Corticosteroid-Binding Globulin – a Targeted Delivery System for Cortisol. Physiology and Responses to Acute and Chronic Inflammation.

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Abstract

Corticosteroid-binding globulin (CBG) is the large glycoprotein principally responsible for transporting the life-sustaining hormone cortisol. Levels of biologically active free cortisol are regulated by the concentration and the cortisol-binding affinity of CBG. A member of the serine protease inhibitor superfamily, CBG has a single high-affinity cortisol-binding site per molecule and an exposed reactive centre loop that acts as a protease bait domain for neutrophil elastase, inducing permanent conformational change upon enzymatic cleavage. Allosteric modulation of the inherently plastic binding site reduces the cortisol-binding affinity of CBG by ten-fold (high cortisol-binding affinity CBG [haCBG] → low cortisol-binding affinity CBG [laCBG]), favouring cortisol release *in vitro*. The targeted deposition of free cortisol at sites of inflammation facilitates down-stream genomic and non-genomic immunomodulatory, anti-inflammatory effects, neurocognitive and metabolic effects.

Novel measurement of haCBG and total CBG by enzyme-linked immunosorbent assay is at the forefront of research into CBG cleavage *in vivo*. The present thesis utilises this recent development in seven clinical studies to translate existing evidence of the cleavage phenomenon to the clinical setting for the first time, with the aim of determining whether haCBG levels were associated with illness severity and clinical outcomes in inflammatory states. We hypothesised that depletion of anti-inflammatory CBG-cortisol is a key mechanism in the pathogenesis of uncontrolled inflammation in systemic inflammatory disorders.

Studies of haCBG and laCBG levels in acute inflammatory conditions showed that increasing illness severity in sepsis and septic shock was associated with markedly reduced circulating haCBG concentrations *in vivo*, with illness severity correlating better with haCBG levels than

either free or total cortisol levels. CBG cleavage also occurred in patients with severe infection, including Pseudomonal infection. Administration of the pro-inflammatory cytokine tumour necrosis factor-α did not induce CBG cleavage or the acute phase response immediately (< 6 hours) post-infusion. These studies suggest that in acute inflammation, depletion of haCBG may limit the availability of cortisol to inflammatory sites, perpetuating inflammation, however greater than six hours is required for CBG cleavage to become effectual. Additionally, the pathogen inciting a systemic inflammatory response may influence the propensity for CBG cleavage. We also present evidence of CBG as a thermocouple early in inflammation to enhance free cortisol levels.

In contrast, chronic inflammatory states displayed reduced CBG cleavage, including the metabolic syndrome and rheumatoid arthritis, in association with worsening disease activity. Thus compromised cleavage may hinder CBG-mediated delivery of anti-inflammatory cortisol. Studies in patients with α1 antitrypsin deficiency showed that paradoxically, proteolytic cleavage of CBG was reduced despite increased neutrophil elastase activity. Our data suggest cleavage is mediated by alternate proteases in some circumstances.

In pregnancy, CBG increases three-fold, and we show that this increase is due to haCBG alone which may provide an increased reservoir of CBG-bound cortisol for immunomodulatory purposes in puerperal infection. In comparison, the rise in CBG in women receiving exogenous oestrogens is due to elevated haCBG as well as laCBG, while neither haCBG nor laCBG levels change following menopause, challenging the mechanisms governing oestrogen-mediated CBG production.

In summary, this pioneering research reveals perturbations in haCBG and laCBG levels in health and disease which had hitherto been unrecognised. The absolute pool of haCBG-cortisol, and the accessibility of that pool to cleavage bears significant influence over inflammatory outcomes. Post-translational modification including glycosylation or genetic CBG variation may contribute to haCBG dysregulation. The role and relevance of CBG as a releasing agent and an anti-inflammatory molecule is more complex and adapted than currently appreciated, and holds great opportunity for diagnostic and therapeutic application.

Declaration

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.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

…………………………………………

Marni Anne Nenke

January 2017

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Publications

Publications related to work presented in this thesis

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2. **Nenke MA**, Lewis JG, Rankin W, Torpy DJ. Evidence of Reduced CBG Cleavage in Abdominal Obesity: A Potential Factor in Development of the Metabolic Syndrome. *Hormone and Metabolic Research*. 2016 Aug;48(8):523-8. doi: 10.1055/s-0042-108728. Epub 2016 Jun 14.

3. **Nenke MA**, Lewis JG, Rankin W, McWilliams L, Metcalf RG, Proudman SM, Torpy DJ. Reduced corticosteroid-binding globulin cleavage in active rheumatoid arthritis. *Clinical Endocrinology (Oxf).* 2016 Sep;85(3):369-77. doi: 10.1111/cen.13081. Epub 2016 May 8.

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illness severity in sepsis and septic shock; clinical implications. *Clinical Endocrinology (Oxf)* 2015 Jun;82(6):801-7 doi: 10.1111/cen.12680. Epub 2015 Jan 8.

<u>Other publications /abstracts produced during candidature</u>

1. Meyer EJ, **Nenke MA**, Rankin W, Lewis JG, Torpy DJ. Corticosteroid-binding globulin: a review of basic and clinical advances. *Hormone and Metabolic Research*. 2016 Jun;48(6):359-71. doi: 10.1055/s-0042-108071. Epub 2016 May 23.

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3. **Nenke MA**, Haylock CL, Rankin W, Inder WJ, Gagliardi L, Eldridge C, Rolan P, Torpy DJ. Low-dose hydrocortisone replacement improves wellbeing and pain tolerance in chronic pain patients with opioid-induced hypocortisolemic responses. A pilot randomized, placebo-controlled trial. *Psychoneuroendocrinology*. 2015 Jun;56:157-67. doi: 10.1016/j.psyneuen.2015.03.015. Epub 2015 Mar 14.

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5. **Nenke MA**, Torpy DJ. Addison's disease. Managing "sick days" to avoid crises. *Endocrinology Today* 2014; 3(1): 26-31.

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1. **Nenke MA**, Nielsen ST, Lehrskov LL, Lewis JG, Rankin W, Moller K, Torpy DJ. Pyrexia's effect on the CBG-cortisol thermocouple, rather than CBG cleavage, elevates the acute free cortisol response to TNF-alpha in humans. *Stress*.

2. **Nenke MA**, Zeng A, Meyer E, Lewis JG, Rankin W, Johnston J, Kireta S, Jesudason S, Torpy DJ. Corticosteroid-binding globulin elevation in human pregnancy is due to increased concentration of the circulating high-affinity form. *Journal of the Endocrine Society*.

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Abbreviations

AAT	α1 antitrypsin
AATD	α1 antitrypsin deficiency
AB	Assay buffer
ACTH	Adrenocorticotropic hormone
ANC	Absolute neutrophil count
ANOVA	Analysis of variance
APACHE II	Acute Physiology and Chronic Health Evaluation II
AUC	Area under the curve
AVP	Arginine vasopressin
BMI	Body mass index
BP	Blood pressure
CAR	Cortisol awakening response
CBC	Corticosteroid-binding capacity
CBG	Corticosteroid-binding globulin
COCP	Combined oral contraceptive pill
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
DAS28	Disease activity score in 28 joints
DMARD	Disease-modifying anti-rheumatic drug
EAC	Early arthritis clinic
ELISA	Enzyme-linked assay
ESR	Erythrocyte sedimentation rate
FN	Febrile neutropaenia
GA	Global assessment
GR	Glucocorticoid receptor
haCBG	High cortisol-binding affinity CBG
HC	Healthy control
HPA	Hypothalamic-pituitary-adrenal
HREC	Human research ethics committee
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
HSD	Hydroxysteroid dehydrogenase
ICS	Inhaled corticosteroids
ICU	Intensive care unit
IL	Interleukin
IL-6sR	Interleukin-6 soluble receptor
laCBG	Low cortisol-binding affinity CBG
LOS	Length of stay
MetS	Metabolic syndrome
MIF	Macrophage migration inhibitory factor
NA	Not applicable

NE	Neutrophil elastase
OGTT	Oral glucose tolerance test
OS	Other shock
PBS	Phosphate buffered saline
RA	Rheumatoid arthritis
RAH	Royal Adelaide Hospital
RCL	Reactive centre loop
S	Sepsis
SD	Standard deviation
SEM	Standard error of the mean
Serpin	Serine protease inhibitor
SLPI	Secretory leukocyte protease inhibitor
SJC	Swollen joint count
SS	Septic shock
SS-NS	Septic shock non-survivors
SS-S	Septic shock survivors
TBG	Thyroxine-binding globulin
TJC	Tender joint count
TNF	Tumour necrosis factor
WC	Waist circumference
WCC	White cell count

Preface

The importance of cortisol to human health cannot be underestimated. As the final effector of the hypothalamic-pituitary-adrenal (HPA) axis, a tightly regulated system entrained by light and food intake, cortisol exerts pleiotropic effects that influence cardiovascular stability, growth, metabolism, neurocognition, inflammation and immunity, acting via the ubiquitous glucocorticoid receptor (GR), to maintain homeostasis (Figure I) (Marik *et al.*, 2008). Stress, defined as a state that threatens homeostasis, often leads to inflammatory cytokines release; these cytokines potently stimulate the HPA axis via corticotropin-releasing hormone (CRH). The stress response is coordinated and stressor-specific (Pacak *et al.*, 1998), and results in an up to ten-fold increase in adrenal cortisol production (Salem *et al.*, 1994).

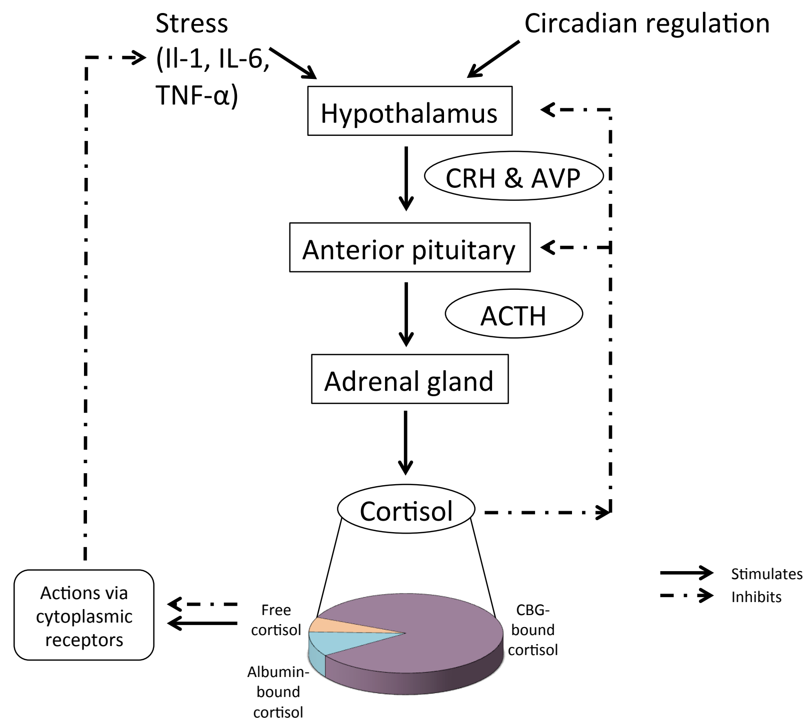


Figure I The hypothalamic-pituitary-adrenal axis. Stress and circadian regulation acting via hypothalamic CRH drives pituitary production of ACTH which stimulates secretion of adrenal cortisol. Cortisol acts via a negative feedback loop to inhibit CRH and ACTH. Cortisol is largely bound to transport proteins. Free cortisol exerts its effects via cytoplasmic receptors.

The free hormone hypothesis posits that only unbound cortisol is biologically active (Mendel, 1989), being able to diffuse across cell membranes to access its cytosolic receptor. Approximately 5–6 % of cortisol circulates free with remaining cortisol bound to the cortisol-binding proteins albumin and corticosteroid-binding globulin (CBG). CBG is a 50 000–60 000 dalton high-affinity but low capacity monomeric α_1-glycoprotein responsible for binding 80–90% of relatively insoluble glucocorticoids. (Slaunwhite and Sandberg, 1959; Dunn *et al.*, 1981; Lewis *et al.*, 2005). Beyond its traditional role in cortisol transport, CBG also provides a buffer to protect tissues from physiologic cortisol fluctuations, as well as reducing cortisol clearance thereby increasing its half-life (Brien, 1981; Bright, 1995). Furthermore, CBG provides a potentially accessible reservoir of stored cortisol to replenish the free fraction in times of stress.

Along with cytokine-mediated activation of the HPA axis, acute inflammation is characterised by a reduction in CBG synthesis (Bartalena *et al.*, 1993; Emptoz-Bonneton *et al.*, 1997; Tsigos *et al.*, 1998), resulting in saturation of CBG and a transient free cortisol spike. In contrast, chronic inflammation is often associated with relative hypocortisolism, that is, attenuated cortisol levels for the given inflammatory stimulus. This may be a protective adaptation or reflect impairment at some level of the HPA axis (Chrousos, 1995; Edwards, 2012). Total CBG has been extensively studied in settings of acute inflammation where levels plummet, for example in burns (Bernier *et al.*, 1998), abdominal surgery (Dimopoulou *et al.*, 2008), cardiac surgery (Gibbison *et al.*, 2015), multi-trauma (Beishuizen *et al.*, 2001), acute pancreatitis (Muller *et al.*, 2007; Gibbison *et al.*, 2015), myocardial infarction (Zouaghi *et al.*, 1984), pre-eclampsia and gamete-recipient pregnancies (Ho *et al.*, 2007), and in septic shock (Beishuizen *et al.*, 2001; Ho *et al.*, 2006) where serum interleukin (IL)-6 levels correlate inversely with CBG levels. Experimental inflammation induced by cytokine infusion

confirms the fall in CBG (Tsigos *et al.*, 1998). In obesity and insulin resistance, states of chronic inflammation, total CBG levels are also reduced (Fernandez-Real *et al.*, 2002; Schafer *et al.*, 2013).

The association between CBG and inflammation is supported by evidence from animal models and humans with CBG deficiency. *Cbg*$^{-/-}$ mice have an increased susceptibility to lipopolysaccharide-induced septic shock, with a significant reduction in survival rates within the first 48 hours being due to the absence of inherent CBG activity rather than a lack of biologically active free corticosterone (Petersen *et al.*, 2006). A second knockout mouse model (Richard *et al.*, 2010) shows blunted corticotropin and corticosterone responses to stress. Additionally, the *SERPINA6* gene has been associated with genetic variation in stress-related HPA axis function in animal models (Solberg *et al.*, 2006). In humans, naturally occurring CBG mutations are associated with a phenotype that includes hypotension and pain-fatigue syndromes (Appendix B; Torpy *et al.*, 2001; Perogamvros *et al.*, 2010; Cizza *et al.*, 2011; Torpy *et al.*, 2012). Pain and fatigue are well known symptoms associated with the phenomenon of hypocortisolism, but available data do not clearly define the mechanism for these and other stress-related disorders (Heim *et al.*, 2000; Fries *et al.*, 2005; Geiss *et al.*, 2012).

The fundamental physicochemical and biosynthetic properties of native human CBG have been well characterised (Reviewed in Meyer *et al.*, 2016). CBG is primarily synthesised by hepatocytes, encoded by the single gene *SERPINA6* encompassing 19 kilobases located on chromosome 14 (q31–q32.1), consisting of five exons, of which four are coding (Underhill and Hammond, 1989; Seralini *et al.*, 1990). The gene is clustered with ten serine protease inhibitor (serpin) genes, including α1 antitrypsin (AAT), that appear to have evolved

relatively recently through gene duplication events (Seralini *et al.*, 1990; Breuner and Orchinik, 2002; Seixas *et al.*, 2007; Moisan, 2010), and produces a 405 amino acid precursor peptide, from which a 22 amino acid hydrophobic leader sequence is cleaved prior to secretion (Hammond *et al.*, 1987). CBG is also produced locally at numerous sites with mRNA having been identified in human endometrium, ovary, fallopian tube, placenta and cardiomyocytes (Misao *et al.*, 1994; Misao *et al.*, 1999a; Misao *et al.*, 1999b; Miska *et al.*, 2004; Schafer *et al.*, 2015). CBG has also been identified in the pituitary, cerebrospinal fluid, hypothalamus, lung, kidney, testis and adipose tissue, although it is unclear whether this is due to local production or internalisation (Perrot-Applanat *et al.*, 1984; Predine *et al.*, 1984; Hammond *et al.*, 1987; Scrocchi *et al.*, 1993; del Mar Grasa *et al.*, 2001; Sivukhina *et al.*, 2006).

However, CBG is a large, complex and highly conserved molecule (Seal and Doe, 1963), adding biological plausibility to the possibility that it may have a more substantial role in cortisol homeostasis, particularly in relation to inflammation. Thus the most insightful commentary over the last three decades has been surrounding the additional roles that CBG may perform. The deduction of the primary structure of CBG in 1987 came with the realisation that CBG shared substantial sequence homology with AAT, the archetypal serpin protein, hence CBG was identified as a clade A serpin, although it does not have inhibitory capacity (Hammond *et al.*, 1987). Pemberton *et al*. (1988) then presented data in a landmark study showing that CBG underwent the conformational "stressed" to "relaxed" change typical of serpins in the presence of neutrophil elastase (NE) at sites of inflammation, *in vitro*, with the post-cleavage CBG molecule (laCBG) having nine-fold reduced cortisol-binding affinity compared with the native, intact form (haCBG; Figure II). Hence widespread views of the function of CBG has progressed towards it being a highly evolved, targeted delivery agent,

capable of supplying anti-inflammatory cortisol to sites of tissue injury, aimed at restraining inflammatory damage (Pemberton *et al.*, 1988; Hammond *et al.*, 1990; Henley and Lightman, 2011; Perogamvros *et al.*, 2012; Chan *et al.*, 2014; Hammond, 2016).

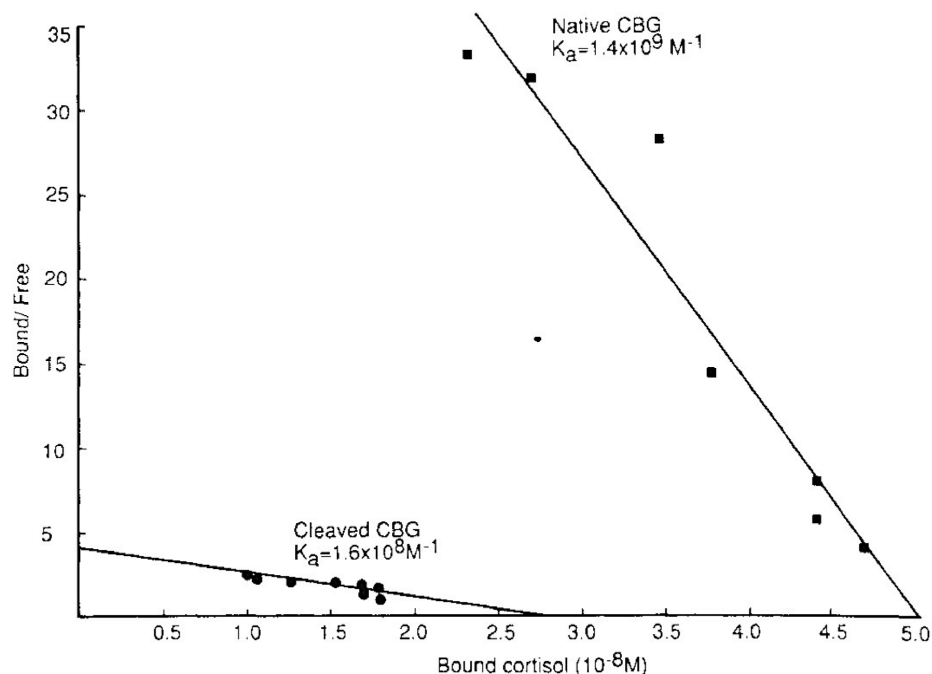


Figure II Binding affinity of native and cleaved CBG. Scatchard plot revealing the fall in cortisol-binding affinity that occurs when CBG is exposed to human neutrophil elastase (Pemberton *et al.*, 1988).

The crystal structures of CBG have only recently been solved, and depict the irreversible molecular transition of haCBG to laCBG following enzymatic cleavage (Figure III). Features of the protein include a fully exposed reactive centre loop (RCL) that acts as a protease "bait" domain and a single hydrophobic glucocorticoid-binding site. Incorporation of the cleaved N-terminal segment of the RCL into the body of the protein triggers rearrangements that influence the flexible binding pocket, efficiently perturbing cortisol binding (Klieber *et al.*, 2007; Zhou *et al.*, 2008; Qi *et al.*, 2011; Gardill *et al.*, 2012). These studies, along with *in*

vitro binding studies and models of cortisol partitioning corroborate the release of cortisol following CBG cleavage, increasing local tissue concentrations of bioavailable cortisol four-fold (Chan *et al.*, 2013; Nguyen *et al.*, 2014).

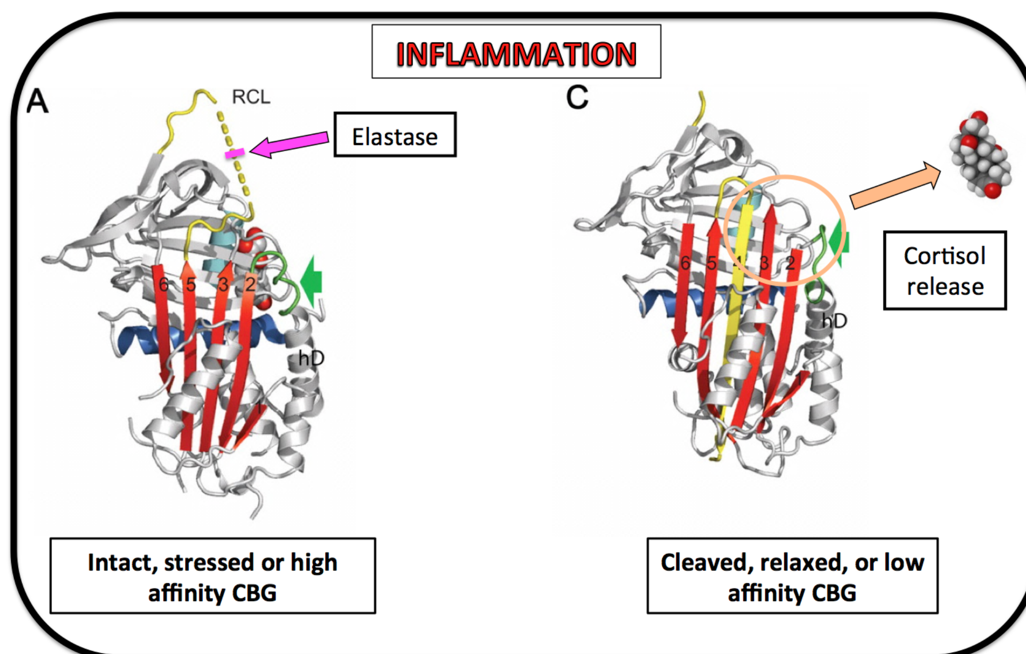


Figure III Molecular mechanism of CBG cleavage and cortisol release. A ribbon depiction of the crystal structure of CBG undergoing conformational change from stressed, haCBG to relaxed laCBG after cleavage and subsequent insertion of the reactive centre loop (RCL; yellow) into the body of the molecule (β sheet A strands numbered 2–6), affecting the plasticity of the binding site and releasing bound cortisol. The steroid-binding pocket is bound by helix H (cyan), helix A (blue) and helix D (hD). Adapted from Qi *et al.*, 2001.

Given the fundamental requirement for cortisol and the clear relevance of CBG to cortisol availability in inflammation, the need to interrogate the haCBG-cortisol → laCBG + free cortisol pathway is obvious. Despite this, the purported cortisol-releasing function has remained an *in vitro* phenomenon despite the recent molecular advancements. In addition, little is known about the properties and functions of haCBG and laCBG (Table I) or about the factors that govern CBG cleavage *in vivo*. If this remarkable process did occur *in vivo*, it

would hold great diagnostic and therapeutic opportunities for inflammatory conditions. On the other hand, consideration would need to be given to the consequences of elevated free cortisol levels, and the ensuing negative feedback effect on both hypothalamic CRH and pituitary adrenocorticotropic hormone (ACTH) as both animal and human studies show that free cortisol, rather than total cortisol, regulates basal cortisol tone (Lewis *et al.*, 2005; Richard *et al.*, 2010).

Table I Known characteristics of haCBG and laCBG.

	haCBG	**laCBG**
Molecular weight (daltons) (Pemberton *et al.*, 1988)	52 000	48 000
Cortisol binding sites (*n*) (Zhou *et al.*, 2008)	1	1
Cortisol-binding affinity (K_A) (Pemberton *et al.*, 1988)	1.4×10^9 M^{-1}	1.6×10^8 M^{-1}
Thermostability (Pemberton *et al.*, 1988)	Decreased	Increased

K_A = Association constant.

We were motivated by clinical conundrums involving relative hypocortisolaemia in acute and chronic inflammation and the lack of information regarding haCBG and laCBG, which together form the major transport system for our body's pivotal stress hormone cortisol. We undertook seven clinical studies to translate the existing basic scientific evidence of the cleavage phenomenon to the clinical setting for the first time, and to determine the relationship of haCBG and CBG cleavage to severity in inflammatory diseases using validated immunoassay techniques further explored in Chapters 1 and 2. The overall aim of this thesis was to determine whether haCBG levels were associated with illness severity and clinical outcomes in inflammatory states. In Chapters 3, 4 and 5 we explore settings of acute inflammation, including septic shock, bacteraemia and experimental cytokine infusion. We compare this to chronic inflammation as seen in the metabolic syndrome and rheumatoid arthritis in Chapters 6 to 8. Finally we examine haCBG levels and cleavage during pregnancy,

a state of sustained hypercortisolaemia, in Chapter 9. We hypothesised that depletion of anti-inflammatory CBG-cortisol is a key mechanism in the pathogenesis of uncontrolled inflammation in systemic inflammatory disorders, in both acute and chronic inflammation.

Chapter 1

Measurement of high-affinity CBG

1.1 Traditional methods for measuring CBG

The discovery of CBG, a protein with high affinity but low capacity for cortisol, was made by three independent groups between 1956–1959 (Brien, 1981), and was soon isolated in 1962 (Seal and Doe, 1962). Initial measurements utilised CBG's binding capacity for cortisol. In these cases, following saturation of CBG with cortisol, free and CBG-bound steroid were separated by one of numerous techniques including gel filtration, equilibrium dialysis, ion-exchange resin and paper electrophoresis (Brien, 1981). The amount of cortisol present in the CBG-cortisol complex was then measured, representing the amount of cortisol required to saturate the ambient CBG, and expressed as binding capacity in μg cortisol/100 mL serum. CBG has also been measured as mg CBG/L serum, by firstly determining cortisol binding capacity and using the knowledge that CBG binds cortisol in a 1:1 ratio and has a molecular weight of approximately 53 000 daltons to estimate the concentration (Doe *et al.*, 1964). These early studies assumed a single binding affinity and were often done at non-physiological temperatures, i.e. 4 °C.

The purification of CBG by affinity chromatography (Rosner and Bradlow, 1971) enabled the development of specific antibodies so that the simpler and more direct techniques of radial immunodiffusion (Van Baelen and De Moor, 1974), radioimmunoassay (Bernutz *et al.*, 1979) and enzyme-linked immunosorbent assay (ELISA) (Fantl *et al.*, 1988) could be employed. In

2003, Lewis *et al.*, developed and validated the first ELISA to use a combination of polyclonal and monoclonal antibodies to CBG (Lewis *et al.*, 2003).

