality independent of acute phase reactant CRP argues against this.

Our septic shock cohort had elevated serum free cortisol levels, and CBG negatively correlated to %free cortisol (proportion of free to total cortisol) (Pearson -0.36, 2-tailed P<0.01). Serum free cortisol concentrations are elevated in association with the lower CBG concentrations in septic shock compared to sepsis alone.[2] This is consistent with the expectation that CBG depletion does have an effect on elevating circulating free cortisol relative to total cortisol (free:total cortisol ratio). In the tissues this effect if likely to be amplified by cleavage at inflammatory sites as shown by Chan et al. JCEM.[3] where abruptly lowered cortisol binding affinity has been shown to elevate interstitial cortisol concentrations 3-4-fold.

CBG knockout mice have an increased mortality in sepsis.[4] In a lipopolysaccharide (LPS) septic shock model $CBG^{-/-}$ mice had significantly reduced 48-hour survival rates. With the administration of LPS, free corticosterone concentrations increased and were higher in $CBG^{-/-}$ mice compared to wild type, suggesting susceptibility to septic shock was not due to reduced circulating free corticosterone.

The lack of association between serum cortisol levels and outcomes are in keeping with previous studies and support the notion that CBG is not simply a proxy for cortisol and are consistent with CBG's role in targeted cortisol-tissue delivery. In addition, the administration of hydrocortisone only has a marginal effect on mortality, and only in the most severely ill.

6.4 Expanding our knowledge of CBG physiology

My work described in this thesis and the work of others has moved CBG beyond the notion of a transport protein for cortisol. CBG is increasingly accepted as a sophisticated and specialised tissue delivery molecule for cortisol. Further steps to elucidate the functions of CBG will be to identify proteases, beyond NE, and study interactions with CBG in the cortisol delivery system. While CBG RCL cleavability by NE[5], chymotrypsin[6], and LasB[7], a protease produced by *Pseudomonas aeruginosa* and virulence factor, have been characterised, other RCL proteases are yet to be discovered. Surface plasmon resonance (SPR) technology may have application here in the characterisation of the effect of novel protease cleavage of the RCL on CBG:cortisol binding affinity.

CBG is predominantly synthesised and secreted by hepatocytes, with its promoter region responding to liver-specific transcription factors.[8,9] CBG and CBG mRNA have been identified in other human tissues: pituitary cells that secrete ACTH—corticotropes; the hippocampus, a key site for cortisol negative feedback; heart; kidney; and placenta[10-14] implying localised synthesis, however the physiological importance of local synthesis of CBG is not understood. It is noteworthy that patients with CBG deficiency or reduced CBG:cortisol binding affinity often have difficulties with chronic unexplained fatigue and pain. The pathophysiology of these symptoms is not known and does not respond to glucocorticoid administration, but this observation points towards a hitherto unknown function of locally synthesised CBG. Cortisol-bound CBG binds to cell membranes[15-17] and activates secondary messenger pathways suggesting intrinsic biological activity, however the CBG receptor has not been characterised or cloned in humans, with only indirect *in vitro* evidence of its existence and partial characterisation in rats.[18,19]

Several heritable *SERPINA6* pathogenic gene mutations have been identified following clinical inquiry into patients presenting with hypocortisolaemia in association with a variety of clinical

features including chronic fatigue, chronic pain, exercise intolerance, depression, hypotension and obesity. Three such *SERPINA6* variants result in a reduction in plasma CBG levels (CBG Null/Adelaide[20,21], CBG Santiago[22] and **CBG Montevideo[23], publication related to this thesis**) and four variants result in a reduction in cortisol-binding affinity and/or capacity [CBG Leuven[24-26], CBG Lyon[20,21,27-30], CBG G237V[31] and CBG Athens[30]]. Additional mutations have been identified through population screening, CBG A51V and CBG E102G within a Han Chinese population resulting in reduced plasma CBG levels and reduced cortisol-binding capacity respectively.[32] A polymorphism, CBG A224S was over-represented in a chronic fatigue cohort.[33] Markers in the CBG gene, but not in other key hypothalamic–pituitary–adrenal axis regulatory genes, are associated with chronic widespread pain syndrome.[33] The CBG gene is expressed in brain regions that are involved in the stress response.[34] CBG knockout mice demonstrate alterations in hippocampal cortisone activity, with elevated levels of free cortisone in the hippocampus, FKBP5 down regulation, high MR expression and increased MR and GR downstream gene expression without local changes in 11BHSD expression or activity.[35] Furthermore, the hippocampus of these CBG—deficient mice demonstrated neurological damage with increased Tau phosphorylation, supporting the importance of the role of CBG on the central nervous system, specifically the hippocampus and its possible involvement in neurological damage. The identification of increasing numbers of individuals with *SERPINA6* variants affecting CBG function (concentration or cortisol binding reducing) and symptoms consistent with attenuated features of hypocortisolism (chiefly fatigue, hypotension, hypoglycaemia) despite normal circulating free cortisol, suggests a specific role for CBG in glucocorticoid function, perhaps involving cortisol regulation of hepatic glucose homeostasis and cortisol-brain communication.