Denaturing electrophoresis has been used to confirm the loss of a 4 000–5 000 dalton C-terminal fragment of protein upon exposure to NE, providing evidence that CBG cleavage occurs *in vitro*. Furthermore, it has been possible to estimate the cortisol-binding affinity of haCBG and laCBG forms for almost 30 years (Pemberton *et al.*, 1988). However, until recently there was no methodology available to measure the individual circulating concentrations of haCBG or laCBG, or any marker to quantitate CBG cleavage in circulation, leaving unanswered questions about the relevance and role of CBG cleavage *in vivo*.

1.2 Development of the high-affinity CBG assay

In 2011, our colleagues in Christchurch, New Zealand, developed parallel monoclonal antibody-based ELISAs that enable the determination of total and haCBG levels *in vivo* (Lewis and Elder, 2011). Using knowledge of the primary sequence of human CBG (Hammond *et al.*, 1987) including the 18 amino acid RCL (Figure 1.1), monoclonal antibodies were raised against the exposed RCL of CBG, incorporating the elastase cleavage site. The consensus epitope sequence that showed complete competition was STGVTLNL (Figure 1.2), corresponding to amino acids 341–348.

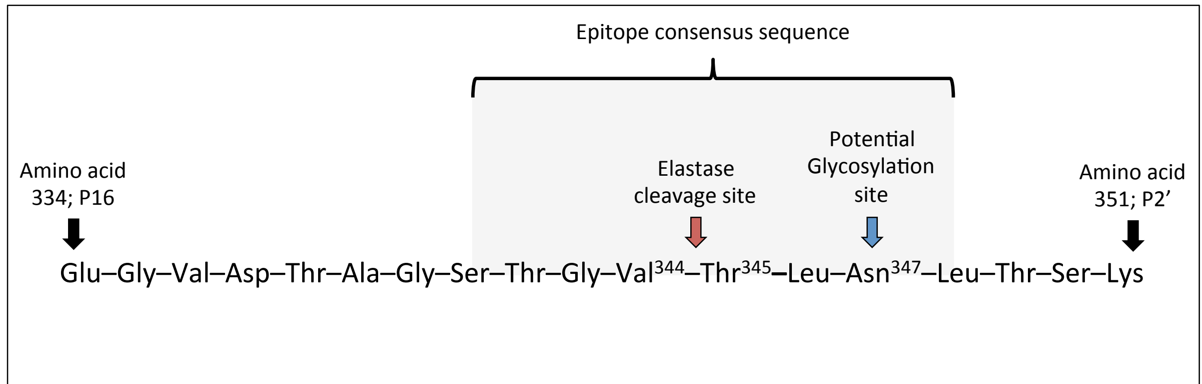


Figure 1.1 Amino acid sequence of the human CBG RCL. The epitope consensus sequence includes the elastase cleavage site and a potential glycosylation site.

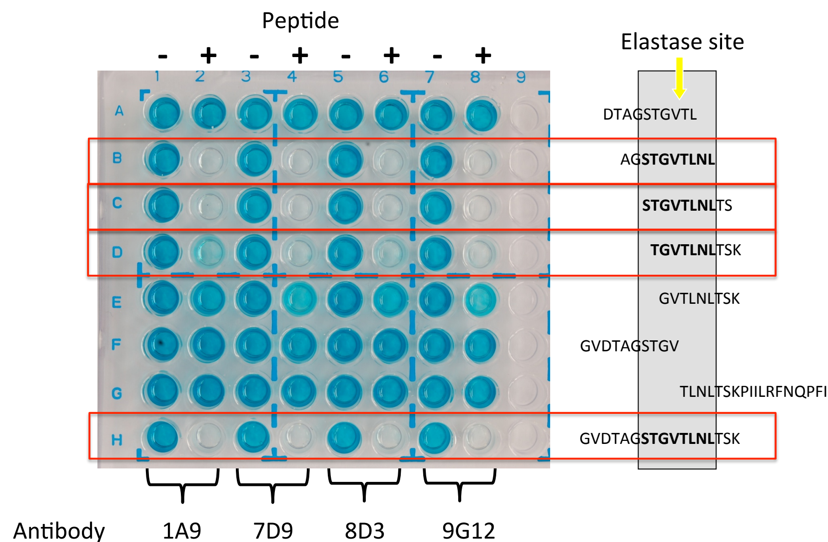


Figure 1.2 RCL monoclonal antibody epitope mapping. Four RCL antibodies 1A9, 7D9, 8D3 and 9G12 were tested in the presence (+) or absence (-) of synthetic RCL peptides. No signal was detected when antibody was applied to a peptide that crossed the elastase cleavage site. Peptide sequences are shown on the right. The consensus sequence that showed complete competition was STGVTLNL (Lewis and Elder, 2011).

Accordingly, treatment of RCL peptide conjugates with human NE resulted in the loss of chromogenic signal from 9G12, supporting cleavage of the protein (Lewis and Elder, 2011). Furthermore, when CBG was measured by ELISA in human serum following timed treatment with NE, immunoreactive CBG levels plummeted rapidly when measured by antibodies spanning the elastase cleavage site (Figure 1.3). These data confirm that the 9G12-based assay detects only intact haCBG. On the other hand, total CBG levels which are measured using 12G2, a separate monoclonal antibody directed against a non-RCL site, did not decline following elastase treatment (Figure 1.3).

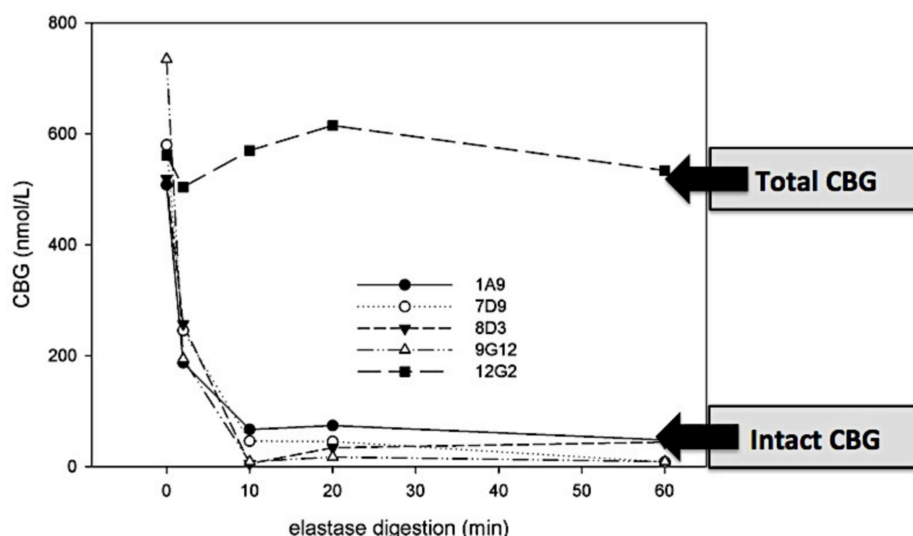


Figure 1.3 CBG measured by ELISA following elastase digestion. RCL monoclonal antibodies 1A9, 7D9, 8D3 and 9G12 lose signal following NE digestion, while total CBG measured by monoclonal antibody 12G2 is unaffected (Lewis and Elder, 2011).

Maintenance of signal following peptide-N-glycosidase F treatment suggests that binding of these antibody is unaffected by glycosylation at the partially utilised consensus site Asparagine^{347}, located within the RCL and 9G12 monoclonal antibody recognition frame

(Lewis and Elder, 2013). Thus using the two monoclonal antibodies 9G12 and 12G2, parallel ELISAs can measure haCBG and total CBG *in vivo* while cleaved, laCBG is deduced by subtraction of intact haCBG from total CBG levels. Notably, haCBG levels become unstable upon a fourth freeze-thaw cycle (Lewis and Elder, 2013).

1.3 Contribution to scientific literature

Using this methodology, our understanding of haCBG and laCBG has grown markedly. Women have higher total and haCBG than men, haCBG has little diurnal variation although there is marked inter-individual variation (Lewis and Elder, 2013). The two affinity forms co-exist in the human circulation under normal physiological conditions, with approximately one-third of CBG being low-affinity (Lewis and Elder, 2013). The half-lives of human haCBG and laCBG in the rabbit are equivalent at approximately 10 hours (Lewis *et al.*, 2015). This was an unexpected finding for two reasons: 1) it had previously been assumed that the cleaved protein would be rapidly removed from the circulation and 2) the half-life of CBG in humans was previously reported to be five days using older radiolabelled techniques (Sandberg *et al.*, 1964). In light of these data, the small residual cortisol-binding affinity of laCBG needs to be considered in settings where laCBG might accumulate. It would provide an extra pool of laCBG-cortisol with added cortisol buffering, but reduce free cortisol levels and the potential for targeted cortisol delivery from haCBG (Nguyen *et al.*, 2014).

The assays have also been used to demonstrate that, in addition to NE, chymotrypsin is able to cleave CBG, resulting in undetectable levels of haCBG while total CBG remains unaffected. A separate group have now found that LasB secreted by *Pseudomonas aeruginosa* is able to cleave CBG, using the older methodologies of Western blotting and cortisol-binding capacity (Simard *et al.*, 2014).

The Coolen's formula: $U^2 \times K(1 + N) + U[1 + N + K(G - T)] - T = 0$, where T is total cortisol, G is total CBG, U is unbound cortisol, K is the affinity of CBG for cortisol at 37 °C and N is the ratio of albumin-bound cortisol (1.74), has traditionally been used to estimate free cortisol, replacing cumbersome direct measurement (Coolens *et al.*, 1987). Gross discordance between total CBG and haCBG levels vastly underestimates free cortisol levels when this equation is applied (Lewis and Elder, 2011; Lewis and Elder, 2013). This emphasises the importance of variable binding kinetics and provided the impetus for a revised equation that takes into account the co-existent pools of measured haCBG and laCBG (Nguyen *et al.*, 2014). The derived model provides an accurate estimation of free cortisol and is also able to demonstrate sequential saturation of haCBG then laCBG, as well as the continual rise in albumin-bound cortisol in states of hypercortisolaemia.

An unexplored potential of the 9G12 antibody is its use in immunohistochemical tissue localisation of haCBG (Lewis and Elder, 2011), to determine whether extravascular CBG remains intact or is cleaved to mediate intracellular glucocorticoid effects (Moisan *et al.*, 2014). Additionally, the identification of patients with either relatively high or very low haCBG, particularly during inflammation, would represent excellent candidates for characterisation of the *SERPINA6* gene looking for mutations which may impact the propensity for CBG cleavage, warranting further study.

1.4 Establishing the assay for research: adversity and adaption

The ELISAs used for this research were developed in Christchurch, New Zealand. Drs John Lewis and Peter Elder provided the methodology and in-house monoclonal and polyclonal antibodies for the studies described in this thesis. Establishing the assay in a new laboratory in

Adelaide, South Australia took considerable time. Adaptions were made to overcome technical difficulties as described herein.

The original standard was prepared from pooled third trimester pregnancy plasma containing phenylmethylsulfonyl fluoride, calibrated against purified intact CBG. The same standard and dilutions were used on each of the 12G2 and 9G12 plates, with the top standard being equivalent to 1280 nmol/L of total CBG and haCBG respectively. When performing the assay here, haCBG levels were consistently and inappropriately higher than total CBG levels.

When routine troubleshooting was unable to rectify this issue, we considered that some cleavage of the standard material may have occurred, effectively increasing the relative level of haCBG in samples tested. We sourced a new standard, a human recombinant CBG (Cat no. 10998-H08H, Sino Biological Inc., Beijing, China). This recombinant CBG (rCBG) includes the full amino acid sequence of CBG, is expressed in human cells and carries a molecular weight of 55 000–60 000 daltons, reflecting its native, glycosylated state. After calculating dilutions to achieve comparable standard concentrations, parallelism was tested successfully (Table 1.1). The new rCBG performs well in the assay, with haCBG being appropriately less than total CBG. Cleavage of the new standard is not expected to occur as the purified rCBG is not exposed to proteases, however, a thorough log of quality controls used in the ELISAs is kept and if the levels of haCBG tend to increase compared to total CBG levels this will be evident and can be addressed promptly.

Table 1.1 Test for parallelism using rCBG as standard. Two independent samples were assessed following serial 1:1 dilution using the 12G2 and 9G12 antibodies against the new commercial rCBG as standard (results in nmol/L).

Sample 1		Sample 2	
Anti-12G2	Anti-9G12	Anti-12G2	Anti-9G12
24	9	12	0
53	37	20	9
111	74	43	23
214	165	87	58
494	315	199	126
847	547	379	234

Two microtitre plates are used in the assay, with the chromogenic signals from samples on each plate being read against the standard on that plate. Ideally, the standard curves produced from each plate would be similar. This proved problematic, with the standard curve from the total CBG/12G2 plate levelling out at higher concentrations, with the apex of the curve being at 400–600 nmol/L where the majority of CBG levels were falling. This resulted in ambiguous and unreliable results above 600 nmol/L. Increasing levels of both the in-house monoclonal 12G2 antibody and the goat anti-mouse IgG_{2a}-horseradish peroxidase (HRP) were tested, as was increasing the incubation times to ensure ligands were fully saturated. As a result the incubation of the 12G2 antibody was increased to 90 minutes and the incubation period for IgG_{2a}-HRP was increased to 30 mins. The pipetting and mixing techniques were checked by an experienced laboratory supervisor and reagents and antibodies were frequently refreshed. Alternative plates were trialled to ensure adequate antibody binding. Plates that were originally washed with an automated washer are now gently hand-washed to avoid displacement. Fresh substrate is made for each assay to avoid substrate exhaustion, but increasing the concentration of the substrate resulted in too rapid colour development (120

seconds) to produce consistent results. Compounding these difficulties, the microtitre plates used in the assay were discontinued, prompting an arduous search for a replacement. The apex for the standard curve has now moved to approximately 1000 nmol/L. (Figures 1.4 and 1.5). Thus, when analysing samples where the expected concentrations exceed this value, the samples are further diluted by ≥1:2 to keep colorimetric detection within the linear segment of the standard curve. A comparison of the new and old methodology is tabulated in Appendix A.

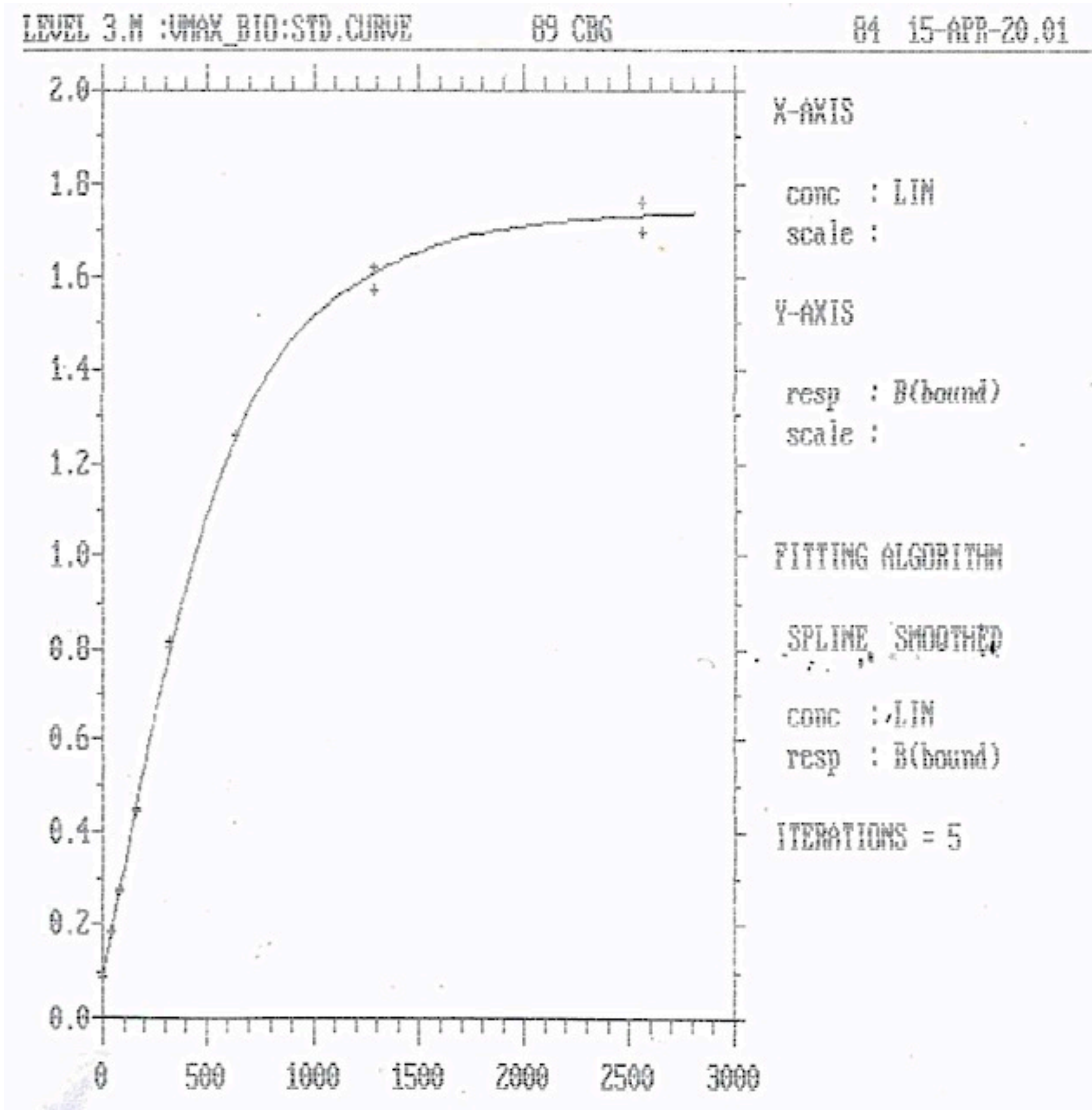


Figure 1.4 Standard curve using commercial recombinant CBG with 12G2 antibody assay

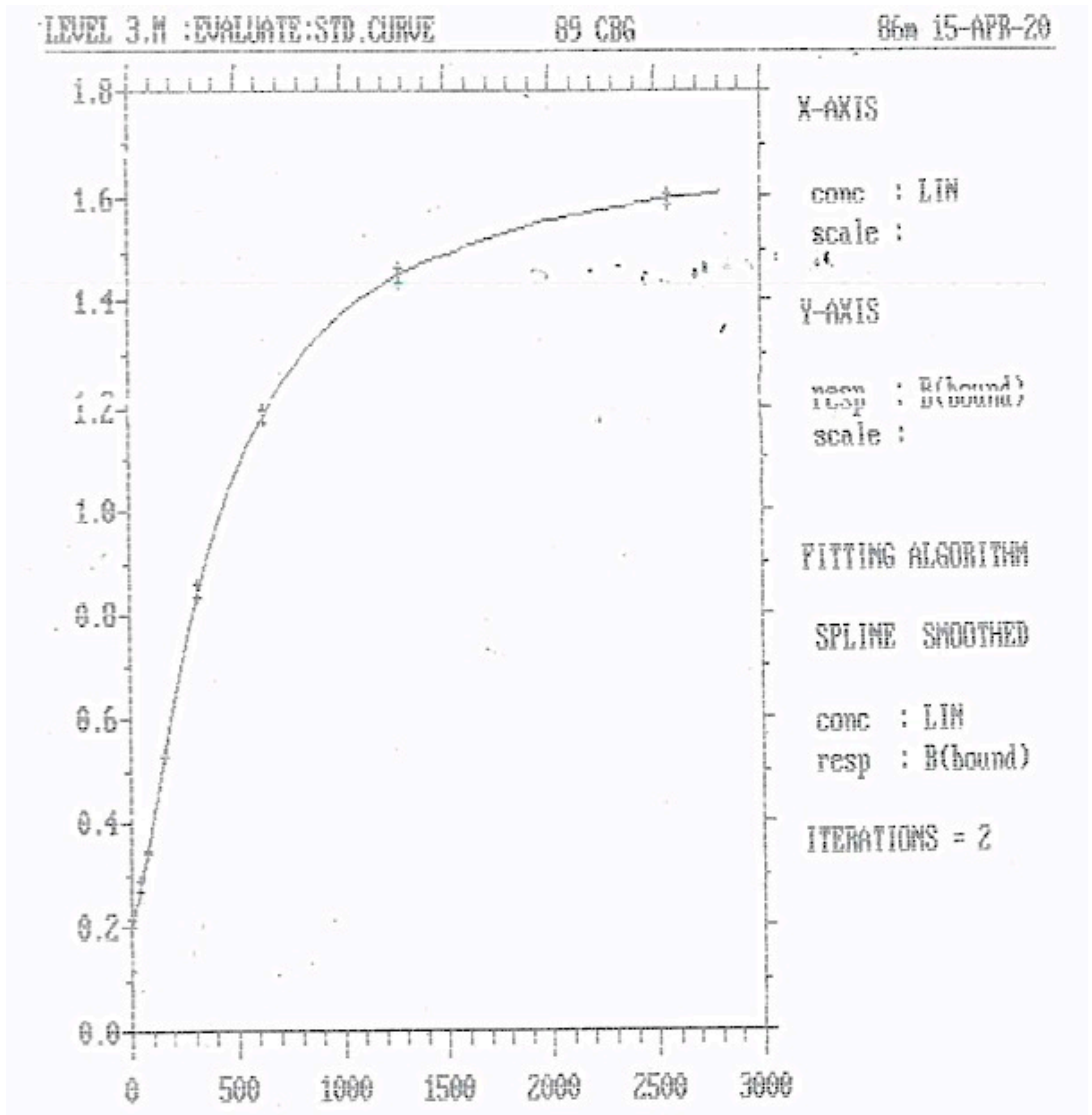


Figure 1.5 Standard curve using commercial recombinant CBG with 9G12 antibody assay

These changes have meant that the assay is more time-consuming and labour-intensive, however the results are more reliable. Additionally, the introduction of a commercially available standard adds greater consistency and sustainability.

1.5 Summary

The novel and well-validated measurement of haCBG and laCBG *in vivo* has facilitated a huge advancement in our understanding of CBG physiology, contributing significantly to peer-reviewed literature over the past five years. It has allowed us to confirm that the haCBG and laCBG coexist, and points toward confirmation of the phenomenon of CBG cleavage as a means of targeted cortisol delivery. The present thesis utilises the modified techniques described in this chapter to translate the *in vitro* evidence of CBG cleavage in inflammation to the clinical setting, including a range of acute and chronic inflammatory conditions, for the first time.

Chapter 2

Methods

2.1 Introduction

This chapter will discuss the methodology for hormone assays that are common to all the studies presented in this thesis. Investigations that were required for only single studies are described in the relevant chapters.

2.2 Hormone assays

2.2.1 Serum total and high-affinity corticosteroid binding globulin

CBG affinity forms were measured in serum using validated two-site non-competitive direct ELISAs. Total CBG and matching haCBG assays were performed in parallel with two internal quality controls to ensure validity. All reagents were firstly brought to room temperature. Two 96-well microtitre plates (Corning® COR3590; In Vitro Technologies, Noble Park North, Victoria) were identically coated with 100µL of the IgG fraction from an in-house rabbit anti-human CBG antibody, diluted in 20 mL phosphate-buffered saline (PBS), and incubated overnight. Plates were washed four times with PBS containing 0.1% Tween–20 (volume/volume) then non-specific binding blocked with 200 µL assay buffer [PBS containing 0.1% Tween–20 (volume/volume) and 0.1% gelatin (weight/volume)] per well. After emptying by inversion, 100 µL of standard or 1:1000 patient serum was added to duplicate wells. Standard was prepared by serially diluting commercially available recombinant human CBG (Cat no. 10998-H08H, Sino Biological Inc., Beijing, China). After

incubation at room temperature for 60 minutes plates were washed and 100 μL/well of appropriate specific in-house monoclonal antibody was added to the respective plates at dilutions of 1:20 and incubated for 90 minutes. Assays with antibody 12G2 gave total CBG levels, and 9G12 gave intact haCBG. After washing, 100 μL/well of diluted peroxidase-conjugated anti-mouse antibodies were added to the respective plates. Goat IgG against mouse IgG_{2a} (GAM/IgG2a/PO, Nordic-MUBio, Susteren, The Netherlands) and rat anti-mouse IgG_1 (Cat No. 559626, BD Pharmingen™, Sydney, New South Wales) were applied to the 9G12 and 12G2 antibodies respectively. After 30 minutes, the plates were washed and 100 μL/well of substrate was added (8.2 g anhydrous sodium acetate and 3.6 g citric acid in 600 mL distilled water, added to 400 mL of methanol containing 270 mg tetramethylbenzadine, with 500 μL of 30% hydrogen peroxide). Colour development was blocked with 100 μL/well 0.9 M hydrochloric acid. Absorbance was read at 450 nm and concentrations were determined by interpolation from the appropriate standard curve generated from antibodies on each plate. laCBG was calculated indirectly by subtraction.

2.2.2 Serum and salivary total cortisol

Total serum cortisol was measured using a commercial electrochemiluminescence immunoassay on a Roche e601 analyser (Roche Diagnostics, Castle Hill, New South Wales).

2.2.3 Serum free cortisol

Free cortisol fraction estimation was performed using an in-house temperature-controlled ultrafiltration/ligand binding method (Lewis and Elder, 2014). [^{3}H]-cortisol (0.1 microcurie, NET396 Perkin Elmer, Boston, Massachusetts) in ethanol was evaporated to dryness in a glass tube. Serum (500 μL) was added and equilibrated for 60 minutes at 37 ºC. A 50 μL

portion was removed for measuring total counts; the remainder was centrifuged for 15 minutes at 18 400 × *g* in an ultrafiltration device (molecular weight cut-off 10 000 Da) that had been preconditioned with assay buffer (VS0102, Sartorius Stedim Biotech, Goettingen). A 50 μL portion of the ultrafiltrate was removed for measurement of free counts. Comparison of the radioactive counts of the free and total portions determined the percentage of free cortisol. Radioactivity was counted in 6 mL of xylene-based scintillant (Irgasafe Plus, Perkin Elmer). Free cortisol was calculated by the equation:

$$\text{Free cortisol} = \text{total serum cortisol} \times \text{free cortisol fraction}$$

This method has previously been validated against the gold standard, equilibrium dialysis, yielding near-identical cortisol values with a correlation coefficient and R^2 for regression of $R = 0.99$ and $R^2 = 0.99$ respectively (Ho *et al.*, 2006).

Chapter 3

Depletion of high affinity corticosteroid-binding globulin in sepsis and septic shock

3.1 Introduction

Sepsis (S), the systemic inflammatory response to infection, and septic shock (SS), involving progression to refractory hypotension and organ hypoperfusion, are critical illness states associated with significant morbidity and mortality (Dellinger *et al.*, 2013). Activation of the HPA axis is crucial in surviving such an episode. Patients with SS have elevated cortisol production and a 50% reduction in cortisol clearance, resulting in increased free and total cortisol levels (Ho *et al.*, 2006; Cohen *et al.*, 2012; Boonen *et al.*, 2013). In addition to restricting inflammation to prevent damage to healthy tissues, cortisol also stimulates the conversion of noradrenaline to adrenaline at the adrenal medulla and enhances the effect of catecholamines on the vasculature (Chrousos, 1995). Recent studies have explored the concept that cortisol levels may predict the circulatory improvements that sometimes occur after exogenous hydrocortisone administration in SS (Annane *et al.*, 2002), however, large trials have found that cortisol levels, whether basal or stimulated, are not predictive of blood pressure or shock reversal (Sprung *et al.*, 2008). Compared with total cortisol, baseline free cortisol measurements are significantly associated with 28-day mortality (Cohen *et al.*, 2012) and may be a better marker of adrenal function in patients with SS (Ho *et al.*, 2006). Current recommendations suggest that "low dose" glucocorticoids (200-300 mg hydrocortisone per day) be considered for patients with SS refractory to fluid resuscitation and vasopressor

agents (Marik *et al.*, 2008), however, the potential for harm including hyperglycaemia and hypernatraemia remains (Dellinger *et al.*, 2013).