6.5 CBG glycosylation

CBG has six potential asparagine (N)-linked glycosylation sites, of which five sites are occupied by N-acetyllactosamine oligosaccharides, three biantennary and two triantennary, per molecule.[36] Glycan processing occurs post-translation and prolongs half-life and influences binding affinity.[3,37,38]

Site specific glycosylation is important for CBG function. Glycosylation at Asn238 within the ligand-binding pocket is proposed to be vital for effective binding capacity [39] and glycosylation

at Asn347 located within the RCL has been shown to influence cortisol binding capacity by modulating protease cleavage, thought to affect protease accessibility to the cleavage site.[40] Eighty percent of circulating CBG has highly branched and there are voluminous glycans at the Asn347 site. Volume enhancing glycation of Asn347 antagonises NE-cleavage of the RCL, slowing the rate of cortisol release. Glycosylation at Asn347 also modulates the RCL cleavage susceptibility by P. aeruginosa-derived *LasB*.[40] (Figure 6.1)

CBG's binding affinity for cortisol is in part glycosylation dependent. Unglycosylated haCBG and laCBG have reduced binding affinity for cortisol decreased 9- and 2.5-fold compared to glycosylated forms, respectively. *In vitro* studies using recombinant human CBG and fluorescence quenching binding techniques showed glycosylation increases CBG's thermosensitivity.[3,41] CBG's affinity for cortisol when glycosylated decreases 4-fold with a rise in temperature from 37°C to 42°C, while affinity of unglycosylated CBG increases only 2-fold. [3,41] This emphasises the notion that CBG is an adapted carrier protein allowing for the varied release of cortisol at target tissues, by increasing the bioavailability of cortisol at sites of inflammation, and with local or systemic pyrexia, adding to the complexity of interpreting plasma cortisol in patients who are critically ill, febrile, or hypothermic.

Glycosylation prolongs CBG half-life[38] and may be involved in potential CBG-receptor interactions[42,43]. Pregnancy is a unique state of prolonged physiological hypercortisolism with a 2-3-fold increase in total and free cortisol by the third trimester stimulated by placental derived corticotropin-releasing hormone (CRH).[44,45] CBG levels also increase 2–3-fold until 36 weeks gestation after which they fall, coupled with a rise in free cortisol at 38 weeks.[44] The increase in CBG during pregnancy is due in part to pregnancy-specific glycosylation which affects metabolism, and in part to oestriol-stimulated hepatic synthesis.[44,46] We have previously demonstrated that plasma CBG concentrations in pregnancy were 1.9 times higher than that in non-pregnant controls, and that this was comparable to a 3.2-fold rise in CBG in women taking the hormonal combined oral contraceptive pill (COCP), consistent with an oestrogen receptor mediated increase in CBG synthesis. In pregnancy the rise in CBG is attributed to haCBG; haCBG levels rise 2.8 times while laCBG remains unchanged compared to controls. Women taking the COCP had elevations in both haCBG and laCBG; this supports the notion that the reduced CBG cleavage in pregnancy may be due to pregnancy-induced

CBG glycosylation altering clearance.[46] The rise in haCBG during pregnancy theoretically provides for an increased anti-inflammatory cortisol reservoir, allowing for the possibility of a puerperal infection. In addition, the higher haCBG levels allows for the high progesterone levels of pregnancy to compete with cortisol for binding, contributing to the increase in free cortisol required for organ maturation, neurodevelopment and neonatal transition.

CBG glycosylation profiles may be an inter-individual and/or a state (sepsis, pregnancy) dependent phenomenon. We have demonstrated CBG deficiency heralds sepsis severity and septic shock mortality and hypothesise there may be an advantageous CBG glycosylation profile.