CBG, the large glycoprotein responsible for binding 80–90% of circulating cortisol, is a negative acute phase reactant in the setting of inflammation with its synthesis by hepatocytes being inhibited by the pro-inflammatory cytokine IL-6 (Emptoz-Bonneton *et al.*, 1997). Correspondingly, total CBG levels decline acutely in sepsis by up to 50%, and correlate negatively with free cortisol (Ho *et al.*, 2006; Cohen *et al.*, 2012). Data suggest that CBG plays a crucial role in the targeted delivery of cortisol to areas of inflammation, by virtue of its serpin structure and characteristic stressed → relaxed conformational transition (haCBG to laCBG) following enzymatic cleavage by NE, to facilitate the important immunomodulatory actions of cortisol (Pemberton *et al.*, 1988; Hammond *et al.*, 1990). Certainly *in vitro*, human CBG incubated with leukocytes from septic patients results in cleavage, suggesting that CBG is targeted by elastases secreted by activated neutrophils in the physiologic setting of inflammation (Hammond *et al.*, 1990). No data exist as to whether systemic inflammation leads to measurable changes in the cleavage forms of circulating CBG *in vivo*. We investigated total CBG, haCBG and laCBG levels, which provide an *in vivo* indication of CBG cleavage, in patients with critical illness for the first time.

3.2 Hypothesis and aims

The aim of this study was to translate *in vitro* knowledge of CBG cleavage by inflammatory factors, and this new method of distinguishing CBG binding affinity conformations, to the clinical setting.

We hypothesised that altered CBG affinity forms could be measured in the circulation in septic states, and that reduced haCBG levels would correspond to sepsis severity.

3.3 Research design and methods

3.3.1 Patients and design

We conducted a prospective, observational cohort study in an adult tertiary level Intensive Care Unit (ICU) in Adelaide, Australia, between May 2013 and April 2014. The protocol was approved by the Royal Adelaide Hospital (RAH) Human Research Ethics Committee (HREC). Inclusion criteria were: age ≥ 18 years, clinical suspicion of S (infection and ≥ 2 signs of systemic inflammation) or SS (S with hypotension despite adequate fluid resuscitation, adequate intravascular volume status and the need for vasopressors) (American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee, 1992). Vasopressors included any infusion of noradrenaline or adrenaline. Written, informed consent was obtained from patients or their next of kin except in the event of death.

Participants had blood drawn at 9–9:30 am and 4–4:30 pm during their ICU stay. Blood was centrifuged and separated with serum stored at –20 °C until analysed. Participants were enrolled at the time they met inclusion criteria, irrespective of admission date, and were followed until the earliest of either inotrope cessation, ICU discharge, death, or to a maximum of five days. Healthy control data was collected in Christchurch, New Zealand (Lewis and Elder, 2013).

3.3.2 Laboratory measurements

Blood samples were collected for serum total and free cortisol as well as total CBG and haCBG. Hormone assays were described in Chapter 2.

3.3.3 Statistical analysis

Data were analysed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc., San Diego, California). Differences between continuous variables were compared using Student's *t* test for paired samples. *P* value <0.05 was considered significant. One-way analysis of variance (ANOVA) was used to compare continuous variables between the four illness categories. *Post-hoc* analyses included in-built post-test for linear trend or Tukey's multiple comparison test. The effect of mortality was assessed by averaging haCBG levels for each patient (excluding controls) and examining the log-odds of death in quartiles. Results are reported as mean (± SD) except where otherwise stated.

3.4 Results

Forty-three patients were identified for the study. Ten declined consent. Finally, 33 patients were enrolled. One hundred and seventeen samples were analysed; seven patients died, seven were discharged from ICU, and six came off inotropes before all planned samples were collected. On a minority of occasions patients were unavailable for blood sampling. Characteristics of the patient groups including controls are detailed in Table 3.1. There were no differences in the age, gender or Acute Physiology and Chronic Health Evaluation II (APACHE II) scores between the excluded participants and the enrolled patient groups. Enrolled patients were grouped into S, SS survivors (SS-S), and SS non-survivors (SS-NS). Nine enrolled patients were later determined to have a non-infective cause of shock, including cardiogenic shock and multi-trauma, and were therefore grouped as other shock (OS). There

was no age difference between the groups (*P* = 0.65). The median number of samples per patient was 3 (mode 1, range 1–10). Patients were 51.5% female (17/33), with median age 61 years (interquartile range 49–69). The length of ICU stay was 8.4 ± 7.8 days and APACHE II score on admission to ICU was 22.2 ± 7.6. Fourteen of the thirty-three patients underwent surgery within two days prior to or during study enrolment. There was no difference in the number of patients having intra-abdominal or other surgery between the illness groups (*P* = 0.2). Three patients received short term hydrocortisone as treatment for their underlying medical condition (chronic obstructive airways disease and bronchospasm), two patients received hydrocortisone due to prior HPA axis suppression from long term, low dose prednisolone use (for psoriasis and systemic lupus erythematosus), and one received hydrocortisone as empiric treatment for refractory septic shock. These patients were excluded from cortisol analyses. The total and haCBG levels of these patients were within two SD of their group means. There was no difference in albumin levels between the groups (*P* = 0.13, *n* = 80). APACHE II score varied significantly between the groups (*P* = 0.012). The mortality rate of patients in septic shock was 50%.

Table 3.1 Characteristics of critically ill patients and controls by group. Mean (± SD).

	Controls	Sepsis severity			Other shock
		Sepsis	Septic shock – survivors	Septic shock – non survivors	
Participants (*n*)	173	8	8	8	9
Samples (*n*)	173	37	38	23	19
Age (years)	46 (14)	57 (17)	57 (9)	61 (13)	65 (16)
Gender (M:F)	93:80	4:4	2:6	4:4	6:3
APACHE II	NA	20.9 (6.3)	16.3 (3.3)	26.3 (8.1)	25 (8.3)
Albumin (g/L)	NA	23.1 (4.0)	23.7 (6.4)	24.4 (5.7)	27.4 (5.0)
Main source of infection (*n*)					
Pulmonary	NA	4	3	2	NA
Intra-abdominal	NA	1	3	2	NA
Genitourinary	NA	2	1	1	NA
Other [a]	NA	1	1	3	NA
Microbiology (*n*)					
Gram positive	NA	1	1	2	NA
Gram negative	NA	3	2	1	NA
Fungi	NA	0	0	1	NA
Multiple	NA	3	3	3	NA
Not identified	NA	1	2	1	NA
Surgery (*n*) [b]					
Intra-abdominal	NA	0	3	1	2
Other [c]	NA	2	1	4	1
ICU LOS (days)	NA	6.1 (3.8)	14.7 (11.9)	4.9 (3.1)	7.9 (6.5)
Corticosteroid use					
Patients (*n*)	NA	1	1	3	1

NA – not applicable. [a] Other includes cutaneous, cerebrospinal and endocardial infections. [b] Surgery within 2 days prior to or during enrolment. [c] Other surgery includes wound debridement, spinal surgery, abscess incision and drainage and intracranial surgery.

3.4.1 Circulating CBG affinity conformations

Serum CBG levels in healthy controls and in the critically ill cohort are detailed in Table 3.2 and Figure 3.1. Total CBG decreased as illness severity increased, with S *vs*. SS-S and S *vs*. SS-NS both $P < 0.0001$. A linear trend existed for total CBG across sepsis severity ($P < 0.0001$, r = –0.44). Compared with healthy control data, total CBG was 28% lower in the sepsis group, 57% lower in the SS-S group and 56% lower in the SS-NS group.

Table 3.2 Serum CBG and cortisol measurements in critical illness. Mean (± SD).

	Controls	Sepsis severity			Other shock	*P* value[*]
		Sepsis	Septic shock – survivors	Septic shock – non survivors		
Total CBG (nmol/L)	612 (131)	443 (132)	262 (132)	269 (96)	320 (137)	*<0.0001*
haCBG (nmol/L)	411(125)	375 (127)	202 (190)	151 (107)	207 (127)	*0.002*
laCBG (nmol/L)	201 (147)	79 (76)	72 (65)	122 (68)	114 (82)	*0.023*
haCBG: total CBG (%)	68.7 (19.4)	85.1 (19.6)	70.7 (33)	53.4 (29.1)	65.4 (24.7)	*0.0002*
Total cortisol (nmol/L)	NA	703 (203)[a]	914 (1154)[b]	1001 (1522)[c]	1099 (1364)[d]	0.68
Free cortisol (nmol/L)	NA	63 (49)[a]	155 (242)[b]	161 (322)[c]	163 (268)[d]	0.35
Free cortisol (%)	NA	8.3 (4.9)[a]	13.9 (8.9)[b]	10.1 (5.0)[c]	10.9 (5.0)[d]	*0.017*

NA – not applicable. Patients receiving hydrocortisone were excluded from cortisol analyses, thus number of analysed samples were [a]*n* = 26; [b]*n* = 36; [a]*n* = 13; [a]*n* = 17; **P* value denotes one-way ANOVA between the four illness groups. Italics indicate statistical significance.

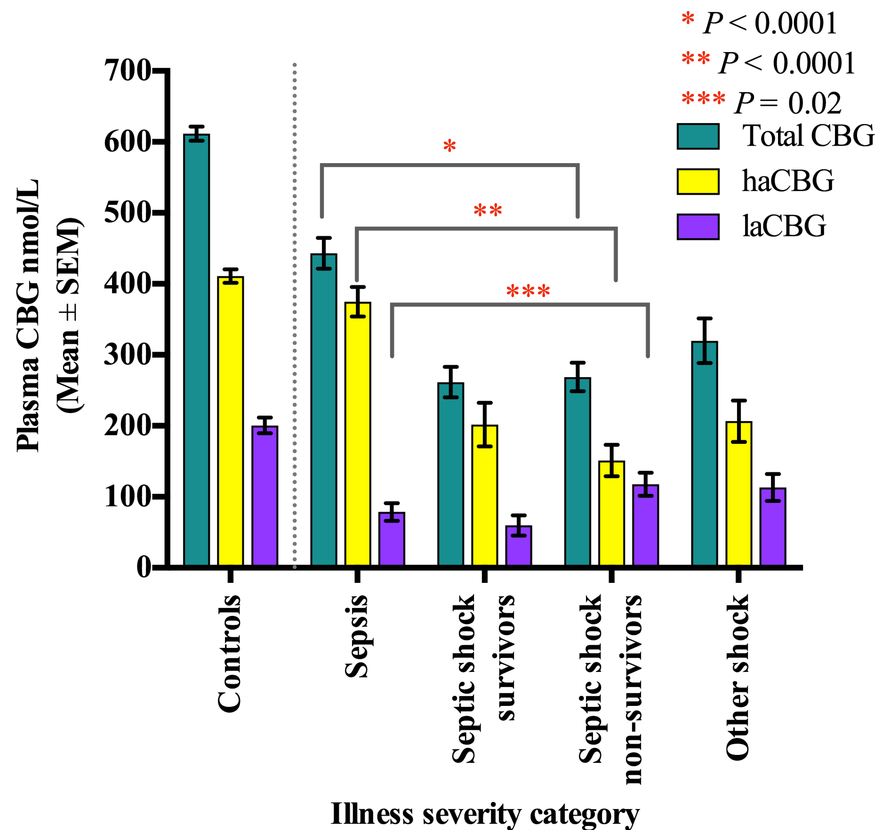


Figure 3.1 Total CBG, haCBG and laCBG in controls and critical illness categories. haCBG decreases as severity of illness increases. [*] For linear trend r = –0.44, P < 0.0001; [**] r = –0.48, P < 0.0001; [***] r = 0.23, P = 0.02.

haCBG levels decreased with illness severity from S → SS-S → SS-NS, with a significant linear trend (P <0.0001, r = –0.48) while laCBG levels increased with a significant linear trend (P = 0.02, r = 0.23). Compared with sepsis alone, those who did not survive septic shock had 39% lower total CBG and 60% lower haCBG. When both categories of septic shock (SS-S and SS-NS) were considered, total CBG and haCBG were 40% and 51% lower than for sepsis alone; 264.3 and 182.7 *vs*. 443.2 and 374.7 nmol/L (for both P < 0.0001). Neither initial haCBG nor lowest haCBG correlated with APACHE II score on admission to ICU (P = 0.63 and P = 0.98 respectively).

The proportion of haCBG decreased (and hence, proportion of laCBG increased) with illness severity in septic patients with a significant linear trend ($P < 0.0001$, r = –0.40). Patients who did not survive septic shock had the lowest proportion of haCBG to total CBG (S *vs*. SS-NS $P < 0.0001$ and SS-S *vs*. SS-NS $P = 0.044$), being significantly lower than controls ($P = 0.0002$).

3.4.2 Serum total and free cortisol concentrations

As opposed to CBG levels, both free and total cortisol concentrations increased non-significantly with illness severity (Table 3.2, Figure 3.2). Patients who survived septic shock had the highest free cortisol fraction (13.9 ± 8.9%), and patients with sepsis alone had the lowest (8.3 ± 4.9%). There was a significant correlation between the proportion of laCBG to total CBG and free cortisol concentrations in the total critically ill cohort ($P = 0.02$, r = 0.24, Figure 3.3). There was no correlation between total CBG and free cortisol ($P = 0.74$).

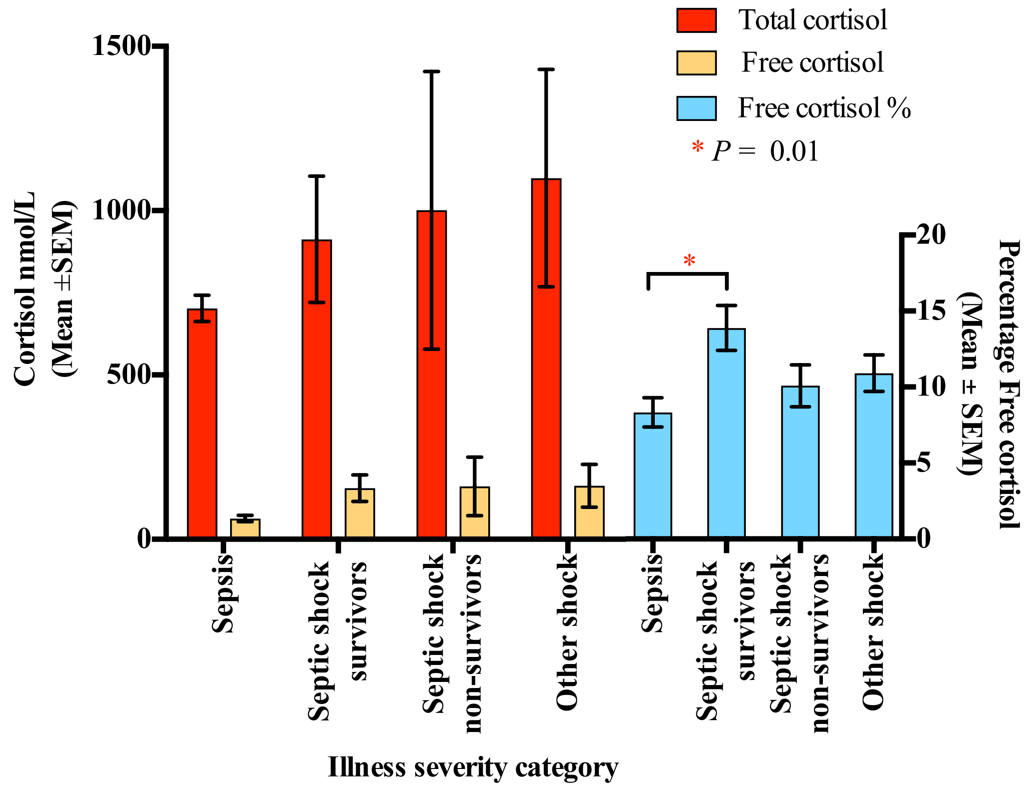


Figure 3.2 Serum free and total cortisol concentrations, with percentage of free cortisol in critical illness categories. Free and total cortisol levels appeared to increase with illness severity although this was not statistically significant. * Percentage free cortisol was significantly higher for SS-S than patients with sepsis alone.

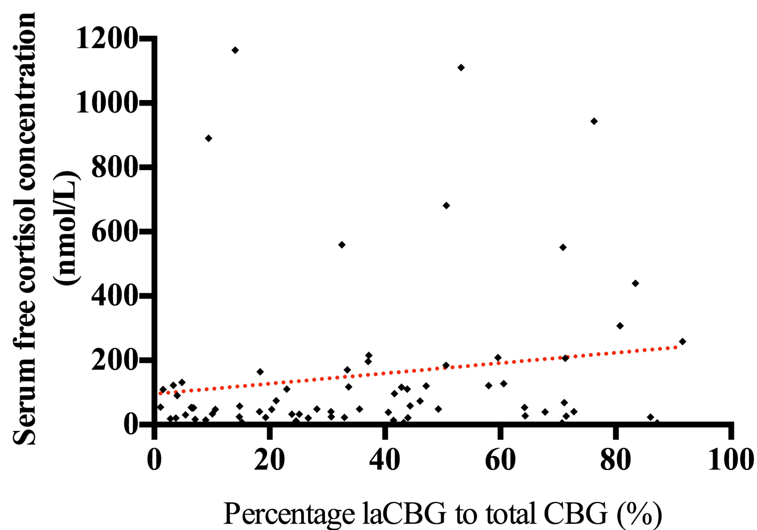


Figure 3.3 Correlation between free cortisol concentration and percentage of laCBG to total CBG in critical illness with line of best fit. For correlation *P* = 0.0008, r = 0.31.

3.4.3 Neutrophils and CBG affinity conformations

We observed significant correlations between the percentage of neutrophils in the white cell count to the percentage of laCBG (P = 0.002, r = 0.60) (Figure 3.4) and to the absolute level of laCBG (P = 0.013, r = 0.50) as determined in a single contemporaneous cross-sectional analysis on the day of enrolment in patients with a septic source. The relation was not seen in OS. There was no difference in the white cell indices between patients with a septic source and those with other shock: white cell count (WCC) 13.7 ± 9.6 *vs*. 11.1 ± 4.0, P = 0.46; absolute neutrophil count (ANC) 11.9 ± 9.1 *vs*. 9.1 ± 3.7, P = 0.39; % neutrophils 85.6 ± 6.9 *vs*. 81.6 ± 10.2, P = 0.2).

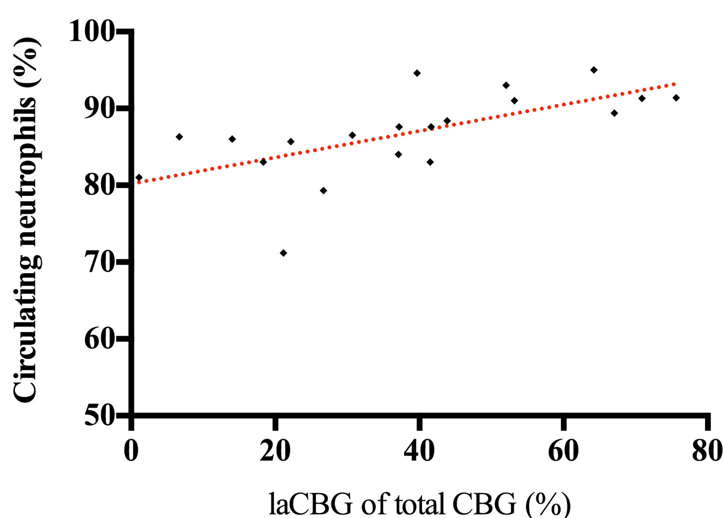


Figure 3.4 Association of the percentage of laCBG to total CBG and percentage of neutrophils to total leukocytes in critical illness with line of best fit. There was a significant and positive correlation between the proportion of laCBG to total CBG and the percentage of circulating neutrophils on the day of enrolment. *n* = 24.

3.4.4 Relationship of haCBG to mortality

An inverse linear relationship is suggested between haCBG levels and mortality.

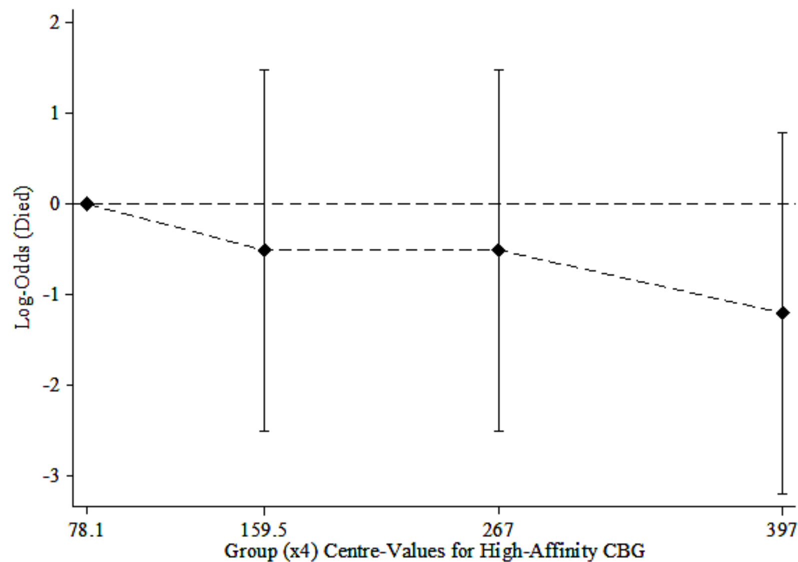


Figure 3.5 Relationship of haCBG to mortality in critical illness. There appears to be an inverse linear relationship between haCBG and mortality. haCBG levels are divided into quartiles.

3.5 Discussion

We have shown for the first time that haCBG levels decrease markedly with sepsis and septic shock, in association with the sepsis severity category; haCBG fell by 9% in sepsis to 51% in septic shock survivors and 62% in septic shock non-survivors relative to controls. These falling haCBG levels concord with *in vitro* data suggesting that CBG is cleaved, converting haCBG to laCBG, in sepsis. Similar effects were seen in non-septic shock, another state of profound stress. High affinity CBG levels correlated much more closely to illness severity than did total or free cortisol, although a significant relationship was seen between CBG affinity forms and free cortisol concentration (Figures. 3.1–3.3). The circulating neutrophil

proportion of total leukocytes was correlated with a rising proportion of laCBG (Figure 3.4). haCBG may also be a potential predictor of mortality (Figure 3.5).

In acute inflammation, the combined effect of reduced haCBG, reduced CBG synthesis and increased cortisol secretion and can be expected to elevate free cortisol levels, facilitating the pleiotropic benefits of cortisol. However, despite generally elevated free cortisol levels, only a small portion may reach the inflamed sites as cortisol is distributed widely. As a specific delivery molecule through its stressed → relaxed conformational change, CBG is able to target the distribution of cortisol to inflammatory sites. Depletion of haCBG and therefore loss of the haCBG-cortisol reservoir, as seen here in septic critical illness, could result in a failure of cortisol supply to meet the local demand thereby perpetuating excessive and ultimately chronic inflammation. Furthermore, studies of individuals with CBG deficiency suggest that it is free rather than total cortisol that is regulated by glucocorticoid feedback (Lewis *et al.*, 2005). Hence, preservation of free cortisol levels by both declining haCBG and reduced cortisol metabolism (Boonen *et al.*, 2013) may inhibit central control of the HPA axis, producing lower HPA axis drive. In this way, during chronic inflammation, both local cortisol delivery and drive to cortisol production may be impaired contributing to uncontrolled inflammation. Given the sharp fall in haCBG that we observed, particularly in those who succumbed to their SS, it is tempting to speculate that there is an absolute level of haCBG below which cortisol is unable to effectively execute its counter-inflammatory functions at the cellular level, due to impaired delivery. If this is the case, haCBG levels may be of utility in predicting benefit from exogenous glucocorticoids given that in SS, cortisol levels *per se* are of limited value for this purpose (Sprung *et al.*, 2008).

We also observed a significant correlation between the percentage of circulating neutrophils and the relative and absolute levels of laCBG. CBG is known to interact with NE on the surface of activated neutrophils *in vitro* (Hammond *et al.*, 1990) resulting in CBG cleavage. Circulating cytokines during S stimulate neutrophil maturation, margination and release from the bone marrow (Brown *et al.*, 2006). Given that white cell indices did not vary between septic and non-septic groups (Figure 3.4), but the correlation between CBG cleavage and neutrophil percentage did, we hypothesise that the neutrophils present in septic patients are more likely to be activated than those in non-septic patients, thus generating greater cleavage. This has not previously been shown *in vivo*, and requires further dedicated analysis.

Surgery, particularly intra-abdominal surgery, is known to activate the HPA axis. Dimopoulou *et al*. showed that CBG levels drop immediately after intra-abdominal surgery, but recover by day 2 post-operatively (Dimopoulou *et al.*, 2008). Post-operative total CBG levels at 474 nmol/L, however, remain higher than the levels seen in our patients with S or SS, thus we do not believe that surgery was a confounding influence in our study. As hydrocortisone therapy is known to increase free and total serum cortisol, patients who received hydrocortisone were excluded from the cortisol analyses. However, *in vitro,* acute glucocorticoid administration does not appear to effect CBG secretion by hepatocytes (Emptoz-Bonneton *et al.*, 1997; Mihrshahi *et al.*, 2006) and *in vivo,* the binding capacity of cortisol for CBG and total CBG is decreased in patients receiving long-term glucocorticoids (Schlechte and Hamilton, 1987; Frairia *et al.*, 1988), but not in those receiving acute glucocorticoids (Frairia *et al.*, 1988), thus we included patients receiving hydrocortisone in our CBG analyses.

The plasma disappearance times of haCBG and laCBG were not known at the time this study was undertaken. More recent investigations have shown that human haCBG and laCBG, when injected into rabbits, have identical half-lives of approximately 10 hours (Lewis *et al.*, 2015). This would suggest that the elevated laCBG levels in SS are maintained and would compound the effect of haCBG-cortisol depletion (Lewis *et al.*, 2015). The persistent pool of laCBG-cortisol then also needs to be considered, including the impact of increased cortisol buffering on HPA axis feedback.

Limitations of our study include the modest number of participants and lack of large sets of serial samples. A greater number of serial samples would have allowed us to observe changes in haCBG over time to the point where a patient recovers from or succumbs to their illness. Furthermore, our study hints towards a relationship between haCBG and risk of death. This needs to be further examined in a dedicated mortality study. Additionally, the number of patients harbouring specific pathogens was small, thus we were unable to comment on the effect of individual infections on CBG cleavage; this requires further research. Once a patient was categorised into one of the four groups, all their samples were analysed as such. Given the continuum that exists between these critical illness states, it is possible that there was some crossover in the samples that cannot be accounted for. In common with other studies of S and SS, the group were heterogeneous in terms of previous and concurrent comorbidities, medications and duration of illness. APACHE II scores are a predictor of mortality and are calculated upon entry to ICU. As they are not a dedicated measure of sepsis severity and given that some of our patients did not develop S or SS until much later in their admission, a lack of correlation between APACHE II score and haCBG is understandable. Finally, when this study was undertaken, it was not known what effect common variables, for example body mass index (BMI), had on haCBG and laCBG levels. Subsequent studies in healthy

volunteers (Chapter 6) show that while total and laCBG levels decrease in patients with markers of obesity, haCBG increases, suggesting CBG cleavage resistance (Nenke *et al.*, 2016c). Anthropometric measures could not be taken in the current study due to the patients' critical condition, however, we do not believe body habitus confounded our results, and if anything, accounting for cleavage resistance would have strengthened the findings.

3.6 Conclusion

In summary, haCBG falls dramatically in critical illness and is related to illness severity, with the lowest levels seen in non-survivors of septic shock. This is the first *in vivo* evidence that CBG cleavage occurs as a result of sepsis in humans. There are possible implications for cortisol transport in sepsis and acute systemic inflammation. The notion that haCBG levels may be a useful predictor of clinical outcomes or response to exogenous glucocorticoids needs to be further explored.

Chapter 4

Mechanisms of CBG depletion in early inflammation

4.1 Introduction

CBG, the major transport protein for glucocorticoids, binds up to 90% of circulating cortisol, with only 5–6 % being free and biologically active (Mendel, 1989; Lewis *et al.*, 2005). Importantly, CBG is able to regulate free cortisol levels in three known ways during inflammation. First, CBG targets the delivery of cortisol to areas of inflammation by NE-based proteolysis (haCBG → laCBG) (Pemberton *et al.*, 1988; Hammond *et al.*, 1990), increasing local bioavailable cortisol four-fold (Chan *et al.*, 2013). Second, hepatic CBG production is down-regulated by IL-6 (Bartalena *et al.*, 1993). Correspondingly, total CBG levels fall within 24 hours following IL-6 infusion (Tsigos *et al.*, 1998) and in septic shock (Nenke *et al.*, 2015). These decreased levels of CBG are more readily saturable, leaving a greater proportion of cortisol free. Finally, steroid-binding is temperature-dependent with pyrexia further accentuating the rise in free cortisol. Hence, cortisol can be released from CBG at increasing physiologic temperatures independent of enzymatic cleavage (Cameron *et al.*, 2010; Chan *et al.*, 2013), with cortisol-binding affinity falling four-fold and free cortisol concentrations increasing exponentially as temperature goes from 37 °C to 42 °C, *in vitro* (Figure 4.1). The mechanism proposed involves a reversible 'flip-flop' of the intact RCL partially into β-sheet A, again inducing disruption of the flexible steroid-binding site (Qi *et al.*, 2011). This provides an additional method to increase cortisol bioavailability during pyrexia.

In contrast, some studies have suggested early hepatic release of CBG following stress, delaying the free glucocorticoid response (Qian *et al.*, 2011).

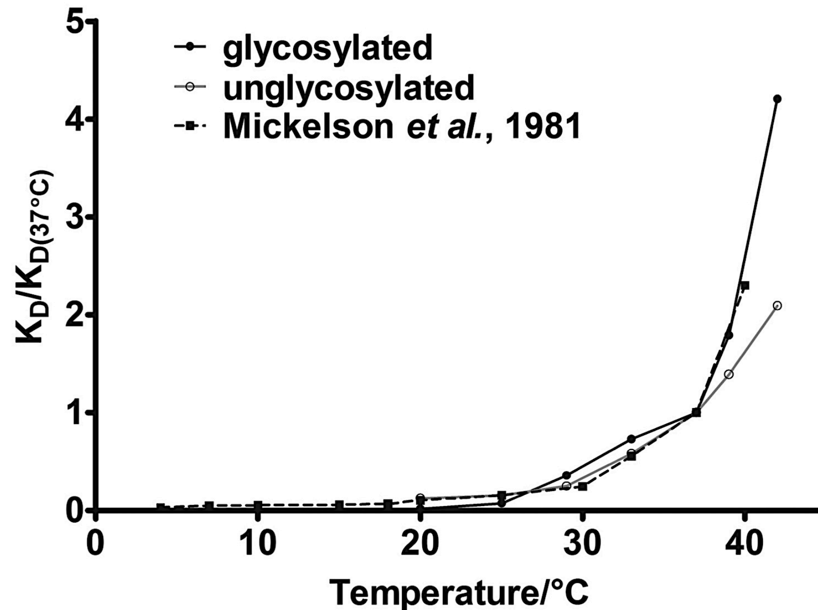


Figure 4.1 The CBG-cortisol thermocouple. The cortisol-binding affinity of haCBG decreases at increasing physiological temperatures, an effect that is partially glycosylation-dependent (Chan *et al.*, 2013).

Infection and tissue damage stimulate the sequential production of pivotal pro-inflammatory and pyrogenic cytokines including tumour necrosis factor (TNF)-α, IL-1β and IL-6 (Figure 4.2) (Elenkov and Chrousos, 2006). IL-6 stimulates the HPA axis at several sites to increase adrenal production of anti-inflammatory cortisol (Chrousos, 1995). Infusion of cytokines, such as TNF-α, can be used in healthy humans as *in vivo* models to mimic systemic inflammation (Michie *et al.*, 1988b). These experiments can provide mechanistic insights into cytokine effects, free of confounding factors encountered in clinical studies of systemic inflammation (as seen in Chapter 3), including concomitant medication administration, comorbidities, variations in duration of inflammatory illness and the risk of mortality. TNF-α

infusion stimulates the production of IL-6 (Nielsen *et al.*, 2013), stimulates the HPA axis (Michie *et al.*, 1988b), reduces CBG levels (Fleshner *et al.*, 1997) and increases NE activity (Owen *et al.*, 1997), potentially activating CBG cleavage.

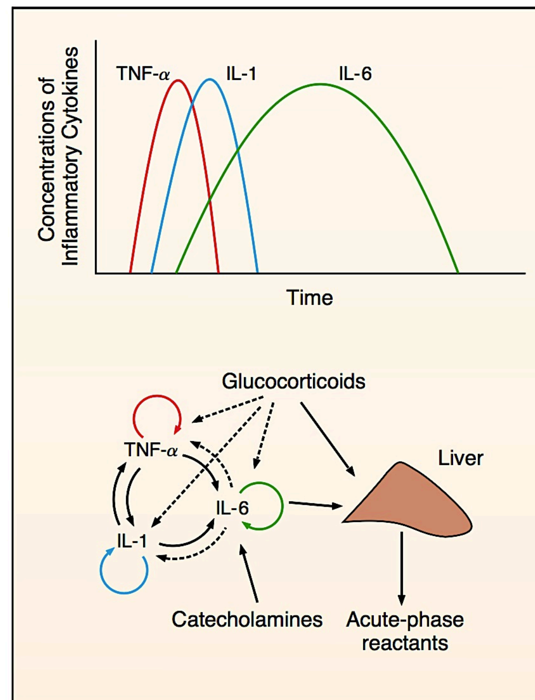


Figure 4.2 Sequential production of cytokines at inflammatory sites. Each cytokine stimulates its own production. IL-6 influences the hepatic production of acute phase reactants. Glucocorticoids are anti-inflammatory and inhibit the production of all three cytokines. Solid lines denote stimulation, dashed lines denote inhibition (Chrousos, 1995).

Levels of haCBG are measurable by ELISA and provide an assessment of CBG cleavage *in vivo*. HaCBG levels fall dramatically during septic shock (Chapter 3, Nenke *et al.*, 2015), however the time kinetics for the depletion of haCBG and the mechanisms of the free cortisol rise immediately following an inflammatory stimulus are unknown. We have used a TNF-α infusion in healthy human volunteers to study the early phase kinetics of CBG cleavage in systemic inflammation and assess the effect of pyrexia on free cortisol levels.

4.2 Hypothesis and aims

The aim of this study was to determine the time kinetics of haCBG reduction following an inflammatory stimulus and whether body temperature or CBG cleavage is more closely associated with increases in free cortisol. We hypothesised that haCBG levels would fall compared to controls, indicating the commencement of inflammation-stimulated CBG cleavage.

4.3 Research design and methods

4.3.1 Participants and Study Design

Details of the enrolment of participants and study design have been published previously (Nielsen *et al.*, 2013). The original protocol was approved by the Scientific Ethics Committee of the Capital Region of Denmark. Twelve healthy male participants underwent a crossover study including a six-hour intravenous infusion of either recombinant TNF-α or normal saline, with the administration of an oral glucose tolerance test (OGTT) at two hours. The TNF-α solution (10 ng/mL) was infused at a rate of 1000 ng/m^2 per hour. Blood samples, tympanic temperature, and data regarding disease symptoms were collected at the commencement of the infusion, at 120 minutes (immediately prior to OGTT), 180, 240, 300 and 360 minutes (serial temperature data was only collected during TNF-α infusion). Samples were stored at – 80 °C until they were shipped between institutions on dry ice.

4.3.2 Measurements

Blood samples were analysed for serum total and free cortisol as well as total CBG and haCBG. Hormone assays were described in Chapter 2. Intra-assay coefficients of variation were 2.8% and 2.7% for total and haCBG respectively.

4.3.3 Statistical Analysis

Data were analysed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc., San Diego, California). Results are expressed as mean ± SEM unless otherwise stated. $P < 0.05$ was considered statistically significant. Repeated-measures two-way ANOVA with matching for both factors was used to evaluate the effect of treatment (saline or TNF-α) and time. For temperature data, repeated-measures one-way ANOVA was used.

4.4 Results

Full data sets were available from twelve male participants and these were used in the CBG analyses. Free cortisol data was unavailable for three subjects because of insufficient sample volumes. Characteristics of the participants include: age 24.3 ± 0.9 years; body mass index 23.7 ± 0.3 kg/m^2; body surface area 2.05 ± 0.03 m^2. TNF-α infusion was associated with a six-fold increase in serum TNF-α (from 3.0 ± 1.2 to 18.0 ± 1.3 pg/mL; time: $F_{3,27} = 43.85$, $P < 0.001$; treatment: $F_{1,9} = 288.0$, $P < 0.001$; interaction: $F_{3,27} = 43.22$, $P < 0.001$ compared to controls) and an eight-fold increase in IL-6 levels (from 3.1 ± 1.7 to 23.2 ± 2.9 pg/mL; time: $F_{3,27} = 30.89$, $P < 0.001$; treatment: $F_{1,9} = 58.83$, $P < 0.001$; interaction: $F_{3,27} = 17.78$, $P < 0.001$ compared to controls; Figure 4.3). Body temperature was normal at the beginning of the saline treatment and no symptoms were reported during the infusion. In comparison, the TNF-α infusion was with associated systemic symptoms of inflammation including a significant increase in body temperature (time: $F_{6,66}=58.3$, $P<0.001$) as well as malaise and discomfort (Nielsen *et al.*, 2013) (Figure 4.4).

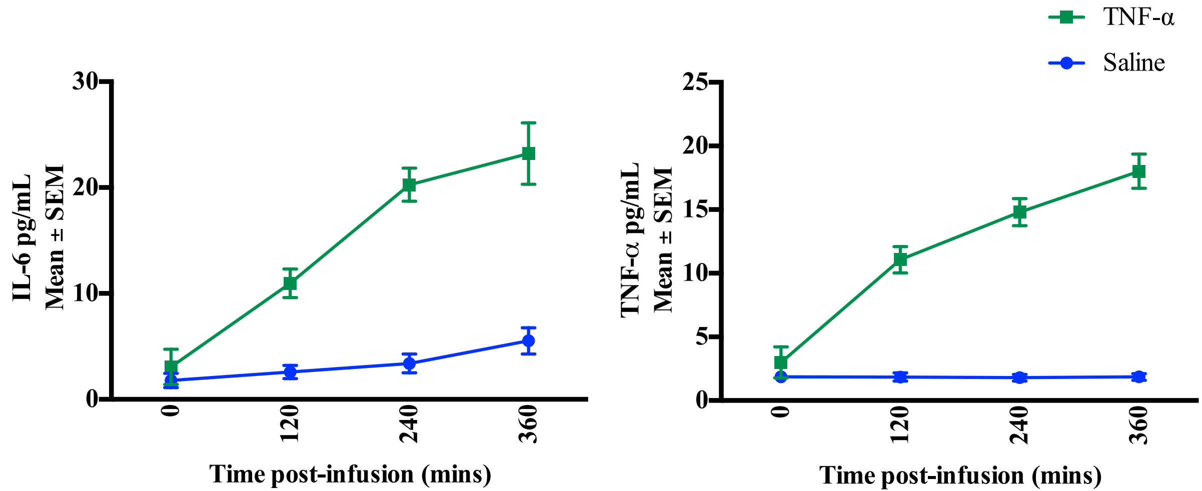


Figure 4.3 **IL-6 and TNF-α concentrations following TNF-α infusion (Nielsen *et al.*, 2013).**

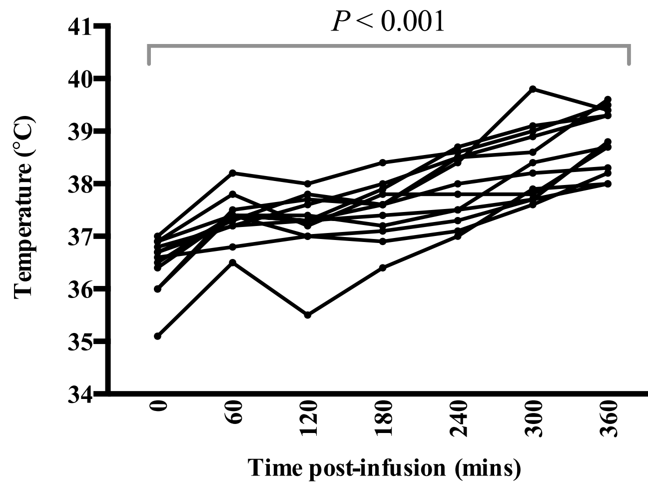


Figure 4.4 **Increase in body temperature over the duration of TNF-α infusion (Nielsen *et al.*, 2013).**

Mean serum CBG and cortisol levels at baseline were not different between the saline and TNF-α infusion groups (Figure 4.5 and 4.6). Total CBG, haCBG and laCBG decreased with

time during both TNF-α and saline infusions ($F_{5,55} = 8.79$, $P < 0.001$; $F_{5,55} = 5.95$, $P < 0.001$; $F_{5,55} = 4.60$, $P = 0.001$ respectively) while there was no change with time for % haCBG:total CBG ($F_{5,55} = 1.72$, $P = 0.15$). There was no effect of TNF-α treatment on CBG or its affinity forms in this time frame.

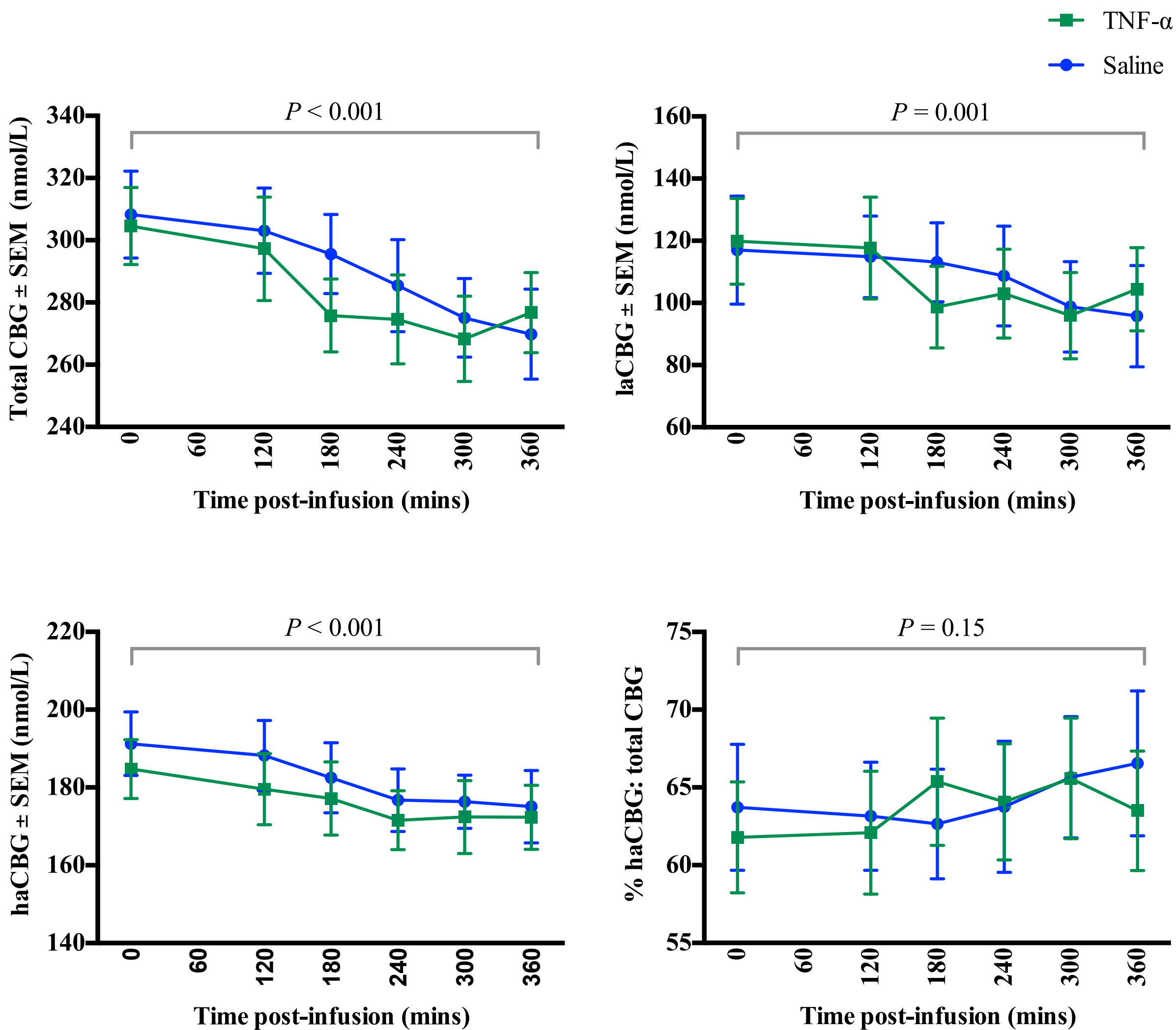


Figure 4.5 CBG levels following TNF-α and saline infusions. Total, haCBG and laCBG levels all fell over time, with no effect of infusion.

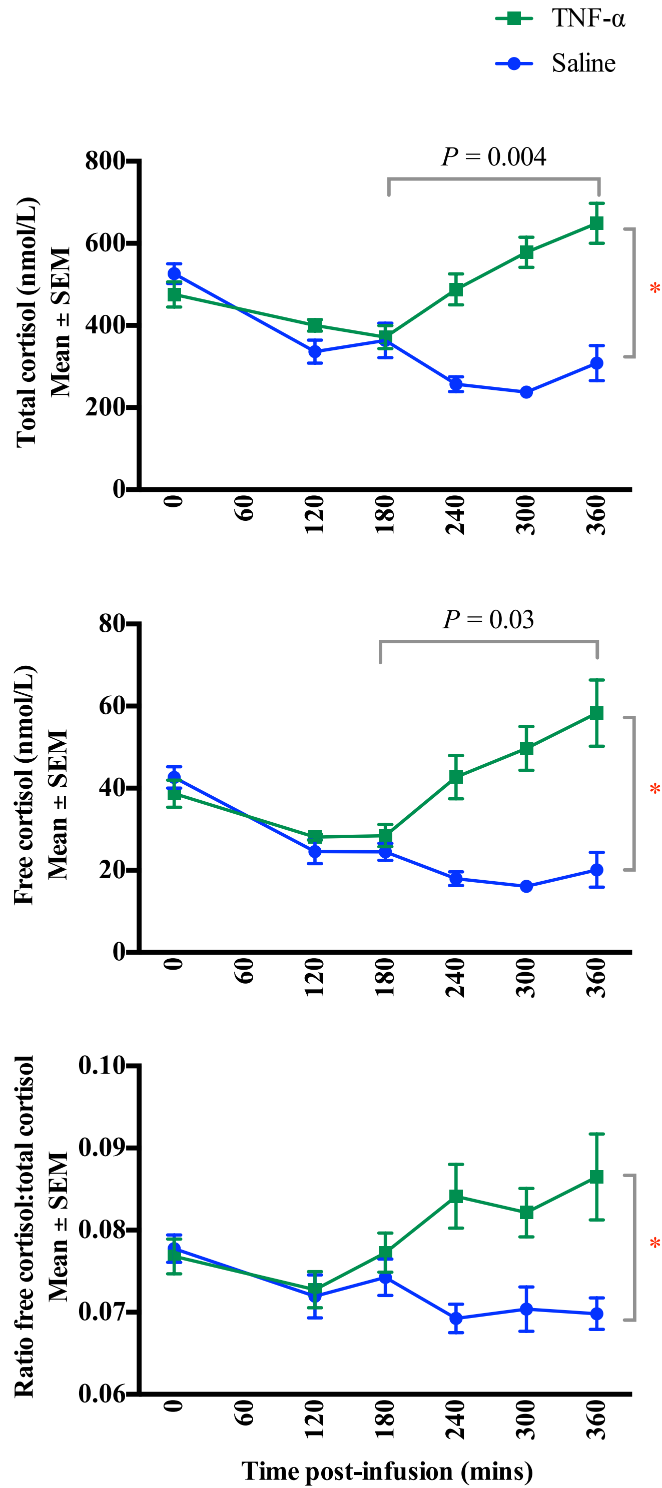


Figure 4.6 Cortisol levels following TNF-α and saline infusions. Total cortisol, free cortisol and the ratio of free:total cortisol increased during TNF-α infusion compared to saline. * $P < 0.01$ for effect of treatment.

Overall, there were differences in total cortisol, free cortisol and the free cortisol fraction across the duration of the infusions (For total cortisol time: $F_{5,55} = 7.93$, $P < 0.001$; treatment: $F_{1,11} = 40.17$, $P < 0.001$; interaction: $F_{5,55} = 16.91$, $P < 0.001$. For free cortisol time: $F_{5,40} = 5.41$, $P < 0.001$; treatment: $F_{1,8} = 29.04$, $P < 0.001$; interaction: $F_{5,40} = 13.79$, $P < 0.001$. For free fraction of cortisol time: $F_{5,40} = 1.26$, $P = 0.30$; treatment: $F_{1,8} = 15.29$, $P = 0.005$; interaction: $F_{5,40} = 7.00$, $P < 0.001$). Total and free cortisol both declined during the first 2–3 hours of the TNF-α and saline infusions. However, from 3–6 hours the TNF-α group exhibited a 2.3 fold rise in free cortisol (time: $F_{3,24} = 3.59$, $P = 0.029$; treatment: $F_{1,8} = 30.45$, $P < 0.001$; interaction: $F_{3,24} = 12.37$, $P < 0.001$) and a 1.9 fold increase in total cortisol (time: $F_{3,33} = 5.44$, $P = 0.004$; treatment: $F = 1.11$, $P < 0.001$; interaction: $F_{3,33} = 17.67$, $P < 0.001$), compared to stable cortisol levels in the saline group (Figure 4.6). The distinct rise in free cortisol (27 to 62 nmol/L) from 3 hours in association with a less marked rise in total cortisol (341 to 645 nmol/L), led to a rise in free:total cortisol ratio (treatment: $F_{1,8} = 20.56$, $P = 0.002$; interaction: $F_{3,24} = 4.20$, $P = 0.016$). This disproportionate rise in free cortisol in the TNF-α group was not associated with a fall in total CBG level (suggesting reduced synthesis), or reduced haCBG (a measure of cleavage). It is also unlikely to be related to cortisol saturation of CBG as CBG was already largely saturated at the time of the precipitous free cortisol rise given its 1:1 molecular binding and the ambient CBG levels of ~ 300 nmol/L. The rise in free cortisol at this early time in inflammation may reflect a weakening of the thermocouple for CBG-cortisol binding as participants were experiencing mild pyrexia at the time.

Following the OGTT, there was a small rise in glucose levels in both saline and TNF-α infusion groups from 5.0 ± 0.1 to 8.7 ± 0.4 nmol/L and 5.1 ± 0.1 to 9.2 ± 0.4 nmol/L. There was no effect of treatment on glucose levels ($F_{1,22} = 0.73$, $P = 0.4$).

4.5 Discussion

This study examines for the first time the early phase of cortisol-CBG kinetics following injection of the pivotal inflammatory cytokine TNF-α. We have shown that total CBG, haCBG and laCBG levels in the first six hours are no different to healthy controls, suggesting that CBG cleavage does not occur in the immediate post-inflammatory period. In addition, the hepatic output of CBG is neither increased nor suppressed within six hours. Total, free and fraction of free cortisol all increase following TNF-α infusion, in association with pyrexia. An elevation of the free:total cortisol ratio may reflect a weakening of the CBG-cortisol thermocouple.

Several studies have shown that total serum CBG decreases in the days-to-weeks after inflammatory stressors such as burns, multi-trauma, septic shock and pancreatitis insult (Pugeat *et al.*, 1989; Bernier *et al.*, 1998; Beishuizen *et al.*, 2001; Muller *et al.*, 2007; Nenke *et al.*, 2015). This is likely due to suppressed hepatic production of CBG, with *in vitro* studies showing that IL-6 inhibits CBG synthesis from Hep G2 cells in a dose- and time-dependent manner (Bartalena *et al.*, 1993), consistent with the presence of a *cis*-regulatory element for IL-6 in the promoter sequence of the rat *CBG* gene (Underhill and Hammond, 1995). *In vivo*, a single injection of IL-6 at 3 μg/kg, a dose known to strongly stimulate the HPA axis and mimic levels reported in sepsis, caused a 50% reduction in CBG levels at 24 hours (Tsigos *et al.*, 1998). Circulating IL-6 levels increased progressively in our study following TNF-α infusion to levels similar to those seen in common infections, but did not reach levels seen in patients experiencing septic shock or major trauma (Araujo *et al.*, 2015; Feng *et al.*, 2016; Mors *et al.*, 2016). Despite this, we did not observe a fall in total serum CBG over 6 hours, perhaps due to the long circulating half-life of CBG. This suggests that lower total CBG is not part of the hyper-acute response to stress which elevates free cortisol. On the other hand,

murine data have suggested an acute 30–40% *increase* in total CBG levels following moderate to severe psychological stress, due to hepatic release of stored protein, resulting in a delayed rise in free corticosterone (Qian *et al.*, 2011). This process of early CBG release from the liver may not apply in the setting of inflammatory stress, as it was not observed in our study. The decline in total CBG and haCBG in both TNF-α and saline infusion groups is likely due to dilution or sequestration/extravasation noting that the volume of fluid infused was the same between TNF-α and normal saline groups, ranging from 1080 mL to 1320 mL over the 6 hour study period. Importantly the OGTT, which was carried out from two hours onwards and was associated with a mild temporary increase blood sugar level of approximately four mmol/L on both trial days (Nielsen *et al.*, 2013), and thus is unlikely to account for a differential affect in the inflammatory response.

TNF-α primes neutrophils and stimulates NE release *in vitro*, in a dose- and time-dependent manner (Owen *et al.*, 1997). However, in a study by Michie *et al.*, neutrophil count did not rise following TNF-α infusion, compared to endotoxin infusion at 6 hours (Michie *et al.*, 1988b), thus NE may not have risen significantly in our 6 hour study period to effect CBG cleavage. Recently Hill *et al*. (Hill *et al.*, 2016) examined changes in corticosterone-binding capacity and hepatic *CBG* mRNA levels in rats following treatment with Freund's adjuvant daily for up to 16 days, surmising that CBG cleavage precedes changes in hepatic CBG production. CBG cleavage would be represented in our study as decreased haCBG, with a corresponding increase in laCBG after TNF-α infusion, compared with saline, however our data suggest no changes in either CBG levels or cleavage within six hours.

In vitro evidence that CBG is a protein thermocouple shows that the cortisol binding affinity of glycosylated haCBG decreases by almost half as body temperature rises from 37 to 39 °C,

amplifying free cortisol levels during pyrexia without requiring enzymatic proteolysis (Fig. 4.1, Chan *et al.*, 2013). The ratio of free cortisol to total cortisol increased early following TNF-α infusion in our study before the HPA axis was stimulated and without evidence of CBG cleavage or any fall in serum CBG concentration. Our antibody detected the intact RCL sequence in the setting of pyrexia and increased free cortisol, supporting the suggestion that the thermocouple mechanism involves more of a subtle "nudge-nudge" of single amino acids from an essentially exposed RCL than a "flip-flop" movement of a large segment in and out of the protein (Henley *et al.*, 2016b; Lewis *et al.*, 2016). Had a "flip-flop" occurred, as previously suggested (Zhou *et al.*, 2008), partial embedding of the RCL would limit the exposure of the amino acid sequence to our antibody, appearing as if the RCL had been cleaved. Instead, we provide *in vivo* evidence that the influence of temperature on free cortisol release is a very early effect of inflammation. IL-1, the most potent and important endogenous pyrogen (Netea *et al.*, 2000), may be involved in the CBG-cortisol thermocouple effect. It typically precedes IL-6 secretion, thereby allowing an immediate boost of free cortisol in the interim before IL-6 is able to induce down-stream effects on the HPA axis and hepatocytes.