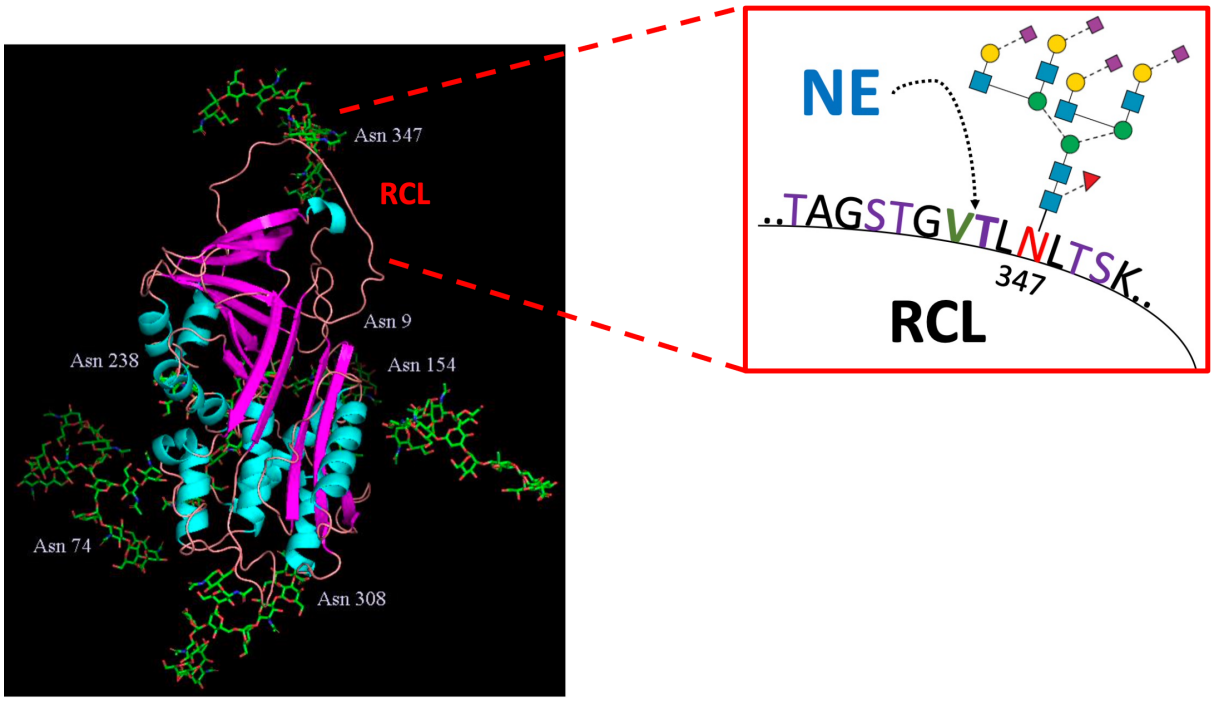


FIGURE 6.1. Glycosylation sites of human corticosteroid binding globulin.[47]

Homology model of uncleaved high affinity (haCBG) human CBG. The β-sheets are shown in magenta, α-helices in light blue and exposed reactive centre loop (RCL). The locations of the six N-glycosylation sites are labelled, and the most common N-glycans are shown in green/red sticks. Red box depicts glycosylation at Asn347 located within the reactive centre loop (RCL) adjacent to the neutrophil elastase cleavage site.

6.6 Non-cortisol applications of CBG therapeutics

CBG has the potential to act as a vehicle for targeted drug delivery with reduced side effects and off-target toxicity. Targeted mutations to the amino acid sequence of the ligand-binding site and RCL allow CBG to carry specific therapeutic molecules and react with desired proteases. This allows for the therapeutic agent to be carried to defined tissues while reducing off-target side effects and such a strategy could allow a lower total dose of the drug to be used while maintaining a higher local concentration of the therapeutic compound at the desired site.

Mutations made at and around the P1 residue of the RCL give rise to two CBG variants that are cleaved by either thrombin or prostate specific antigen (PSA). PSA, which is over produced in prostatic hyperplasia, is most advantageous for the targeting hyperplastic prostrate tissues. Thrombin, released during clot formation, is consequently ideal for conferring specificity to thrombotic sites.[48] While thyroxine-binding globulin (TBG) is structurally similar to CBG, site-directed mutation of tryptophan 371, located within the binding pocket and important for CBG:cortisol binding, to arginine changes ligand specificity of CBG such that it preferentially binds thyroxine[48]. In principle this has wide potential application, most obviously in oncology.

6.7 Conclusion

CBG acts via a 4-phase process (pyrexia, acidosis, reduced CBG hepatic synthesis, NE-cleavage) to deliver cortisol to sites of inflammation to quell the inflammatory process and obviate multiorgan failure in septic shock. I have verified these phenomena *in vitro* using the SPR model. My clinical studies have shown that CBG deficiency (< 200 nmol/L) in septic shock independently predicts ICU mortality (3.2-fold risk ratio), disease severity events and cardiovascular dysfunction. CBG deficiency is potentially reversible and we hypothesize CBG supplementation in septic shock may be of benefit and should be subject to a clinical trial.

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