The impact of temperature on cortisol release would appear to have a more dramatic influence over cortisol availability than NE-mediated cleavage. In modelling experiments, an enclosed compartment with a physiologic free cortisol concentration of 13 nM where CBG is nominally 29% saturated, total cleavage by NE (reducing cortisol-binding affinity by nine-fold) would result in a four-fold increase in free cortisol to 54 nM (Chan *et al.*, 2013). This takes into consideration that cleaved laCBG would assume an important buffering role, becoming increasingly saturated. The effect of temperature, however, increases exponentially, and decreases the cortisol binding affinity of both haCBG and laCBG, although less so in the latter, so that a four-fold reduction in binding affinity at 42 °C results in a three-fold increase

in ambient free cortisol to 37 nM (Chan *et al.*, 2013). Furthermore, the temperature effect is not reliant upon NE concentration and its accessibility to the CBG RCL.

An alternative explanation for the rise in free:total cortisol is a saturation effect of CBG, however this seems unlikely as it is exclusive to the pyrexial cohort despite total CBG falling equally in the control group and does not fit with the data as CBG levels were relatively low compared to circulating cortisol. Some data have shown that passive hyperthermia alone is sufficient to increase circulating cortisol concentrations (Jimenez *et al.*, 2007), but given that the rise in free:total cortisol is observed prior to elevation of total cortisol, an effect of temperature *per se* on cortisol production does not account for our findings. Unavoidable temperature changes during sample handling could potentially attenuate the effect of fever. If this were the case, our findings in fact underestimate the thermocouple impact, which could translate to a more exaggerated rise in free cortisol in fever *in vivo*.

Our study utilised samples collected in a previous experiment. The original randomised crossover trial was designed to examine the effect of systemic inflammation on the incretin effect of a glucose load (Nielsen *et al.*, 2013). Despite not being purpose-designed, the current study makes judicious use of appropriately collected and handled samples and assert that our hypothesis was adequately tested and fit with the interest and motivation of the original experiment; hormonal perturbations in systemic inflammation.

4.6 Conclusion

The earliest change in CBG-cortisol delivery dynamics after administration of the pivotal early pro-inflammatory cytokine, TNF-α, appears to be pyrexia-induced release of cortisol from CBG-cortisol. This precedes HPA axis stimulation. Cleavage of CBG to locally release

additional cortisol and IL-6 mediated suppression of CBG synthesis, as shown in previous studies of established inflammatory processes, are likely to be later phenomena in inflammatory illness.

Chapter 5

CBG cleavage in bacterial infection

5.1 Introduction

Microbiological infection directly activates the inflammatory response, counterbalanced by stimulation of the HPA axis, resulting in the increased production of anti-inflammatory and immunomodulatory cortisol, which is also needed to maintain cardiovascular, metabolic and neurocognitive homeostasis (Webster and Sternberg, 2004; Rhen and Cidlowski, 2005). The balance of anti- and pro-inflammatory activity is a determining factor in the outcome of an immune response, and an intact HPA axis is necessary for host survival (Webster and Sternberg, 2004; Andreasen *et al.*, 2008). Many bacterial pathogens secrete proteases as part of their armamentarium against host defences (Lebrun *et al.*, 2009). The virulence factor LasB, a protease secreted by the opportunistic pathogen *Pseudomonas aeruginosa*, has recently been shown to independently cleave CBG (haCBG → laCBG) releasing free cortisol *in vitro* (Simard *et al.*, 2014), a process previously only attributed to NE (Figure 5.1). Contention exists around whether this capability will augment (Simard *et al.*, 2014) or possibly attenuate (Sumer-Bayraktar *et al.*, 2016) the effects of NE-mediated CBG cleavage. CBG cleavage has not yet been investigated *in vivo* in patients with pseudomonal or other infections. The type of microbial infection may be a crucial factor in CBG cleavage during inflammation, the subsequent delivery of cortisol and the host's ability to eradicate a pathogen.

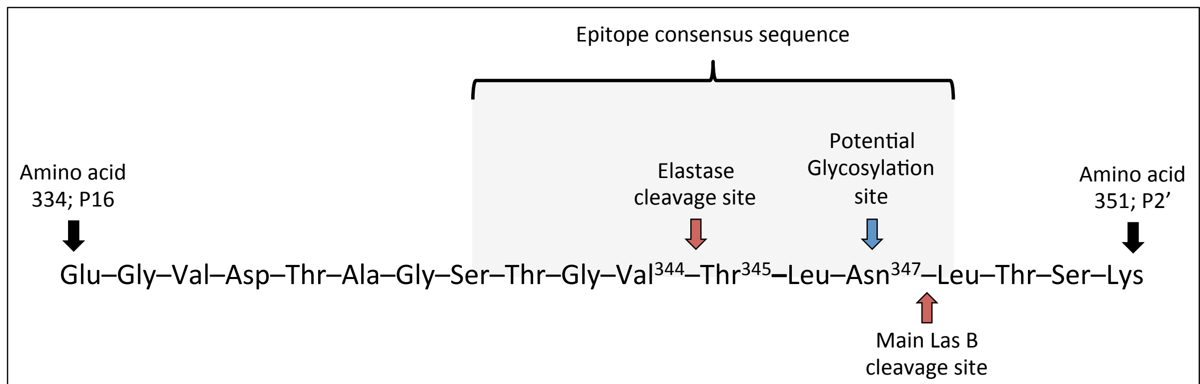
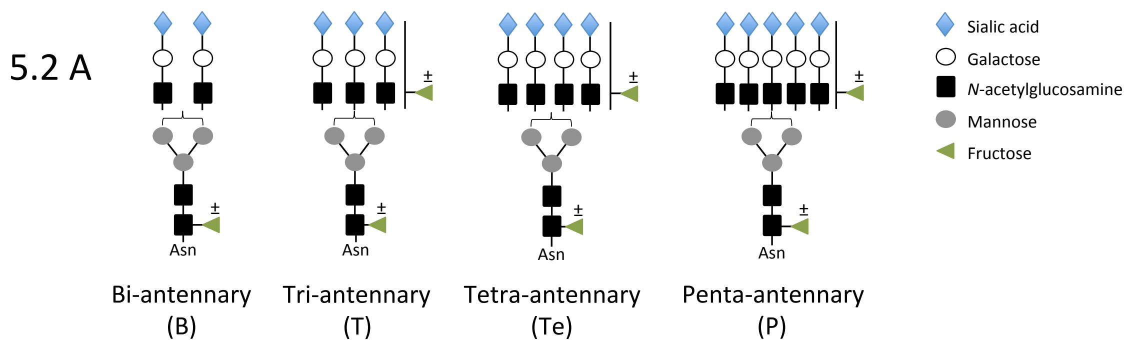


Figure 5.1 Amino acid sequence of the human CBG RCL. Sites of proteolytic cleavage, glycosylation and monoclonal antibody detection are illustrated. *P. aeruginosa* cleaves CBG between Asparagine^{347} and Leucine^{348} separate to the site of NE-based cleavage.

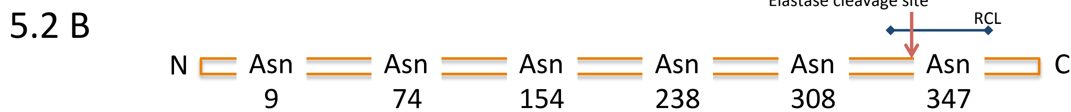
Importantly, CBG is a heavily glycosylated protein with six consensus sites (Asn-Xaa-Ser/Thr) available for attachment of *N*-acetyllactosamine-type oligosaccharides, of which five are usually occupied (Figure 5.2) (Strel'Chyonok *et al.*, 1982). Glycan processing is site-specific but marked intra-individual carbohydrate moiety heterogeneity is seen, contributing to variable molecular size and sialyation (Sumer-Bayraktar *et al.*, 2011). Typically, bi-antennary and tri-antennary oligosaccharide chains are present in a 3:2 molar ratio (Akhrem *et al.*, 1982), however branching increases significantly during pregnancy (Avvakumov and Strel'chyonok, 1987). Glycosylation at the consensus site Asparagine^{238} may be crucial for the synthesis of CBG with steroid-binding capacity (Avvakumov and Hammond, 1994) and may influence the accessibility of steroid to its binding site (Sumer-Bayraktar *et al.*, 2011), while the partially utilised site at Asparagine ^{347} within the RCL may affect availability of the cleavage sites to proteases (Sumer-Bayraktar *et al.*, 2011; Gardill *et al.*, 2012). Glycosylation prolongs CBG half-life (Hossner and Billiar, 1981) and may impair CBG-receptor interactions, particularly with syncytiotrophoblasts (Avvakumov and Strel'chyonok, 1988;

Sumer-Bayraktar *et al.*, 2011). In addition, thermosensitivity and steroid binding are glycosylation-dependent with non-glycosylated haCBG and laCBG having 9- and 2.5- fold reduced cortisol-binding affinity respectively (Chan *et al.*, 2013). Glycosylation profiles are known to vary under different health and hormonal conditions (Mihrshahi *et al.*, 2006) and the energy expenditure required for this post-translational modification suggests it bears considerable biological significance.

5.2 A



5.2 B



Site	1	2	3	4	5	6	Avg
Glycan occupancy	96.7%	99.5%	89%	70.5%	96.3%	84.7%	5.4 [a]
Branching distribution (B:T:Te:P)	48:50:2:0	21:35:41:3	76:21:2:0	79:21:0:0	71:29:0:0	18:71:9:2	51:38:10:1
Fucosylation	17.2%	13.7%	14.1%	0.0%	4.7%	34.9%	0.76 [b]

Figure 5.2 Simplified schematic of CBG glycosylation. 5.2 A) *N*-linked oligosaccharides display variable branching. 5.2 B) Six consensus sites for glycosylation are found in the CBG protein. Each displays variable occupancy, branching and fucosylation. [a] mol glycan/mol CBG; [b] mol Fucose/mol CBG. Adapted from Sumer-Bayraktar *et al.* (2011). Asn – Asparagine.

Furthermore, CBG-cortisol acts as a thermocouple within the range of physiologic body temperatures, with reduced CBG-cortisol binding seen in pyrexia (Chapter 4), a process dependent on adequate glycosylation (Cameron *et al.*, 2010; Qi *et al.*, 2011; Chan *et al.*, 2013).

5.2 Hypothesis and aims

Given the doubt over whether *P. aeruginosa* infection increases or decreases CBG cleavage *in vivo*, we undertook a study in patients with infection to clarify this issue. We hypothesised that *P. aeruginosa* infection would not be associated with decreased cleavage; hence haCBG levels would not be increased. In addition, we extended our investigation to a range of alternative pathogens to determine whether cleavage was differentially affected, thereby altering the availability of anti-inflammatory cortisol and the pursuant inflammatory response independent of inflammatory severity, as measured by inflammatory markers and validated bacteraemia severity scores.

5.3 Research design and methods

5.3.1 Participants and Design

We performed a prospective observational study in a public pathology institution and tertiary hospital in Adelaide, South Australia between February and July 2015. The protocol was approved by the RAH HREC. Inclusion criteria were: positive blood cultures as confirmed by the attending Microbiologist; inpatient admission at the RAH; contemporaneous full blood examination and C-reactive protein (CRP) levels; >1 mL serum or plasma available. Exclusion criteria were: use of hormonal contraception or hormone replacement therapy (HRT). A database of positive blood cultures received and processed at SA Pathology was

used to identify suitable specimens, and concordant samples were de-identified, aliquoted and stored at –20 ºC until analysed. Healthy controls were recruited from outpatient clinics and community advertising. Following the collection of samples, a retrospective analysis of medical records of the infection cohort patients was performed to document inclusion suitability, clinical findings at the time of blood collection, glucocorticoid use, in-hospital mortality and hospital length of stay. Data were then re-evaluated with consideration of confounding medications and severity indices. Sepsis was diagnosed on the basis of systemic inflammatory response syndrome criteria including ≥ two of the following: WCC <4 or >12 × 10^9/L, temperature <36 or >38 °C, respiratory rate >20 breaths per minute or heart rate >90 beats per minute, where all included participants had suspected or proven infections. The Pitt bacteraemia severity score was calculated using temperature, blood pressure, use of mechanical ventilation, cardiac arrest and mental status in the two days proceeding or one day following positive blood culture (Chow and Yu, 1999). Possible scores range from 0 to 14, with > 4 indicating critical illness.

5.3.2 Laboratory measurements

Blood samples were collected for serum total and free cortisol as well as total CBG and haCBG. The intra-assay coefficients of variation for the total CBG and haCBG assays were 4.4% and 3.2% respectively. Hormone assays were described in Chapter 2.

White cell count and CRP were requested by the treating physician as part of clinical care and measured by SA Pathology.

5.3.3 Statistical analysis

Data were analysed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc., San Diego, California). Results are expressed as median (range). Comparisons between groups were performed using the Mann-Whitney U test. Chi-squared testing was used to determine differences between categorical variables. Comparison between three or more groups was performed by Kruskal-Wallis test of ranks. Correlations between continuous variables were assessed with Spearman's rank correlation coefficient. Non-parametric statistics were used given the small numbers in the comparator groups. $P < 0.05$ was considered statistically significant.

5.4 Results

109 positive blood culture samples were collected. Of these, seven patients were excluded for taking HRT or oral contraceptives, and medical records were unobtainable for two patients. After exclusions, 100 infected patient samples were analysed and their characteristics are detailed in Table 5.1. Patients found to be receiving glucocorticoid preparations ($n = 26$) were excluded from cortisol analysis. Samples were also collected from 100 healthy controls. The control patients were younger than the infected patients ($P < 0.0001$), although gender distribution and BMI were no different ($P = 0.16$ and $P = 0.37$ respectively). Sixty-seven patients met criteria for the diagnosis of sepsis at the time of sampling. The distribution of bloodstream isolates was similar to other published Australian cohorts (Aung *et al.*, 2012). Forty-seven percent of patients had their blood samples taken in the Emergency Department prior to being admitted to hospital. For the remaining patients, median time from admission to sample collection was 23 (0.25–953) hours. Their length of hospital stay was 11 (0–137) days.

Table 5.1 Baseline characteristics in the infection cohort. Median (range).

	Infection cohort		Controls
Gender (M:F)	56:44		45:55
Age (years)	67 (20–101)		52 (18–80)
BMI (kg/m^2)	26.4 (13.1–83.9)		26.3 (18.5–59.2)
WCC (× 10^9/L)	11.2 (0.04–57.5)		
Neutrophils (%)	86.5 (5–96.6)		
CRP (mg/L)	140 (1.1–490)		
Pitt score	1.0 (0–10)		
Temp (°C)	38.1 (34.5–40.5)		
SBP (mm Hg)	120 (90–208)		
DBP (mm Hg)	67 (40–97)		
Heart rate (bpm)	92 (53–158)		
Respiratory rate (bpm)	18 (12–48)		
Bloodstream isolates (*n*)	Gram negative bacilli	41	
	• *Escherichia coli*	• 15	
	• *Pseudomonas aeruginosa*	• 11	
	• *Klebsiella pneumoniae*	• 7	
	• *Enterobacter* spp.	• 5	
	• *Bacteroides fragilis*	• 2	
	• *Providencia stuartii*	• 1	
	Gram negative cocci	5	
	• *Neisseria meningitides*	• 2	
	• Other *Neisseria* spp.	• 1	
	• *Acinetobacter*	• 1	
	• Failed to grow on culture	• 1	
	Gram positive cocci	40	
	• *Staphylococcus aureus*	• 15	
	• Coagulase negative staphylococcus	• 12	
	• *Streptococcus* spp.	• 12	
	• *Micrococcus* spp.	• 1	
	Gram positive bacilli	9	
	• Diptheroid	• 4	
	• Propionibacterium	• 3	
	• *Clostridium difficile*	• 1	
	• *Bifidobacterium longum*	• 1	
	Yeast	5	
	• *Candida* spp.	• 5	
Source of infection [a] (*n*)	Genitourinary	26	
	Unknown	16	
	Skin	7	
	Gastrointestinal	7	
	Musculoskeletal	6	
	Line-related	6	
	Lung	6	
	CNS	3	
	Cardiac	1	

LOS – length of hospital stay; WCC – white cell count (reference interval 4.0–11.0 × 10^9/L); ANC – absolute neutrophil count (1.8–7.5 × 10^9/L); CRP – C-reactive protein (<8 mg/L); SBP – systolic blood pressure; DBP – diastolic blood pressure. [a] For culture positive samples only.

5.4.1 Infection cohort vs. *controls*

Patients with infection had 23% lower serum total CBG, 18% lower haCBG and 31% lower laCBG, with an 11% higher % haCBG:total CBG than healthy controls (Figure 5.3). Total cortisol was 2.6 times greater and free cortisol 4.0 times greater in infected patients than controls [848 (230–5103) *vs*. 321 (121–840) nmol/L, $P < 0.0001$; 72 (15–958) *vs*. 18 (7–70) nmol/L, $P < 0.0001$ respectively]. The ratio of free cortisol to total cortisol was also higher in the infected group [0.093 (0.043–0.487) *vs*. 0.061 (0.046–0.083), $P < 0.0001$]. Within the infection cohort, 22% of patients cultured organisms suspected to be contaminants only but were clinically likely to have infection (culture-negative), while 78% cultured pathogenic organisms (culture-positive). There was no difference in total CBG, haCBG, or free cortisol between culture-positive and culture-negative samples (Table 5.2). There was, however, significantly higher % haCBG:total CBG and total cortisol in the culture-positive group along with higher markers of inflammation including % neutrophils and CRP [87 (5–97 *vs.* 83 (6–93), $P = 0.002$) and 150 (1.3–490) *vs*. 74 (1.1–360) mg/L, $P = 0.007$], but similar WCC and ANC. Patients with sepsis had higher Pitt scores than infected patients without sepsis [1.0 (0–10) *vs*. 1.0 (0–4), $P = 0.034$], however sepsis was not associated with altered CBG affinity forms or cortisol levels relative to the non-sepsis group. In the overall infection cohort, haCBG correlated negatively with laCBG ($P = 0.008$, r = –0.256).

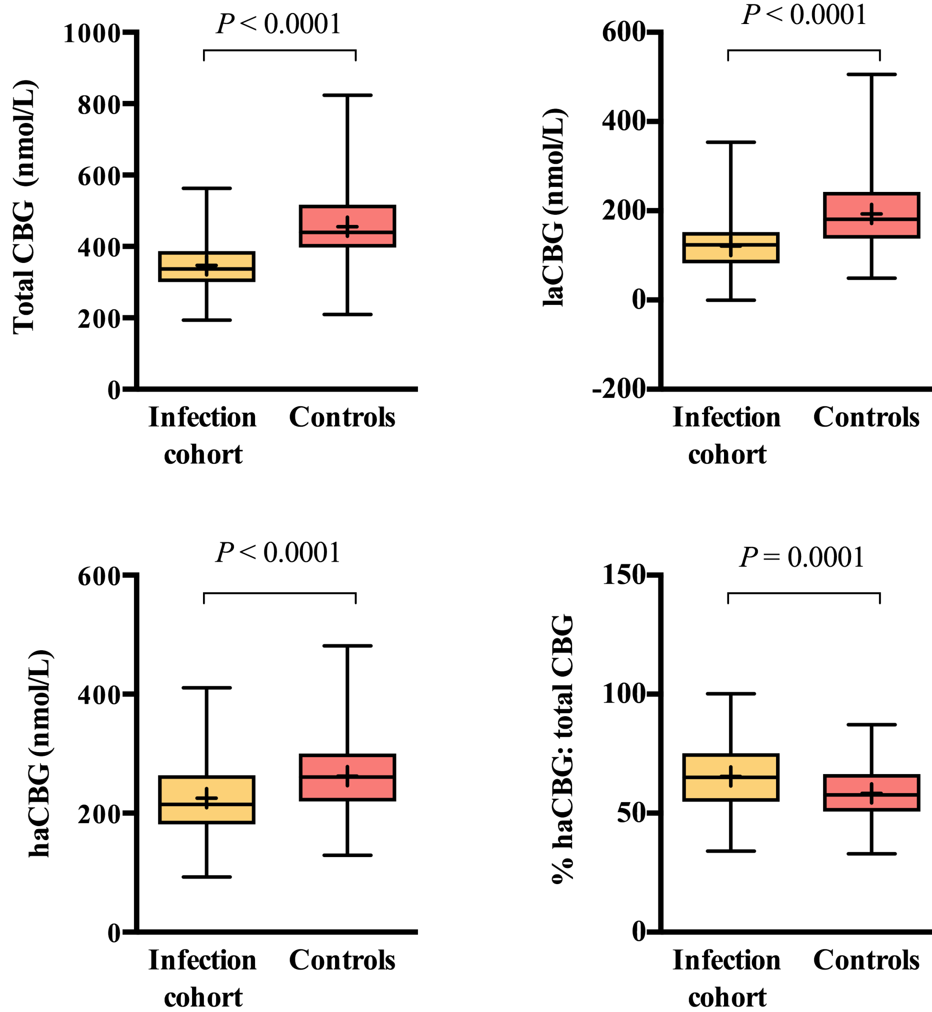


Figure 5.3 CBG affinity forms in the infection cohort and controls. Patients with infection had lower total CBG, haCBG and laCBG, with higher % haCBG:total CBG than healthy controls. Line at median; + at mean; box extends from 25th to 75th percentiles; whiskers from maximum to minimum.

Patients who died during their admission (n = 10) had lower total CBG than those who survived [n = 90; 312 (219–368) *vs*. 339 (194–563) nmol/L, P = 0.06], although this did not reach statistical significance.

Table 5.2 CBG and cortisol levels in controls and infected samples. Median (range).

	Controls	Culture positive $n = 78$	P value *vs.* controls	Culture negative $n = 22$	P value *vs.* controls	P value culture + *vs.* culture-	Sepsis $n = 67$	P value *vs.* controls	Non-Sepsis $n = 33$	P value *vs.* controls
Total CBG	440 (210–824)	337 (194–563)	*<0.0001*	332 (246–537)	*<0.0001*	0.92	339 (204–563)	*<0.0001*	326 (194–485)	*<0.0001*
haCBG	261 (129–481)	221 (93–411)	*0.0003*	187 (112–360)	*0.0002*	0.11	215 (112–411)	*0.0002*	215 (93–320)	*0.0005*
laCBG	181 (49–506)	121 (0–261)	*<0.0001*	137 (53–354)	*0.0011*	0.15	125 (14–354)	*<0.0001*	118 (0–261)	*<0.0001*
haCBG:total CBG (%)	57.7 (32.9–87.2)	65.4 (38.7–100)	*<0.0001*	57.7 (34.1–85.4)	0.64	*0.04*	65.3 (34.1–95.6)	*0.0004*	63.2 (38.7–100)	*0.012*
Total cortisol	321 (121–840)	956 (318–5103)	*<0.0001*	624 (230–2122)	*<0.0001*	*0.046*	958 (230–5103)	*<0.0001*	752 (268–2698)	*<0.0001*
Free cortisol	18.2 (7.2–69.8)	83.6 (21.8–958)	*<0.0001*	52.2 (14.6–230)	*<0.0001*	0.10	85.5 (15.3–567.3)	*<0.0001*	76.1 (20.4–320.4)	*<0.0001*

All units are nmol/L. Italics indicate statistical significance.

5.4.2 Comparison between pathogenic isolates

CBG affinity forms were compared between the commonly encountered bacteraemic isolates *P. aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Candida* spp., *Staphylococcus aureus*, *Streptococcus* spp. and culture-negative samples (Table 5.3). *P. aeruginosa* bacteraemia resulted in the lowest total CBG and laCBG levels of all the pathogen groups and these were all significantly lower than healthy controls, with total CBG, haCBG and laCBG being 1.5, 1.2 and 2.3 times lower than controls respectively. *P. aeruginosa* was also associated with the lowest inflammatory marker levels, including CRP, % neutrophils and ANC. There was no overall difference between total CBG, haCBG or laCBG across pathogen groups. haCBG levels were not reduced in patients with *E. coli*, *K. pneumoniae, Candida* spp. or *Streptococcus* spp. compared with controls despite lower total CBG levels, which may indicate decreased CBG cleavage in these pathogen groups.

Table 5.3 Comparison of CBG affinity forms and cortisol levels between pathogenic isolates. Median (range).

	Pseudomonas aeruginosa **(n = 11)**	***Escherichia coli*** **(n = 15)**	***Klebsiella pneumoniae*** **(n = 7)**	***Candida*** **spp. (n = 5)**	***Staphylococcus aureus*** **(n = 15)**	***Streptococcus*** **spp. (n = 12)**	**Culture negative (n = 22)**	***P*** **value**[a]
Total CBG (nmol/L)	303 (194–386)[‡]	329 (204–477)[‡]	336 (283–425)[¶]	339 (315–412)[¶]	349 (272–461)[‡]	360 (233–563)[¶]	332 (246–537)[‡]	0.40
haCBG (nmol/L)	221 (118–264)[¶]	234 (93–411)	199 (172–345)	288 (198–301)	216 (141–351)[†]	225 (175–376)	187 (112–360)[¶]	0.34
laCBG (nmol/L)	80 (35–136)[‡]	130 (0–170)[‡]	119 (81–154)[†]	118 (14–125)[¶]	135 (18–203)[¶]	128 (58–188)[¶]	137 (53–354)[¶]	0.12
haCBG:total CBG (%)	70.8 (57.1–83.6)[¶]	65.5 (38.7–100)[¶]	63.1 (53.4–81.0)	69.8 (62.6–95.6)[¶]	64.8 (42.7–93.4)	65.3 (53.1–81.3)[†]	57.7 (34.1–85.4)	0.22
Total cortisol (nmol/L)	827 (318–1416)[‡]	1103 (474–2698)[‡]	1215 (582–5103)[‡]	975 (555–1031)[‡]	747 (343–2620)[‡]	691 (84–2256)[‡]	624 (230–2122)[‡]	0.09
Free cortisol (nmol/L)	113 (27.8–203.4)[‡]	118 (40–454)[‡]	131 (50–958)[‡]	86 (40–103)[‡]	71 (20–657)[‡]	51 (4–333)[‡]	52.2 (14.6–230)[‡]	0.22
CRP (mg/L)	64 (1–170)	210 (9–340)	222 (36–450)	200 (11–400)	165 (48–490)	108 (3–210)	74 (1–360)	*0.006*
WCC (× 10^9/L)	4.7 (0.04–57.5)	17.2 (3.5–45.8)	9.0 (0.4–24.1)	10.3 (0.3–15.9)	11.9 (4.3–19)	10.1 (0.04–26)	12 (2.9–22.7)	0.11
% neutrophils	78 (5–93)	88 (86–97)	90 (50–93)	86 (8–14)	89 (63–94)	87 (78–95)	83 (6–93)	*0.003*
Pitt score	2 (0–3)	1 (0–3)	1 (0–4)	3 (0–6)	0 (0–3)	0.5 (0–4)	0.5 (0–10)	0.07

[a] Kruskal-Wallis test of ranks across pathogen groups; [†] $P < 0.05$ *vs*. controls; [¶] $P < 0.01$ *vs*. controls; [‡] $P < 0.0001$ *vs*. controls; for control data see Table 5.2. CRP – C-reactive protein (<8 mg/L); WCC – white cell count (4.00 – 11.0 × 10^9/L); ANC – absolute neutrophil count (1.80 – 7.5 × 10^9/L).

5.4.3 Neutrophil count and CBG cleavage

Eight patients had febrile neutropaenia (FN; ANC < 0.5 × 10^9/L). In most cases the ANC was too low to quantify. Accordingly, total WCC was lower in patients with FN [0.4 (0.04–2.1) *vs.* 11.9 (2.5–57.5) × 10^9/L, *P* < 0.0001]. There was no difference between total CBG, haCBG, laCBG cortisol levels, CRP or temperature compared to patients without FN. Patients with FN had higher free cortisol levels but this was not statistically significant [104 (49–958) *vs*. 71 (15–523) nmol/L, *P* = 0.25]. For the total infection cohort, WCC, ANC and % neutrophils correlated with total cortisol (*P* = 0.03, r = 0.26; *P* =0.006, r = 0.33; *P* = 0.0001, *r* = 0.45), but not total CBG, haCBG or laCBG. Free cortisol correlated with % neutrophils, but not ANC (*P* = 0.002, r = 0.38; *P* = 0.27, r = 0.14), as did the free cortisol fraction (*P* = 0.04, r = 0.26; *P* = 0.54, r = –0.08).

5.4.4 Clinical parameters and CBG

Total CBG, haCBG and laCBG were reduced in association with systolic blood pressure (SBP) and diastolic blood pressure (DBP) (Total CBG *P* < 0.0001, r = 0.39 and *P* = 0.001, r = 0.33; haCBG *P* = 0.049, r = 0.20 and *P* =0.059, r = 0.19; laCBG *P* = 0.003, r = 0.30 and *P* = 0.02, r = 0.23). Total CBG correlated negatively with Pitt Score (*P* = 0.003, r = –0.29) while haCBG correlated positively with CRP (*P* = 0.027, r = 0.22). Body temperature at time of blood sampling ranged from 34.5–40.5 ºC and correlated with free cortisol (*P* = 0.047, r = 0.24).

5.5 Discussion

This is the first *in vivo* study examining CBG cleavage in patients with a range of bloodstream infections. Total CBG, haCBG and laCBG are all reduced in infection,

regardless of culture status, compared with healthy controls. In light of recent *in vitro* data suggesting *P. aeruginosa* infection may independently influence CBG cleavage, we studied CBG levels between common pathogenic groups and found low total and haCBG levels in pseudomonal infection despite milder inflammation, suggesting against organism-related attenuation of CBG cleavage. Additionally, infection with neutropaenia was not associated with reduced CBG cleavage. The data may support a cleavage-enhancing effect of *P. aeruginosa in vivo* and points generally to non-neutrophil elastase factors contributing to CBG cleavage.

Infection is likely to decrease total CBG through IL-6 mediated suppression of hepatic production. In this study, we found total CBG reduced in the setting of significant infection as expected. haCBG was also reduced in the overall infection cohort, as well as in *P. aeruginosa*, *S. aureus*, and culture negative groups, and negatively correlated with laCBG levels. Falling haCBG corresponding to rising laCBG suggests CBG has undergone transition from the stressed to relaxed forms, corroborating enzymatic cleavage, and suggesting that laCBG is not immediately cleared from the circulation (Lewis *et al.*, 2015). haCBG was not reduced in *E coli*, *K pneumoniae, Candida spp.* or *Streptococcus* spp. groups, which may suggest protection from cleavage in these infections. In a study of severe sepsis and septic shock (Nenke *et al.*, 2015), patients with sepsis had lower total CBG, haCBG and laCBG, but higher % haCBG:total CBG than healthy controls, but as the systemic inflammation worsened to septic shock, CBG cleavage increased, with haCBG plummeting, particularly in those who died. Taking these studies together, it may be that in the early stages of particular infections, cortisol reserves are judiciously regulated, with the aim of supplying only adequate glucocorticoid to control the immediate infection. As systemic inflammation progresses to become life threatening, however, cleavage may be stimulated to exhaustion in an attempt to

maintain blood pressure, depleting haCBG-cortisol stores. In the septic shock study, laCBG increased in association with increased illness severity and increased cleavage. We did not see this increase in laCBG levels in the current study, which may be due to improved clearance of the residual protein in early as opposed to end-stage systemic inflammation.

Bacteria secrete numerous proteases, enhancing their pathogenicity by various mechanisms (Maeda, 1996; Lebrun *et al.*, 2009). The exposed RCL of serpin proteins, a poorly conserved sequence, is susceptible to proteolytic attack by such bacterial proteases (Sires *et al.*, 1994; Maeda and Yamamoto, 1996; Rapala-Kozik *et al.*, 1999; Weiss *et al.*, 2014). For example, proteases from *P. aeruginosa*, *S. aureus, Streptococcus pyogenes* and *E. coli* are capable of cleaving the RCL of α1 antitrypsin, resulting in protein inactivation (Morihara *et al.*, 1984; Potempa *et al.*, 1986; Rapala-Kozik *et al.*, 1999; Weiss *et al.*, 2014). Simard *et al.* examined a number of bacteria including *S. aureus* and *E. coli* and found that *P. aeruginosa* alone was able to disrupt the cortisol binding activity of CBG, exclusive of NE *in vitro* (Figure 5.1) (Simard *et al.*, 2014). This may combine with NE to amplify CBG cleavage and free cortisol release during infection. The zinc-metalloprotease LasB (pseudolysin; *P. aeruginosa* elastase) was determined to be the virulence factor responsible for CBG cleavage at a separate cleavage site within the RCL, with approximately 50% of the CBG cortisol binding capacity being lost after 16 hours of protease exposure. However, the rate at which CBG lost its cortisol-binding activity was slower than with NE, suggesting that *P. aeruginosa* may be relatively inefficient in its cleavage. The decreased potency of LasB was recently confirmed in a study that revealed the importance of glycan characteristics, showing that when the glycosylation consensus site within the RCL is occupied (Figures 5.1 and 5.2), LasB is unable to cleave CBG, restricting its access to about 15% of CBG (Sumer-Bayraktar *et al.*, 2016). This is because glycosylation, particularly with tri-antennary oligosaccharides, causes perturbation of

the RCL structure (Sumer-Bayraktar *et al.*, 2016). Furthermore, the purpose of the relatively slower cleavage instigated by LasB may be to constrain cortisol release to below the bacteriostatic threshold while still suppressing host immunity (Sumer-Bayraktar *et al.*, 2016). In our study, we saw significantly lower haCBG concentrations in *P. aeruginosa* infection than in healthy controls. Given the relatively low level of inflammation seen with CRP and WCC, these results do not support impairment of NE-mediated CBG cleavage by LasB *in vivo*. The lack of overall difference in CBG affinity forms between pathogen groups may be the result of a type 2 error given the small sample sizes. Given that the CBG assay used in this study provides results within hours, the combined finding of low total CBG and low laCBG in patients suspected of infection may be an early indication of the need to treat with antibiotics that provide pseudomonal cover while blood cultures, which can take days, are still pending. The preliminary data shown here that low total CBG may indicate poor prognosis needs to be explored in dedicated mortality studies, but suggests that total CBG could be used as an early biomarker of infection severity. The decision to assess the degree of inflammation using CRP and WCC is pertinent here, as these are measurements used commonly in clinical practice and have more relevance than research-based measurement of inflammatory cytokines.

NE, a serine protease secreted by activated neutrophils at sites of inflammation, is strongly stimulated by lipopolysaccharide in the outer membrane of gram-negative bacteria (Webster and Sternberg, 2004). NE was the first endogenous protease identified to participate in CBG cleavage (Pemberton *et al.*, 1988), although several more, including chymotrypsin and LasB, are now implicated (Gardill *et al.*, 2012; Lewis and Elder, 2014; Simard *et al.*, 2014). NE may not be the only factor mediating cortisol release from CBG in infection given the lack of difference in CBG cleavage between patients with and without FN. NE is expected to markedly decrease in FN but in our study this had no effect on haCBG or laCBG levels

compared to infected patients with sufficient and even elevated neutrophil counts. Additionally, there was increased cleavage in the culture-negative infection group despite having the same WCC and ANC as the culture-positive group. Rather than CBG cleavage predominating in infection in our study, an alternative process such as local CBG synthesis, sequestration (Kuhn, 1988), or altered clearance due to glycosylation changes may take precedence. This is similar to our findings in α1 antitrypsin deficiency (described in Chapter 8) where excessive NE due to deficient α1 antitrypsin did not affect CBG cleavage in the basal, non-infective setting (Nenke *et al.*, 2016a).

Given the recent data showing the intricate but significant impact of glycosylation on CBG, we favour altered glycan formation as the major mechanism responsible for altered CBG cleavage seen in this study (Sumer-Bayraktar *et al.*, 2016). Examination and comparison of glycoform profiles in infection should be extended beyond patients with pseudomonal infection alone. While a recent study of human CBG affinity forms (in rabbit circulation) suggests equivalent half-lives of 10 hours (Lewis *et al.*, 2015), this was performed under basal physiological conditions. Additional studies of serum CBG disappearance in sepsis would complement the glycan analysis. Finally, our monoclonal antibodies have the potential to be used in immunohistochemical studies (Lewis and Elder, 2011), to determine whether CBG found in tissues is present as haCBG or laCBG, indicating localised cleavage of CBG within tissues which could facilitate the identification of unrecognised tissue proteases.

Finally, CBG is known to act as a protein thermocouple, with its cortisol-binding affinity decreasing as body temperature increases within the physiological range, thereby releasing cortisol in pyrexial states independent of proteolytic cleavage (Cameron *et al.*, 2010; Chan *et*

al., 2013), although the molecular mechanism for this is contentious (Lewis *et al.*, 2016). We found a positive correlation between body temperature at the time of blood sampling and free cortisol levels, supporting the *in vitro* data, and agreeing with our findings in Chapter 4.

5.6 Conclusion

This study has revealed evidence of increased CBG cleavage in infection. In particular, low haCBG was seen in *S. aureus* and in *Pseudomonas* infection, consistent with the recent findings of LasB-associated CBG cleavage *in vitro*. Taken with a lack of influence of neutrophil count, this suggests that factors other than NE may be important in CBG cleavage and hence tissue cortisol delivery.

Chapter 6

Reduced CBG cleavage in abdominal obesity and the metabolic syndrome

6.1 Introduction

The previous three chapters dealt with haCBG depletion following enzymatic cleavage in acute inflammation and infection. Chronic inflammation, however, represents a separate entity with differential effects on the HPA axis and adaption of the stress response. It was hitherto unclear what role haCBG plays in chronic inflammation and whether the process involving targeted delivery of anti-inflammatory cortisol via proteolytic cleavage is as efficient and effective as it is in acute inflammation.

Sixty-three percent of Australian adults are overweight or obese, contributing to increasing morbidity and mortality. Visceral adipose tissue is a pro-inflammatory endocrine tissue, associated with systemic insulin resistance, inflammation and endothelial dysfunction (Figure 6.1) (Kershaw and Flier, 2004). Overweight and obesity, therefore, are considered chronic inflammatory states (Monteiro and Azevedo, 2010). Cortisol secretion is increased in obesity, however, cortisol metabolism is also increased and thus circulating cortisol levels remain normal (Mussig *et al.*, 2010; Incollingo Rodriguez *et al.*, 2015). As glucocorticoid exposure promotes adipocyte differentiation, visceral adiposity and insulin resistance (Geer *et al.*, 2014), "functional hypercortisolism" (Anagnostis *et al.*, 2009) is implicated in the development of the metabolic syndrome (MetS) which comprises abdominal obesity, hypertension, dyslipidaemia and glucose intolerance; a condition conferring high

cardiovascular risk (Alberti *et al.*, 2009; Anagnostis *et al.*, 2009). Studies of the relationship between total CBG and anthropometric measurements or markers of metabolic derangement demonstrate an inverse relationship between CBG and both BMI (Lapidus *et al.*, 1986; Fernandez-Real *et al.*, 2002; Lewis *et al.*, 2009; Lewis *et al.*, 2010; Schafer *et al.*, 2013) and insulin resistance (Fernandez-Real *et al.*, 2002), implicating increased cellular availability of free cortisol in the pathogenesis of fat accumulation (Fernandez-Real *et al.*, 2002). CBG levels also negatively correlate with glycated haemoglobin, insulin resistance, SBP and DBP, but not fasting glucose (Fernandez-Real *et al.*, 2002; Schafer *et al.*, 2013).

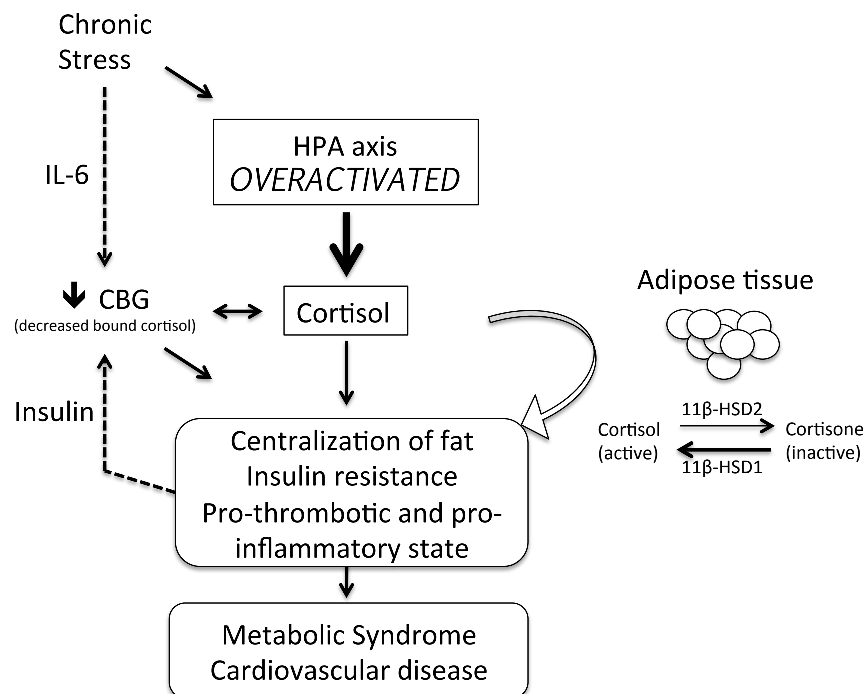


Figure 6.1 The HPA axis in obesity. The HPA axis is hyperactive in obesity – a maladaptive response to chronic stress exposure. 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 is upregulated in adipose tissue, increasing active cortisol conversion. Insulin and IL-6 inhibit hepatic CBG production which may contribute to an increase in free cortisol exposure. Solid line represents stimulation; dashed line represents inhibition.

Additional evidence links CBG variation to obesity (Meyer *et al.*, 2016); CBG mRNA has been identified in rat adipocytes, with local production potentially affecting glucocorticoid access to adipose tissue (del Mar Grasa *et al.*, 2001); CBG polymorphisms influence fat deposition in pigs (Ousova *et al.*, 2004) and may be related to cortisol-mediated metabolic disruptions in obese pre-menopausal women (Barat *et al.*, 2005). Finally, CBG deficiency in humans is associated with increased preadipocyte proliferation and enhanced differentiation *in vitro* (Joyner *et al.*, 2003), and an obese phenotype (Appendix B) (Torpy *et al.*, 2001; Hill *et al.*, 2012).

With regards to normal physiology, women have been found to have higher total CBG levels than men in most previous studies (Fernandez-Real *et al.*, 2002; Lewis *et al.*, 2004; Lewis *et al.*, 2010) with levels increasing following menopause (Wilson *et al.*, 1981) despite CBG generally correlating inversely with age (Fernandez-Real *et al.*, 2002; Lewis *et al.*, 2010; Schafer *et al.*, 2013). As yet, however, analysis of the effects of common clinical variables on the different CBG affinity forms has not been undertaken.

6.2 Hypothesis and aims

We performed an observational study to determine the normal range and distribution of haCBG and laCBG in the general population, and assess the relationship of age, menopause, blood pressure and anthropometric characteristics to CBG affinity forms for the first time. We hypothesised that abdominal adiposity and MetS would be associated with altered CBG cleavage.

6.3 Research design and methods

6.3.1 Participants

We performed a prospective cross-sectional study between October 2014 and July 2015 at a single tertiary teaching hospital in Adelaide, South Australia. The protocol was approved by the RAH HREC. Inclusion criteria: age ≥ 18 years old. Exclusion criteria: known disease of the HPA axis, pregnancy, use of the combined oral contraceptive pill (COCP) or HRT, and active inflammatory or infective condition. Advertising was placed in the community and online. Patients were also invited to participate by their treating doctor in outpatient clinics. Interested participants were screened after written informed consent. Anthropometric measurements and blood sampling were completed between 8 am – 6 pm. Ethnicity of participants matched the local population with the large majority being of European decent while <15% were of Chinese or south Asian descent. As the purpose of the study was to assess CBG affinity forms, cortisol was only measured to determine marked changes in the total/free cortisol ratio in association with altered CBG affinity form levels, rather than to rigorously assess cortisolaemia which would require several integrated cortisol measurements under various conditions.

6.3.2 Anthropometric measurements

Demographic details, medical information and anthropometric measures were taken by the same principal investigator. Participants had seated blood pressure (BP) measured twice using an aneroid sphygmomanometer, with the average recorded. Height was measured using a stadiometer and weight was recorded wearing indoor clothing without shoes. Waist circumference (WC) was determined using a soft measuring tape placed horizontally around the abdomen with the bottom edge of the tape measure at the uppermost border of the iliac

crest at end of normal expiration. Absolute and percentage of body fat was measured by bioimpedence analyser (Omron HBF-302W; Omron Corp, Japan).

Assessment of MetS was based on measured WC and BP, as well as patient-reported diagnosis of diabetes, hypertriglyceridemia and/or use of anti-hypertensive or anti-diabetic medication, fenofibrates or nicotinic acid. Three out of five criteria (elevated WC, BP, triglycerides, impaired fasting glucose or reduced high-density lipoprotein cholesterol) were sufficient for diagnosis (Alberti *et al.*, 2009).

6.3.3 Laboratory methods

Blood samples were collected and stored at –20 °C for serum total and free cortisol as well as total CBG and haCBG. The intra-assay coefficients of variation for the total CBG and haCBG assays were 4% and 2% respectively. Hormone assays were described in Chapter 2.

6.3.4 Statistical analyses

Data were analysed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc., San Diego, California). Results are expressed as mean ± SEM unless otherwise stated. Comparisons between two groups were performed using two-tailed unpaired Student's *t* test. Chi-squared testing was used to determine differences between categorical variables. Correlations between continuous variables were assessed with Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant.

6.4 Results

Baseline demographic and anthropometric characteristics of the participants are detailed in Table 6.1. One hundred healthy volunteers were enrolled. Fifty-five were women, and of these 23 were post-menopausal. Ages ranged from 18 to 80 years with no difference between women and men. Thirty-five participants had a normal BMI (< 25 kg/m^2), 34 were overweight (25 ≤ BMI < 30) and 31 were obese (BMI ≥ 30). Mean WC, height, weight, and percentage of body fat were higher for men than women. There was no difference in the proportion of women and men who were classified as having abdominal obesity according to gender specific cut offs [65.5% of females (WC ≥ 80 cm) *vs.* 68.9% of males (WC ≥ 94 cm), *P* = 0.83] (Alberti *et al.*, 2009). Seventeen percent had the MetS. The median time of sample collection was 10:45 am (range 8:15 am – 5:55 pm).

Table 6.1 Baseline characteristics of healthy volunteers. Mean (± SD).

	Females	**Males**	***P* value**
Number	55	45	
Age (years)	50 (15)	50 (20)	0.94
Systolic BP (mm Hg)	125 (16)	131 (15)	0.07
Diastolic BP (mm Hg)	75 (9)	77 (11)	0.24
Waist circumference (cm)	88 (14)	102 (19)	< *0.0001*
Height (m)	1.63 (0.07)	1.75 (0.07)	< *0.0001*
Weight (kg)	71.1 (16.3)	90.9 (26.2)	< *0.0001*
BMI (kg/m^2)	26.9 (6.1)	29.5 (7.5)	0.06
Body fat (%)	35.1 (7.0)	27.5 (8.6)	< *0.0001*
Body fat (kg)	25.6 (9.9)	26.6 (15.7)	0.69
Menopausal status (*n* = 55)			
• **Pre/peri**	• 58%	NA	
• **Post**	• 42%	NA	
Cigarette smoking			
• **Current smoker**	• 6%		
• **Ex-smoker**	• 10%		
• **Never smoker**	• 84%		

6.4.1 CBG affinity forms in relation to adiposity

Levels of CBG affinity forms in relation to anthropometric measurements are shown in Figure 6.2. CBG concentrations showed a right-skewed distribution. The percentage of haCBG to total CBG was normally distributed. Means (± 95% confidence interval) are detailed in Table 6.2. Total CBG, haCBG and laCBG levels did not vary with the time of sampling. Women had 11% higher total CBG and haCBG than men. Total and haCBG levels were higher in post-menopausal than pre/peri-menopausal women but this was not statistically significant. There was no correlation between age and CBG affinity forms in the total cohort, however men alone showed a negative correlation between total CBG and laCBG and age ($P = 0.04$, $r = –0.31$ and $P = 0.04$, $r = –0.30$ respectively). There was no association between CBG levels and SBP or DBP in the total cohort, however both showed a positive relationship to total CBG ($P = 0.04$, $r = 0.31$ and $P = 0.05$, $r = 0.30$) and haCBG ($P = 0.05$, $r = 0.30$ and $P = 0.05$, $r = 0.29$) in men alone.

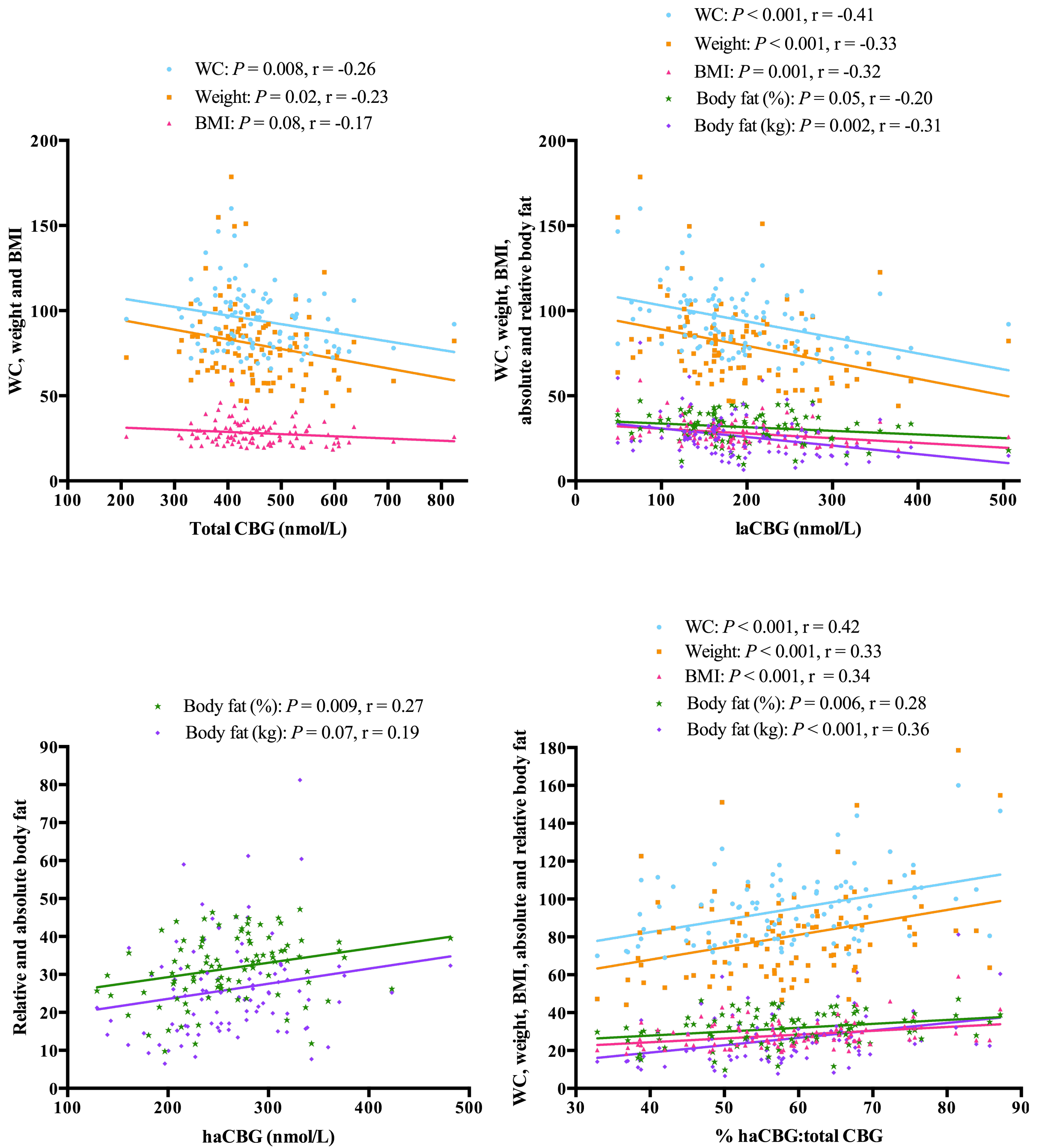


Figure 6.2 Correlations between CBG affinity forms and anthropometric measurements in healthy volunteers. The obese phenotype was associated with decreased total CBG and laCBG, and an increase in haCBG and % haCBG:total CBG.

Table 6.2 CBG and cortisol levels among healthy volunteers. Mean (± 95% CI).

	All	**Women (n = 55)**	**Men (n = 45)**	***P* value [a]**	**Pre/peri-menopausal (n = 32)**	**Post-menopausal (n = 23)**	***P* value [b]**
Total CBG	455 (437–474)	479 (459–500)	426 (395–456)	*0.004*	472 (450–493)	490 (449–532)	0.39
haCBG	262 (250–274)	275 (260–290)	246 (227–265)	*0.02*	264 (245–283)	292 (267–316)	0.07
laCBG	193 (178–209)	204 (186–223)	180 (153–206)	0.12	208 (185–231)	199 (165–232)	0.62
haCBG:total CBG (%)	58.4 (56.1–60.7)	58.0 (55.1–60.8)	58.9 (55.0–62.7)	0.71	56.3 (52.2–60.4)	60.3 (56.4–64.2)	0.17
Total cortisol	328 (302–355)	326 (286–365)	331 (295–367)	0.85	308 (255–361)	350 (288–412)	0.30
Free cortisol	20.6 (18.6–22.7)	20.4 (17.3–23.5)	20.9 (18.3–23.6)	0.79	19.2 (14.8–23.5)	22.1 (17.5–26.7)	0.35

Units are nmol/L. Students t test between [a] women and men, and [b] pre/peri-menopausal and post-menopausal. Italics indicate statistical significance.

For the total cohort (Figure 6.2), WC, weight and BMI correlated negatively with total CBG and laCBG, and positively with % haCBG:total CBG. Absolute and percentage of body fat correlated with positively with haCBG and % haCBG:total CBG, and negatively with laCBG, but did not correlate with total CBG. Gender had a significant impact on these associations.

6.4.2 CBG affinity forms; men and women

Total and haCBG levels were significantly (11%) higher in women; laCBG was non-significantly higher (Table 6.2). Menopausal status had no significant effect on CBG levels.

6.4.3 CBG affinity forms and the metabolic syndrome

Participants with MetS had lower total CBG and lower laCBG. There was a trend towards a higher haCBG:total CBG ratio in the MetS group. Taken together these results are indicative of decreased CBG cleavage in MetS (Table 6.3).

In obese participants without the MetS (BMI>30, non-MetS) there was no correlation between CBG levels and weight parameters, nor was there any difference between total CBG, haCBG, laCBG or % haCBG:total CBG compared to participants with normal BMI ($P = 0.4$; $P = 0.4$; $P = 0.09$ and $P = 0.1$ respectively) (Table 6.3). There was a trend toward the BMI>30, non-MetS participants having higher total CBG and lower % haCBG:total CBG compared to their counterparts with MetS ($P = 0.7$ and $P = 0.8$ respectively) (Table 6.3).

Table 6.3 CBG and cortisol levels among volunteers with and without the metabolic syndrome. Mean (± 95% CI).

	MetS (***n*** **= 17)**	**Non-MetS** (***n*** **= 83)**	***P*** **value** [a]	**BMI >30, MetS** (***n*** **= 13)**	**BMI>30, non-MetS** (***n*** **= 18)**	**BMI <25** (***n*** **= 35)**	***P*** **value** [b]
Total CBG	407 (375–439)	465 (444–486)	*0.02*	419 (384–453)	463 (428–499)	482 (451–513) [c]	*0.05*
haCBG	253 (222–285)	264 (251–277)	0.52	273 (243–302)	269 (231–306)	253 (233–273)	0.51
laCBG	153 (126–181)	201 (184–219)	*0.02*	146 (111–181)	195 (163–227) [c]	229 (204–254) [c]	*0.001*
haCBG:total CBG (%)	62.1 (55.6–68.6)	57.6 (55.1–60.1)	0.15	66 (59–72)	58 (52–64)	53 (50–56) [c]	*0.002*
Total cortisol	350 (273–427)	324 (295–352)	0.46	352 (253–452)	332 (274–390)	347 (294–401)	0.92
Free cortisol	21.8 (16.5–27.2)	20.4 (18.1–22.7)	0.61	21.6 (14.6–28.6)	21.3 (16.5–26.0)	22.4 (18.0–26.8)	0.94

Units are nmol/L. MetS – metabolic syndrome. [a] Students *t* test between MetS and Non-MetS; [b] One way ANOVA between BMI >30, MetS *vs*. BMI>30, non-MetS *vs.* BMI <25; [c] $P < 0.05$ compared with BMI >30 with MS group on Students *t* test. Italics indicate statistical significance

6.4.4 Free and total cortisol levels

Altered CBG affinity forms had no obvious effect on total:free cortisol ratios in these studies (Table 6.2 and 6.3).

6.5 Discussion

The major findings of this study are lower total and laCBG in obesity and higher haCBG, particularly when distributed centrally and associated with the MetS. The extent of the changes and their consistency over several measures of obesity, despite modest participant numbers, suggests that the findings are of biological significance. Existing knowledge about the cortisol delivery function of CBG, particularly at inflammatory sites leads us to propose that CBG cleavage may be impaired in central obesity/MetS, hence cortisol's anti-inflammatory action may not be realised, perpetuating inflammation (Geer *et al.*, 2014).

Previous studies have shown that total CBG levels are reduced in central obesity (Fernandez-Real *et al.*, 2002; Lewis *et al.*, 2009; Schafer *et al.*, 2013). This proposedly enhanced free cortisol levels, promulgating the central obesity process (Fernandez-Real *et al.*, 2002). Obesity and insulin resistance are associated with chronic, low-grade inflammation (Geer *et al.*, 2014) with evidence of elevated levels of IL-6, CRP and most importantly NE, which appears to directly contribute to inflammation-induced insulin resistance (Vgontzas *et al.*, 1997; Visser *et al.*, 1999; Mansuy-Aubert *et al.*, 2013). The finding of lower CBG levels in central obesity may have been due to increased inflammation-driven cleavage in these studies as measurement of circulating CBG affinity forms *in vivo* was not possible at the time (Fernandez-Real *et al.*, 2002; Lewis *et al.*, 2009; Schafer *et al.*, 2013). This is the first study of CBG affinity forms in obesity and although we confirm reduced total CBG, which may be due to the known effect of adipocyte-derived inflammatory cytokines on CBG synthesis

(Bartalena *et al.*, 1993; Tsigos *et al.*, 1998), our results also suggest reduced CBG cleavage. Hence, the net effect of altered CBG levels may be pro-inflammatory rather than anti-inflammatory.

The mechanism of reduced CBG cleavage in central obesity is unknown. In health, approximately 60–70% of CBG is intact haCBG and 30–40% is cleaved laCBG (Lewis and Elder, 2013). The dominant mechanism of cleavage in health is also unknown; traditionally recognised NE at sites of inflammation (Pemberton *et al.*, 1988) and now chymotrypsin and LasB (Lewis and Elder, 2014; Simard *et al.*, 2014) are known to cleave CBG, but other tissue enzymes may also act on CBG's RCL to effect cleavage (Nenke *et al.*, 2016a). Impaired cleavage may result from glycosylative changes to the CBG protein (Nenke *et al.*, 2016a), stimulated by the extensive inflammatory or hormonal milieu of adipose tissue (Kershaw and Flier, 2004; Wensveen *et al.*, 2015). Alternatively, polymorphisms in the CBG gene (Appendix B) may introduce resistance to cleavage (Simard *et al.*, 2015), as discussed in Chapter 7, predisposing to increased adiposity. While the definitive glycosylation profile of CBG has not been studied in patients with obesity or MetS, Fernandez-real *et al.* found that the molecular weight of CBG by Western blot and its interaction with lectin, Con A was different in obese glucose-tolerant patients compared to obese, glucose-intolerant patients, suggestive of altered glycosylation (Fernandez-Real *et al.*, 1999). Furthermore insulin reduces the ratio of acidic CBG glycoforms *in vitro* (Mihrshahi *et al.*, 2006).

The regulation of hepatic CBG biosynthesis by cytokines and hormones has been investigated. Oestrogens, as well as mitotane and selective oestrogen-receptor modulators increase CBG production, possibly via oestrogen α receptor-dependent mechanisms (Sakai *et al.*, 1978; Nader *et al.*, 2006), while IL-6 and insulin inhibit CBG production *in vitro* and *in*

vivo (Bartalena *et al.*, 1993; Crave *et al.*, 1995; Tsigos *et al.*, 1998; Fernandez-Real *et al.*, 1999) and could account for the reduced total CBG in obesity. Furthermore, along with the plethora of genomic and non-genomic effects mediated by glucocorticoids, prolonged exposure to glucocorticoids inhibits CBG gene expression via the GR involving the transcription factor CCAAT-enhancer-binding protein (C/EBP), a process similar to the regulation of other acute phase proteins (Verhoog *et al.*, 2014). Hence during periods of stress, elevated cortisol may suppress CBG synthesis, further enhancing free cortisol levels. As such, glucocorticoid-mediated CBG gene repression could contribute to the low CBG levels seen in our study, perpetuating the obese phenotype.

Impaired CBG cleavage may be a feature of chronic inflammatory states as we have described this finding in rheumatoid arthritis and α1 antitrypsin deficiency (Chapters 7 and 8) (Nenke *et al.*, 2016a; Nenke *et al.*, 2016b). Measures of CBG cleavage have not yet been reported in other chronic inflammatory conditions. This contrasts sharply with the situation in severe sepsis and septic shock (Chapter 3), as well as bacterial infection (Chapter 5) where total and haCBG levels are markedly reduced, consistent with increased cleavage, likely through increased activity of tissue proteases such as NE and potentially bacterial proteases (Simard *et al.*, 2014; Nenke *et al.*, 2015). The measurement of haCBG is dependent on recognition of the intact RCL by antibody 12G2; lack of recognition is interpreted as RCL cleavage with laCBG levels inferred from the difference between total CBG and haCBG. Hence a possible caveat could arise where the RCL is intact but "masked" from antibody access which would be interpreted as low levels of haCBG. In this event the RCL would presumably also be "masked" from cleavage and in effect CBG would sequester cortisol from inflammatory release. We consider this scenario unlikely as glycosylation on Asparagine^{347}, as an obvious antibody "masking" candidate, does not appear to alter antibody recognition (Lewis and

Elder, 2013). Another putative candidate may be the proposed “flip-flop” of the intact RCL into the protein structure but we consider this option also questionable (Lewis JG *et al.*, 2016). These considerations tend to rule out this caveat although it remains possible that structures distal to the RCL could still mask antibody recognition.

In our study, women had 11% higher total CBG than men, consistent with previous reports (Fernandez-Real *et al.*, 2002; Lewis *et al.*, 2004; Lewis *et al.*, 2010), but also had 11% higher haCBG and laCBG levels with no apparent gender difference in % haCBG:total CBG. Multiple studies have also noted gender dimorphism in the relationship of total CBG levels to anthropometric measurements (Lewis *et al.*, 2009; Lewis *et al.*, 2010; Schafer *et al.*, 2013). Despite gender differences having previously been attributed to an oestrogen effect, we believe the mechanism of sexual dimorphism in CBG levels is less clear. We show here that menopause, with its recognised reduction in oestrogen levels, is not associated with a fall in circulating CBG. One previous study found a 25% increase in total CBG levels following menopause (Wilson *et al.*, 1981) using CBG levels calculated from corticosteroid-binding capacity (CBC), a method commonly employed before direct quantitation, as employed in our study, was possible. CBC could be affected by variations in CBG binding affinity, explaining the discrepancy between our findings. Other studies show no variation in CBG concentrations or binding capacity despite variation in oestrogen levels throughout the menstrual cycle (Moore *et al.*, 1978a; Wilson *et al.*, 1981). These data call into question the influence of physiologic oestrogen levels on CBG production. Pharmacologic oestrogen doses, however, do appear to increase CBG production (Musa *et al.*, 1967). Pregnancy is also associated with increased CBG levels (Ho *et al.*, 2007), although this complex state is associated with a plethora of hormonal and immunologic changes that may have pregnancy-specific effects on CBG (Mitchell *et al.*, 2004), so that the effect of hyper-oestrogenaemia alone cannot be

discriminated. Contrastingly, in rats oestrogen decreases hepatic CBG gene expression and serum CBG levels (Smith and Hammond, 1992; Grasa *et al.*, 1998) and oestrogen treatment decreases CBG secretion by Hep G2 cells *in vitro* (Mihrshahi *et al.*, 2006). These data may not be relevant to humans or to physiological range oestrogen and do not aid in clarifying the mechanism by which pharmacologic oestrogen doses lead to elevated total CBG levels. This is explored in more detail in Chapter 9 where we show that differences exist between the increase in CBG affinity forms in the oestrogenic states of pregnancy and oral contraceptive use, potentially due to the altered glycosylation profiles induced by these conditions.

We did not find a correlation between age and total CBG which is consistent with some (Moore *et al.*, 1978a; Lapidus *et al.*, 1986; Lewis *et al.*, 2004) but not all previous reports (Fernandez-Real *et al.*, 2002; Lewis *et al.*, 2010; Schafer *et al.*, 2013). Two of the studies that showed a negative correlation, however, were undertaken in patients with medical comorbidities, which tend to accumulate with age and could confound the findings (Fernandez-Real *et al.*, 2002; Lewis *et al.*, 2010; Schafer *et al.*, 2013). The only study in healthy participants found a modestly significant negative correlation with age in the overall cohort that was not sustained when the genders were separated (Fernandez-Real *et al.*, 2002). In all studies the degree of correlation was low ($r = -0.097$ to -0.168).

6.6 Conclusion

This study has revealed lower total CBG levels in obesity, in accordance with previous findings, and new evidence of reduced CBG cleavage, especially in central obesity. Resistance to cleavage of CBG may hamper cortisol delivery to inflamed tissues and perpetuate the inflammation seen in central obesity.

Chapter 7

CBG cleavage in rheumatoid arthritis

7.1 Introduction

So far we have shown that the process by which CBG undergoes NE-mediated proteolytic cleavage (Pemberton *et al.*, 1988; Hammond *et al.*, 1990) occurs *in vivo*, particularly in acute inflammation where depletion of haCBG may limit the delivery of cortisol to tissues (Chapters 3 and 5; Nenke *et al.*, 2015; Nenke *et al.*, 2017). On the other hand, the ability of CBG to deliver cortisol during chronic inflammation, for example in MetS, may be inadequate due to cleavage resistance (Chapter 6; Nenke *et al.*, 2016c).

Cytokine-mediated activation of the HPA axis is a key feature of acute inflammation, stimulating cortisol secretion from the adrenals which acts at multiple sites to suppress and redirect inflammation, and to protect healthy tissues (Chrousos, 1995). In chronic inflammation, however, despite the elevation of key pro-inflammatory cytokines, ACTH and cortisol levels are unexpectedly normal. In addition, flattened diurnal variations and a blunted stressor-specific response is seen, indicating a degree of HPA axis dysfunction, which has been attributed to adaption or desensitisation of the HPA axis (Edwards, 2012). The hypofunctioning HPA axis in such chronic inflammatory diseases, including rheumatoid arthritis (RA), coronary artery disease, multiple sclerosis, Sjögren's syndrome, atopic dermatitis and systemic lupus erythematosus, fails to restrict inflammatory activity, predisposing to or increasing the severity of disease (Figure 7.1) (Harbuz, 1999; Nijm and

Jonasson, 2009). The aetiology of the HPA defect in these chronic inflammatory conditions, however, remains elusive.

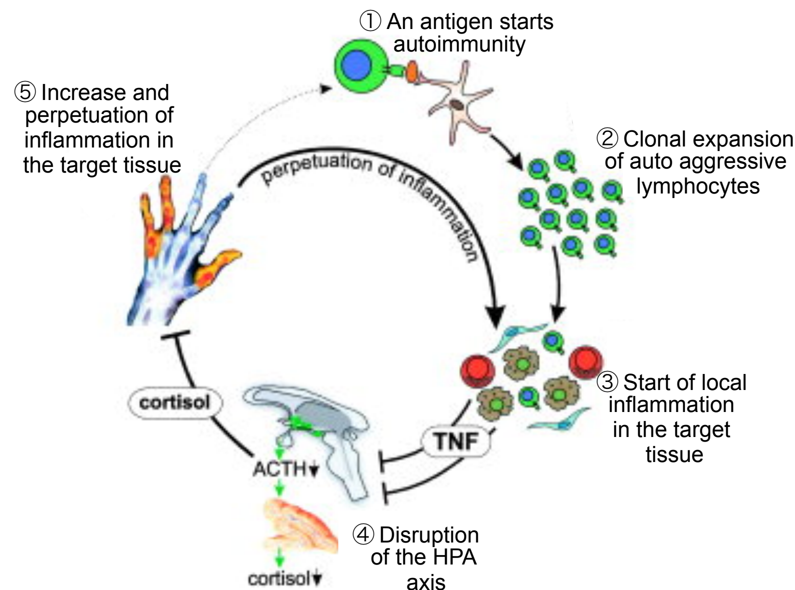


Figure 7.1 The HPA axis in the pathophysiology of chronic inflammatory RA. A vicious double cycle occurs when exposure to an initiating trigger instigates autoimmunity, which is not adequately challenged by the host's immune system. Cortisol production is insufficient to overcome subsequent inflammation (Straub *et al.*, 2008).

RA is a common, chronic inflammatory arthritis characterised by persistent synovitis, articular destruction and systemic inflammation (McInnes and Schett, 2011) where elevated levels of pro-inflammatory cytokines such as macrophage migration inhibitory factor (MIF) and the soluble receptor for IL-6 (IL-6sR) contribute to RA pathogenesis, correlate with disease activity, and predict response to some therapies (Jones *et al.*, 2001; Ayoub *et al.*, 2008; Nishina *et al.*, 2014).

The endogenous cortisol response in RA is subdued, with cortisol levels often being normal despite marked inflammation (Figure 7.2) (Eijsbouts *et al.*, 2005). This suggests an HPA axis defect in RA, of unknown basis, that may predispose to uncontrolled, chronic inflammation. RA has been recognised as a steroid-responsive disease since 1949 (Neeck, 2002) with oral glucocorticoids successfully slowing the progression of radiographic erosions (Kirwan *et al.*, 2007). Long-term low doses (7.5 mg prednisolone daily) in combination with other disease-modifying anti-rheumatic drugs (DMARDs) are often used despite the well-known dose-dependent accrual of toxicity (Hoes *et al.*, 2010).

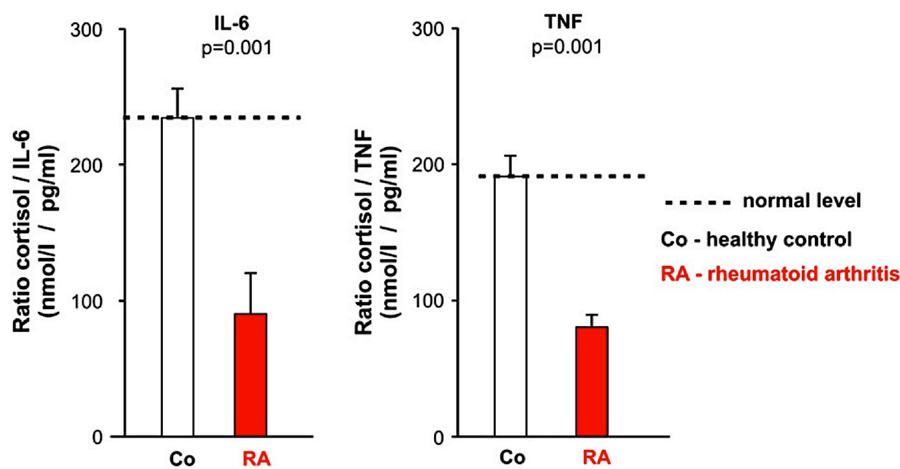


Figure 7.2 Ratio of cortisol to inflammatory cytokines in RA. Secretion of cortisol is inadequately low in relation to inflammatory markers in patients with RA compared with healthy controls (Capellino and Straub, 2008).

We investigated the role of CBG cleavage in RA, *in vivo*, for the first time, by performing a prospective study of patients with a range of clinical disease activity to determine whether CBG cleavage relates to RA activity.

7.2 Hypothesis and aims

Our objective was to determine whether CBG cleavage relates to RA disease activity. We hypothesised that impaired CBG cleavage may contribute to defective HPA axis function in RA, by impairing endogenous cortisol delivery to inflamed joints.

7.3 Research design and methods

7.3.1 Participants

We performed a prospective observational study at a single tertiary centre in Adelaide, South Australia from 2014–2015. Ethical approval was granted from the RAH HREC. Medical records of patients attending the Early Arthritis Clinic (EAC) at the RAH were reviewed for eligibility. The EAC is a clinic for patients following a new diagnosis of RA which provides long-term treatment according to a pre-defined algorithm incorporating disease activity and standardised monitoring as previously published (Proudman *et al.*, 2007). Healthy volunteers were recruited from outpatient clinics and community advertisements. Inclusion criteria were: age ≥ 18 years with either a diagnosis of RA (Arnett *et al.*, 1988), or no known RA. Exclusion criteria included use of oral or regular inhaled corticosteroids, intra-articular or intra-muscular corticosteroids within two weeks, COCP/HRT use or pregnancy. Eligible RA patients were invited to participate by their treating physician. Written, informed consent was obtained for all participants by the principal investigator.

7.3.2 Clinical measurements

Disease activity measurements for the RA cohort included patient-reported 100 mm visual analogue scale assessments of pain, global assessment of disease activity (patient GA) and fatigue, and physician-based global assessment of disease activity (physician GA) as well as

tender joint count (TJC28) and swollen joint count (SJC28) in 28 joints. Clinical data, laboratory tests including erythrocyte sedimentation rate (ESR) and CRP as well as use of DMARDs were recorded as per clinic protocol. Disease activity score (DAS28) was recorded after assessing TJC28, SJC28, ESR and patient GA (Fransen and van Riel, 2005). DAS28 scores <2.6 represent remission, while scores of 2.6 to ≤ 3.2, 3.2 to ≤ 5.1 and > 5.1 represent low, moderate and high disease activity respectively.

7.3.3 Sample collection and analysis

Upon enrolment, blood samples were collected, centrifuged, aliquoted and stored at –20 °C. Written and verbal education for salivary cortisol collection via Salivette® (Sarstedt Australia, Technology Park, South Australia) upon waking, 30 minutes post-waking, before lunch and before retiring to bed was provided to RA patients. RA patients performed salivary cortisol collections the following day, returning them to be centrifuged and stored at –20 °C until analysed.

Serum samples were analysed for total CBG, haCBG, laCBG, total and free cortisol, described in Chapter 2. Salivary samples were analysed for cortisol using the same commercial immunoassay as for serum cortisol. The functional sensitivity of the salivary cortisol assay is 8 nmol/L. The intra-assay coefficients of variation for the total CBG and haCBG assays were 4.6 and 3.0% respectively.

7.3.4 Macrophage migration inhibitory factor and interleukin-6 soluble receptor

Raybio® Human MIF ELISA (Cat no. ELH-MIF, Jomar Life Research, Welland SA) and eBioscience Human sIL-6R Instant ELISA (Cat no BMS214INST, Jomar Life Research,

Welland SA) kits were used to measure MIF and IL-6sR by immunoassay respectively. Intra-assay coefficients of variation were 6.8 and 7.5% respectively.

7.3.5 Statistical Analyses

Data were analysed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc., San Diego, California). Results are presented as mean ± SEM unless otherwise stated. Comparisons between two groups were performed using two-tailed unpaired Student's *t* test. Chi-squared testing was used to determine differences between categorical variables. Comparison between three or more groups was performed by one-way ANOVA, with multiple comparisons for trend where needed. Correlations between continuous variables were assessed with Pearson's correlation coefficient. Salivary cortisol area under the curve (AUC) was calculated by the trapezoidal method. $P < 0.05$ was taken as statistically significant.

7.4 Results

7.4.1 Participants

We enrolled 53 RA patients. Their baseline characteristics are detailed in Table 7.1. One patient withdrew before any blood or saliva samples were taken and clinical disease activity data were missing for one patient: both are included in Table 7.1. Ages ranged from 30–88 years with 60% of participants being female. Fifty-three percent of patients were obese and a further 28% were overweight. The time from RA diagnosis ranged from 4 months to 16 years. Most patients were receiving three DMARDs for the treatment of their RA. The majority of patients (57%) were past or present smokers, a known risk factor for RA. The participants had a broad range of disease activity with the number of swollen and tender joints (in 28 joints)

ranging from 0–21 and 0–22 respectively; DAS28 score range 1.24–6.37. Mean ESR was elevated: range 2–120 mm/hour (reference interval <20). Seventy-three healthy controls, matched for age, BMI and gender were also enrolled.

Table 7.1 Baseline characteristics of RA patients. Mean (± SD).

Patient Characteristics	RA cohort (*n* = 53)	Controls (*n* = 73)	*P* value
Gender (M:F)	21:32	30:43	0.87
Age (years)	62 (13)	58 (13)	0.08
BMI (kg/m^2)	30.7 (7.4)	28.3 (6.9)	0.07
Seropositive:seronegative	43:10		
Years since diagnosis	5.3 (4.4)		
DMARDs			
Methotrexate	44 (83%)		
Sulfasalazine	30 (57%)		
Hydroxychloroquine	36 (68%)		
Leflunomide	13 (25%)		
Gold	4 (8%)		
Azathioprine	3 (6%)		
Biological	8 (15%)		
Tocilizumab	1 (2%)		
Abatacept	1 (2%)		
Adalimumab	6 (11%)		
Number of DMARDs used per patient			
0	3 (6%)		
1	10 (19%)		
2	11 (21%)		
3	21 (40%)		
4	2 (4%)		
5	6 (11%)		
Current smoker	17%		
Former smoker	40%		
Never smoker	43%		
Disease activity scores			
Patient pain [a]	28.0 (27.3)		
Fatigue [a]	28.2 (28.4)		
Patient GA[a]	23.9 (22.8)		
Physician GA [a]	22.0 (22.0)		
TJC28	4.9 (6.5)		
SJC28	4.0 (5.2)		
ESR [b]	23.9 (24.5)		
CRP	8.6 (13.6)		
DAS28 [b]	3.6 (1.6)		

Reference range for CRP <8 mg/L; ESR <20 mm/hour. [a] Assessed on 100 mm visual analogue scale. [b] results not available for 3 patients: *n* = 50.

7.4.2 Serum corticosteroid-binding globulin affinity forms: RA vs. *controls*

CBG affinity form levels are shown in Table 7.2, with significant findings depicted in Figure 7.3. Mean total CBG was lower in the RA cohort than healthy controls, although haCBG levels did not differ, hence the percentage of haCBG to total CBG was higher in RA compared with controls. laCBG levels were also significantly lower in the RA cohort. Collectively, these findings are consistent with lower CBG cleavage rates in patients with RA.

Table 7.2 CBG affinity forms and cortisol levels in RA patients. Mean (± SEM).

	Control $n = 73$	**RA cohort** $n = 52$	***P* value** [a]	**RA disease activity**				***P* value** [b]
				Remission $n = 17$	Low $n = 5$	Moderate $n = 16$	High $n = 11$	
Total CBG (nmol/L)	459 (11)	421 (12)	0.02	379 (20)	422 (41)	441 (17)	457 (32)	0.02
haCBG (nmol/L)	268 (7)	268 (10)	0.99	239 (21)	263 (12)	274 (16)	307 (15)	0.02
laCBG (nmol/L)	191 (8)	153 (9)	0.003	139 (11)	159 (34)	166 (16)	153 (29)	0.58
haCBG:total CBG (%)	59 (1)	64 (2)	0.02	62 (3)	64 (5)	63 (3)	68 (4)	0.25
Total cortisol (nmol/L)	329 (14)	293 (14)	0.09	331 (25)	294 (38)	259 (27)	285 (28)	0.19
Free cortisol (nmol/L)	21 (1)	19 (1)	0.29	22 (2)	17 (3)	18 (2)	16 (2)	0.12
Salivary cortisol AUC (nmol/L per 14 hours)	NA	140 (7) [c]	NA	144 (11) [d]	129 (13) [e]	142 (14) [f]	135 (20) [g]	0.85

[a] Student's *t* test for controls *vs*. total RA cohort. [b] One-way ANOVA with multiple comparisons post-test for linear trend across RA disease activity (Remission: DAS28 <2.6, Low: 2.6 < DAS28 ≤ 3.2, Moderate: 3.2 < DAS28 ≤ 5.1, High: DAS > 5.1). [c] $n = 43$. [d] $n = 15$. [e] $n = 4$. [f] $n = 13$. [g] $n = 9$. NA – not applicable.

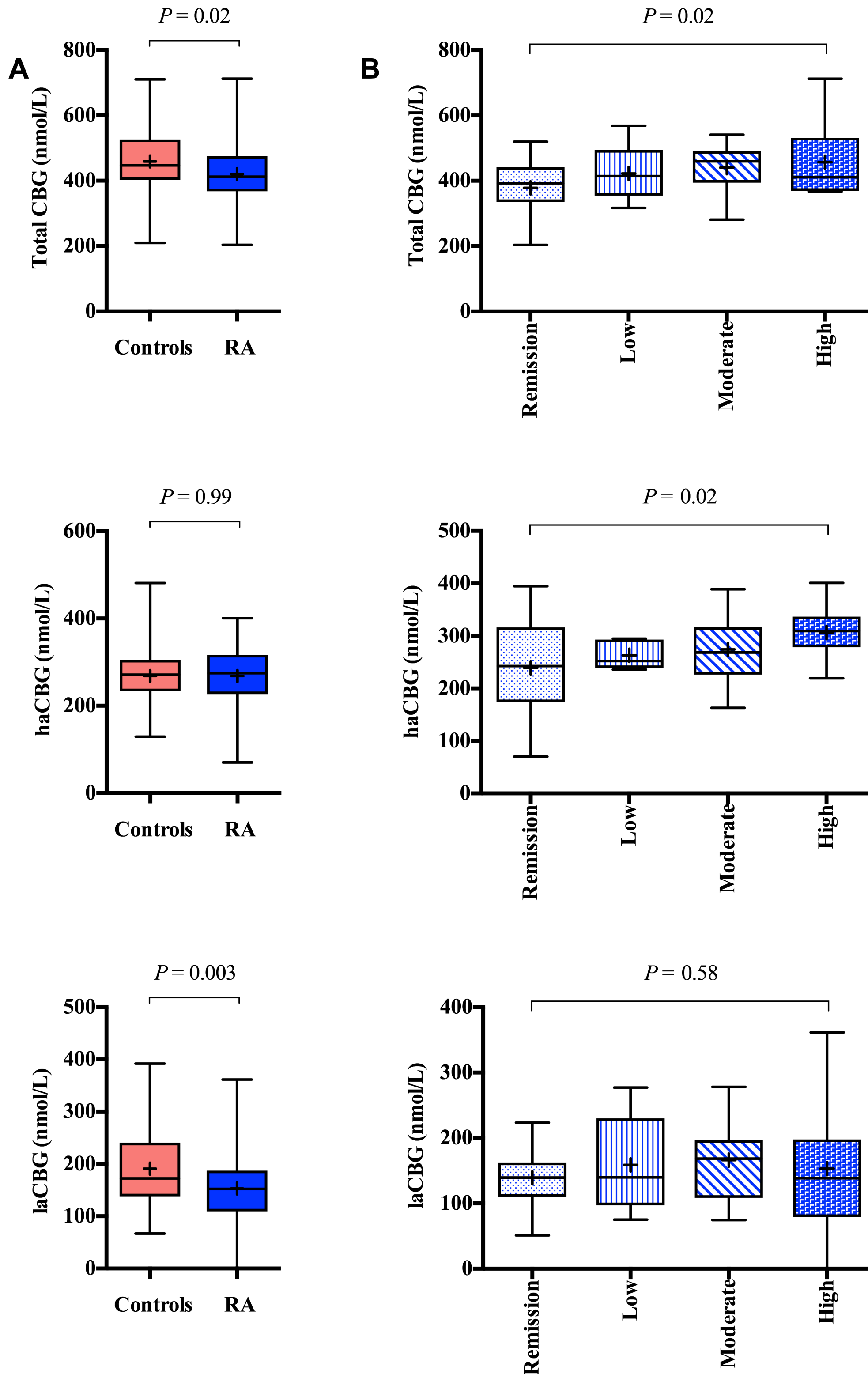


Figure 7.3 **CBG affinity forms in RA. Panel A: CBG affinity forms between controls and RA patients. Total CBG and laCBG levels were higher in controls than RA patients. Panel B: CBG affinity forms according to RA disease activity. There was a significant trend towards increasing total and haCBG with increasing RA disease activity. Box plots include 25th – 75th percentiles; line at median and ✚ at mean; whiskers extend to minimum and maximum. Remission – DAS < 2.6; low – 2.6 < DAS ≤ 3.2; moderate – 3.2 < DAS ≤ 5.1; high – DAS > 5.1.**

CBG levels were also analysed according to RA disease duration. There was a significant positive correlation between haCBG levels and disease duration (Figure 7.4). On the other hand, there was no difference in total, haCBG, laCBG levels or haCBG:total CBG when patients with RA of disease duration < 1 year (n = 11) were compared to those with disease duration > 1 year (n = 41); 416 ± 19 *vs*. 421 ± 15, P = 0.86; 263 ± 16 *vs*. 269 ± 12, P = 0.81; 153 ± 18 *vs*. 152 ± 11 nmol/L, P = 0.98 and 64 ± 3 *vs*. 64 ± 2 %, P = 0.95 respectively.

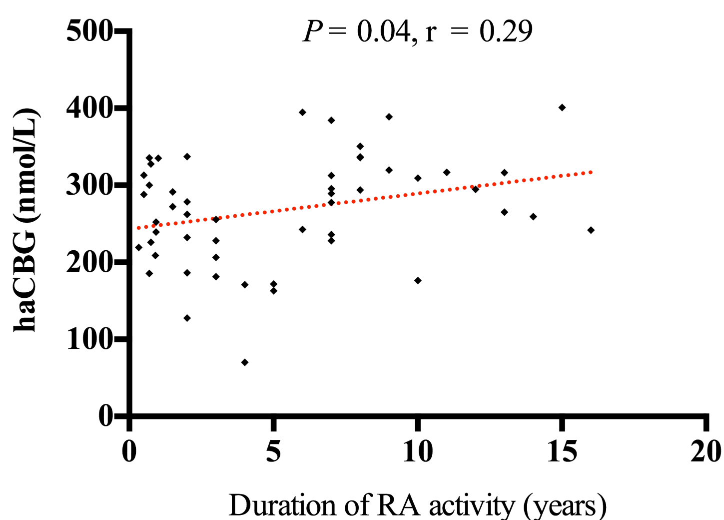


Figure 7.4 Association between haCBG levels and RA disease duration. Patients with shorter RA disease duration had lower levels of haCBG.

7.4.3 Serum CBG levels and RA disease activity

CBG affinity forms and cortisol levels were analysed according to RA disease activity as assessed by DAS28 (Table 7.2, Figure 7.3). The lowest total CBG and haCBG levels and haCBG:total CBG ratio were seen in RA patients in remission (DAS28 < 2.6). Hence, it can be inferred that the highest CBG cleavage rate occurred in the remission group. Patients with the greatest disease activity (DAS28 > 5.1) had higher total CBG, higher haCBG and higher haCBG:total CBG percentage, consistent with the lowest CBG cleavage rates.

There was a positive relationship between clinical markers of disease activity and haCBG levels for patient pain scores ($P = 0.021$, r = 0.32), fatigue scores ($P = 0.024$, r = 0.32), patient GA ($P = 0.023$, r = 0.32), physician GA ($P = 0.037$, r = 0.32), TJC28 ($P = 0.016$, r = 0.36), and DAS28 ($P = 0.017$, r = 0.34).

7.4.4 Serum cortisol: RA **vs.** *controls*

Total and free cortisol levels were similar in RA patients and controls (Table 7.2). Samples were taken between 8:30 am and 1:30 pm reflecting the times of EAC visits. There was no correlation between time and serum cortisol for RA patients ($P = 0.45$, r = −0.11), but there was for controls for the same time period ($P = 0.03$, r = −0.28). A significant correlation between age and total cortisol was seen in the RA cohort ($P = 0.04$, r = 0.29).

7.4.5 Serum cortisol and RA disease activity

There was no difference in total or free cortisol levels when RA patients were analysed by disease activity categories (Table 7.2), although significant negative associations did exist (Figure 7.5, Panel A). Total and free cortisol were lower in patients with higher disease activity scores including TJC28 ($P = 0.084$, r = −0.24 and $P = 0.046$, r = −0.28; data not shown) and DAS28 ($P = 0.048$, r = −0.28 and $P = 0.018$, r = −0.34 respectively; Figure 7.5, Panel A). Furthermore, free and total cortisol:IL-6sR ratios were inversely correlated to disease activity suggesting insufficient cortisol production for the degree of inflammation (Figure 7.5, Panel B).

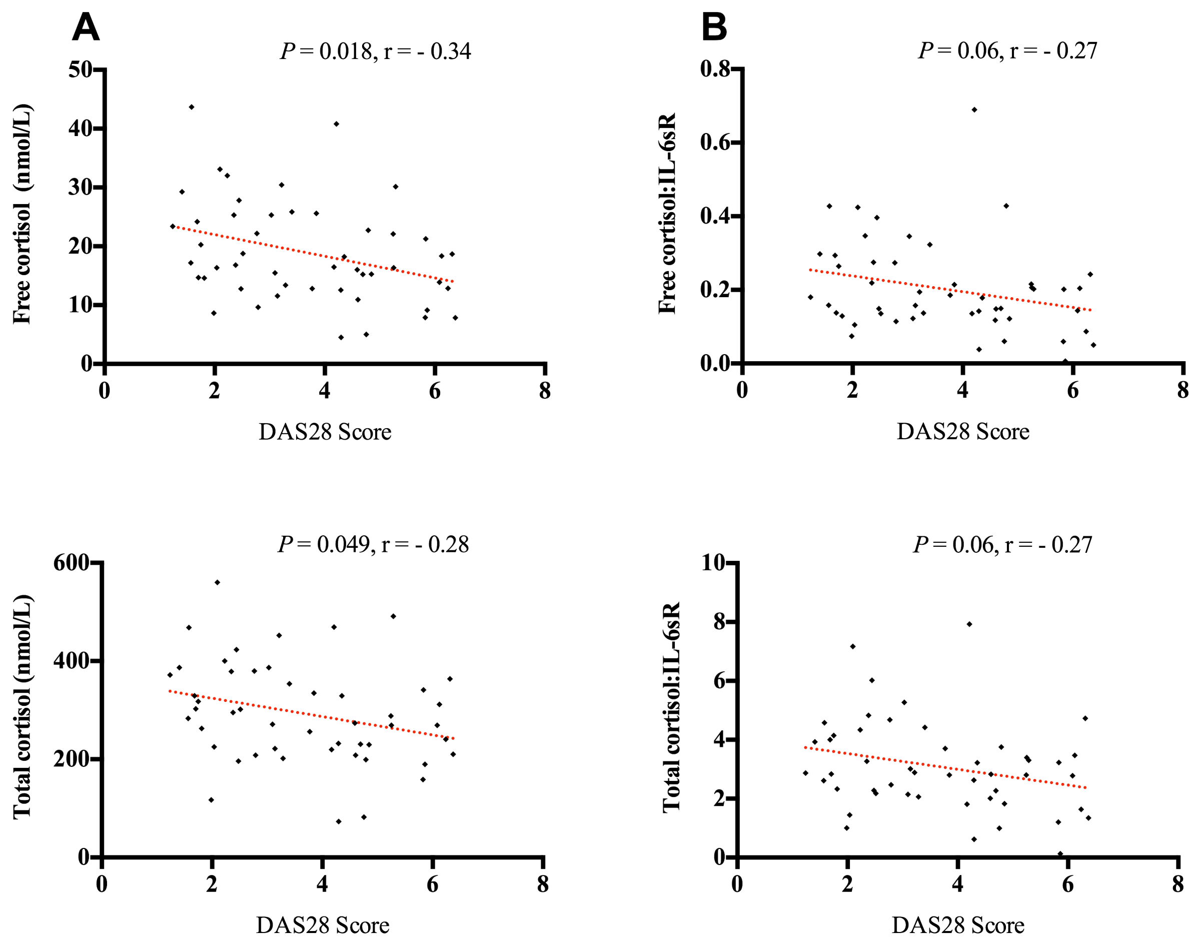


Figure 7.5 Total and free cortisol, with ratio to IL-6sR in relation to RA disease activity. Panel A: Correlations between serum free and total cortisol levels and DAS28 score. Levels of free and total cortisol are inversely correlated with disease activity. Panel B: Correlations between ratios of free and total cortisol to IL-6sR and DAS28 score.

7.4.6 Diurnal salivary cortisol in RA

Forty-three patients returned diurnal salivary cortisol samples. The mean salivary cortisol level over the day for RA patients was 12.1 ± 0.6 nmol/L. Area under the curve for cortisol day curves ranged from 55–265 nmol.hr/L, mean 141 ± 7 nmol.hr/L per 14.5 hours. The diurnal rhythm was maintained with a significant decline in levels throughout the day (Figure 7.6; $P < 0.0001$). A waking cortisol response was not observed. Median (range) times for the collection of samples were 7:25 am (4:00–11:45 am; upon waking), 8:17 am (4:30 am – 1:00

pm; 30 minutes post-waking), 12:30 pm (11:00 am – 6:30 pm; pre-lunch), and 10:10 pm (4:00–11:59 pm; pre-bed).

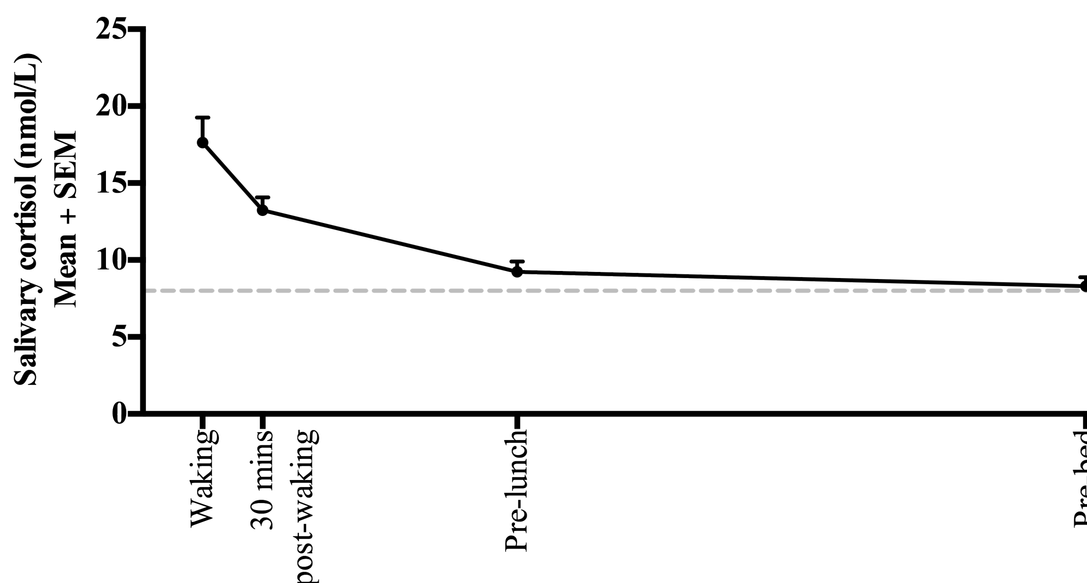


Figure 7.6 Mean salivary cortisol levels at four set collection time-points in RA patients. Levels declined across the day (*P* < 0.0001). Dashed line represents the functional sensitivity of the assay. Waking; *n* = 43, 30 mins post-waking; *n* = 42, pre-lunch; *n* = 43, pre-bed; *n* = 42.

7.4.7 Interleukin-6 soluble receptor and macrophage migration inhibitory factor

IL-6sR levels ranged from 53–1497 ng/mL, median 102 ng/mL, with 90% of results being within the assay reference range (66–203 ng/mL). IL-6sR levels correlated with haCBG, laCBG and haCBG:total CBG (*P* = 0.034, r = 0.30; *P* =0.046 r = -0.28; *P* = 0.009, r = 0.36) as well as BMI and three disease activity indices; physician GA, SJC28 and TJC28 (*P* = 0.018, r = 0.33; *P* = 0.001, r = 0.44; *P* = 0.001, r = 0.47; *P* = 0.011, r = 0.36 respectively).

MIF levels ranged from 0.14–6.0 ng/mL, median 0.73 ng/mL. In all patients MIF levels were lower than the assay reference range (15–52 ng/mL). MIF correlated with physician GA and SJC28 (P = 0.03, r = 0.31 and P = 0.013, r = 0.35).

7.5 Discussion

We investigated cleavage of CBG *in vivo* in RA patients with a range of disease activity. There were no previous studies of CBG cleavage in RA. We found that CBG cleavage was lesser in RA patients than in healthy controls, with lower laCBG and higher haCBG:total CBG ratio in RA patients. We have also shown that RA patients with low levels of CBG cleavage, as evidenced by higher haCBG levels and higher haCBG:total CBG ratios, had greater disease activity as measured by DAS28, while higher (near normal) rates of CBG cleavage were seen in patients able to achieve remission. This suggests that an inability to appropriately cleave CBG to release free cortisol is related to increasing disease activity and represents a previously unidentified abnormality of the HPA axis in patients with RA.

RA patients with low haCBG levels and higher free and total cortisol levels had lower disease activity. We found similar total cortisol levels among patients with RA and controls, with maintenance of circadian variation, consistent with many previous studies (Eijsbouts *et al.*, 2005). Furthermore, a lower ratio of cortisol to IL-6sR, suggesting an insufficient cortisol response to the inflammatory stimulus, was associated with higher disease activity. Thus our study supports the concept of an "abnormally normal" HPA axis in RA with respect to total cortisol levels, but identifies decreased CBG cleavage as a feature, implying impaired delivery of cortisol to inflamed sites, thereby perpetuating chronic inflammation. These results concord with our findings in obesity and the metabolic syndrome, also representing chronic inflammation, where patients with MetS have lower total CBG and laCBG than

controls and where haCBG increases with markers of abdominal obesity implicating decreased CBG cleavage in disease progression (Chapter 6; Nenke *et al.*, 2016c)

In 1989, Sternberg (Sternberg *et al.*, 1989) reported an animal model in which the inability of the HPA axis to mount an adequate cortisol response increased susceptibility to chronic inflammatory arthritis. The inbred Lewis (LEW/N) rat exhibits inadequate HPA axis responsiveness and cortisol production in response to antigenic stimuli and is thus susceptible to experimental autoimmune disease, such as RA-like arthritis, while Fischer (F344/N) rats have hyperactive HPA-axes and are relatively resistant to disease induction. Dexamethasone administration to Lewis rats reverses their susceptibility to arthritis implicating insufficient glucocorticoids in the pathogenesis of their autoimmune phenotype (Sternberg *et al.*, 1989). This concept is thought to apply to RA in humans, although a clear defect of the HPA has hitherto not been identified. The study presented here suggests that an HPA axis-related abnormality— insufficient delivery of cortisol, via CBG —exists in the counter-regulatory response to inflammatory stimuli in RA.

CBG polymorphisms that alter the sensitivity of CBG to cleavage may predispose patients to RA. Until recently, CBG deficiency was considered rare, however genetic variations are increasingly emerging (Appendix B). Of interest, two naturally occurring polymorphisms in the *SERPINA6* gene, CBG I179V and CBG I279F, have been identified which may alter the propensity for CBG to be cleaved by proteases, with these two variants being resistant to cleavage following exposure to NE, chymotrypsin and *P. aeruginosa, in vitro* (Simard *et al.*, 2015). Mutagenesis studies have identified a further potential mutation, T342, which is resistant to NE proteolysis, although there is no clinical correlate as yet (Braun *et al.*, 2010). Importantly, a number of other mutations resulting in loss of CBG production or loss of

steroid-binding ability have been found in kindreds after index cases presented with chronic pain and fatigue syndromes (Torpy *et al.*, 2012). Furthermore, in a population-based study examining HPA axis-related susceptibility to musculoskeletal pain, two CBG haplotypes showed the only significant relationship to risk of chronic widespread pain (fibromyalgia) (Holliday *et al.*, 2010). Hence, CBG is thought to play a role in stress system regulation and the risk of development of idiopathic fatigue/pain syndromes states of relative hypocortisolism (Heim *et al.*, 2000; Torpy *et al.*, 2012);. As recently recommended, further studies should screen for CBG mutations in patients with inflammatory diseases such as RA to confirm whether CBG alterations/deficiencies play a role in the pathogenesis of such conditions (Simard *et al.*, 2015).

Post-translational modifications to CBG, particularly glycosylation, could also increase susceptibility to RA or perpetuate chronic inflammation following disease onset. Cytokine-induced glycosylation changes of acute phase reactants and other glycoproteins in inflammatory conditions have been well documented (McCarthy *et al.*, 2014). In particular, RA is known to be associated with pro-inflammatory protein glycosylation patterns which have been implicated in RA pathogenesis (McCarthy *et al.*, 2014; Mastrangelo *et al.*, 2015) with normalisation of these changes being associated with a good response to therapy (Mastrangelo *et al.*, 2015). Pooled human plasma contains up to 26 glycoforms suggesting significant individual variation *in vivo* (Sumer-Bayraktar *et al.*, 2011). Native glycosylation of CBG in health (Sumer-Bayraktar *et al.*, 2011) and in pregnancy (Mitchell *et al.*, 2004) have been documented, but changes due to inflammation have not. Glycosylation of CBG appears to be important for protein stability, regulation of degradation, binding affinity, thermosensitivity and half-life (Hossner and Billiar, 1981; Sumer-Bayraktar *et al.*, 2011; Chan *et al.*, 2013). Alterations in glycoforms may also be important for the binding of CBG to

specific receptors in target cells or accessibility of cortisol to the GR (Sumer-Bayraktar *et al.*, 2011). Furthermore, changes at the consensus glycosylation site at P3 of the CBG RCL (Asparagine^{347}) could interrupt protease access to and cleavage of CBG (Sumer-Bayraktar *et al.*, 2011; Gardill *et al.*, 2012; Simard *et al.*, 2014; Sumer-Bayraktar *et al.*, 2016). Hence, altered CBG glycosylation in RA may reduce CBG cleavage and therefore cortisol delivery to inflamed tissues.

One previous study measured total CBG by commercial radioimmunoassay in RA (Eijsbouts *et al.*, 2005), finding similar levels in healthy controls and patients with recent onset RA (<1 year). In our study, CBG affinity forms were similar regardless of whether RA disease duration was <1 year or >1 year, however overall there was a positive correlation between haCBG levels and disease duration. This discrepancy could be due to lower numbers in the <1 year group, or the fact that patients with longer disease duration have likely developed multiple co-morbidities which may independently influence CBG. Notably, the mean duration of disease activity for our study was 5.3 years (range 4 months – 16 years), emphasising the chronic nature of inflammation in RA. Alternatively, the findings could suggest a depletion of haCBG and cortisol delivery in the initial stages of RA. It is interesting to note that total CBG is not decreased in RA as would be expected of a negative acute phase reactant under such inflammatory conditions (Eijsbouts *et al.*, 2005).

We acknowledge some limitations to our study. We did not show a cortisol awakening response (CAR) in the salivary cortisol day curves although the circadian rhythm was still clearly demonstrated. To our knowledge, CAR has not previously been studied in RA patients, but fatigue, burnout and exhaustion, psychosocial factors often seen in RA, are associated with an attenuated CAR (Chida and Steptoe, 2009). It is possible that waking

cortisol response is blunted in RA due to such concomitant factors, but a study with electronically timed saliva samples to exclude the possibility of poor adherence to the protocol should be performed for verification. Additionally, we are unable to exclude a possible drug effect of DMARDs on CBG cleavage. The use of these drugs was either too frequent, for example methotrexate, or too infrequent, to permit reliable subgroup analyses to examine for possible effects. There is no evidence, however, to suggest that this may occur. Finally, when stratified according to disease activity, patient numbers in each category were modest.

7.6 Conclusion

We have shown that CBG cleavage, the process by which cortisol is delivered to inflamed tissues, is reduced in RA compared with controls, with increased CBG cleavage rates and enhanced delivery of cortisol being associated with remission. These results need to be confirmed in a longitudinal study looking at CBG cleavage in individuals over time in relation to disease improvements and flares, but could be useful in directing therapy by predicting which patients will benefit from low-dose exogenous glucocorticoid administration. Alternatively, engineered CBG with better cleavage characteristics may be useful in ensuring adequate delivery of endogenous cortisol in RA.

Chapter 8

Paradoxical reduction in CBG cleavage in α1 antitrypsin deficiency

8.1 Introduction

CBG is a serpin protein (Hammond *et al.*, 1987) with no inhibitory capacity. NE, a destructive serine proteinase with broad substrate specificity, offers the most recognised mechanism for CBG cleavage resulting in conformational transition that disrupts the cortisol-binding site and decreases CBG's affinity for cortisol by 9−10 fold, effectively releasing cortisol at inflammatory sites (Pemberton *et al.*, 1988; Hammond *et al.*, 1990; Klieber *et al.*, 2007; Zhou *et al.*, 2008; Chan *et al.*, 2013). We have found that in basal states including in healthy volunteers, where an abundance of NE is not expected, CBG cleavage still occurs with laCBG accounting for up to 45% of circulating CBG (Chapter 6; Nenke *et al.*, 2016c). Furthermore, the absence of neutrophils does not prohibit cleavage during infection (Chapter 5; Nenke *et al.*, 2017). These data suggest that non-NE cleavage may be an important factor in CBG physiology. However to date, only pseudomonal LasB and chymotrypsin have been identified as alternate proteases able to cleave CBG (Lewis and Elder, 2014; Simard et al., 2014).

Alpha-1 antitrypsin (AAT), also a serpin protein, is the major inhibitor of NE, working in concert with other endogenous proteinase inhibitors to protect epithelial tissues from excessive proteolysis. AAT is produced by hepatocytes in large quantities such that NE is saturated in the general circulation. AAT is also secreted locally by macrophages, monocytes

and epithelial cells in response to inflammatory cytokines and lipopolysaccharide (Knoell *et al.*, 1998; Henriksen, 2014). When NE is released in concentrated bursts from activated neutrophils at sites of inflammation, a process described as "quantum proteolysis", it momentarily overwhelms inhibitory AAT (Liou and Campbell, 1996; Campbell *et al.*, 1999), allowing proteolysis, including CBG cleavage, to occur at sites of inflammation (Pemberton *et al.*, 1988; Hammond *et al.*, 1990). AAT, produced by the *SERPINA1* gene, has a wide spectrum of protein variants (Pi), distinguishable by isoelectric focussing. Pi M alleles have normal AAT levels, while Pi S and Pi Z alleles have serum levels of 60% and 15% of normal levels respectively (Table 8.1). Patients with AAT deficiency (AATD) have increased NE activity which varies according to AAT levels and which leads to a recognised proteinase-antiproteinase imbalance (Weitz *et al.*, 1992; Campbell *et al.*, 1999; Carter *et al.*, 2011). At localised inflammatory sites in AATD, the area and duration of tissue exposed to attack by NE is increased 10-fold due to the lack of inhibiting AAT (Campbell *et al.*, 1999). The resulting AATD phenotype is early onset basal emphysema, with increased proteolysis of lung parenchymal elastin due to the inherent proteinase-antiproteinase imbalance and specifically NE excess (Stockley, 2014). This theory is the basis for the administration of AAT augmentation therapy, which has been shown to decrease markers of elastin degradation due to NE (Ma *et al.*, 2013).

Table 8.1 Serum concentrations of AAT according to genotype. Adapted from de Serres and Blanco (2014).

AAT Pi genotype	AAT serum levels (g/L)	Risk for pulmonary emphysema
MM	1.0−2.0	Normal
MS	1.0−1.8	Normal
SS	0.7−1.05	Possible
MZ	0.66−1.2	Possible
SZ	0.45−0.8	Slightly increased
ZZ	0.1−0.4	Very increased
Null	Undetectable	Almost certain

It was previously thought that NE-mediated CBG cleavage did not occur systemically due to the presence of saturating levels of AAT (Hammond *et al.*, 1990). However, we have shown that haCBG and laCBG coexist and can be measured in the circulation, with sometimes up to 45% of CBG in apparently healthy individuals being of the cleaved laCBG affinity form (Lewis and Elder, 2013; Nenke *et al.*, 2016c). Thus systemic cleavage of CBG may be occurring continuously in the general circulation, rather than exclusively at inflammatory sites. It may be that a sufficient reservoir of haCBG is advantageous in providing a pool of cortisol for immune modulation at inflammatory sites. It is not known if NE mediates systemic CBG cleavage under low-inflammatory conditions.

In vitro, incubation of human CBG with activated leukocytes results in protein cleavage, a process that is blocked when cells are pre-incubated with an excess of AAT (Hammond *et al.*, 1990). Sepsis and septic shock, conditions associated with elevated NE (Endo *et al.*, 1995), demonstrate increased CBG cleavage *in vivo,* (Nenke *et al.*, 2015). It is not known, however, whether AAT deficiency is associated with increased CBG cleavage in patients without active acute inflammation.

8.2 Hypothesis and aims

We hypothesised that the uninhibited NE activity in AATD would result in increased cleavage of CBG, as shown by reduced levels of circulating haCBG and elevated laCBG. Hence, we undertook an observational study to determine the levels of haCBG and laCBG in patients with AATD *in vivo*.

8.3 Research design and methods

8.3.1 Participants and Design

This was a prospective, observational cross-sectional study, conducted between December 2014 and April 2015 at a single tertiary centre in Adelaide, South Australia. The protocol was approved by the RAH HREC. Participants were identified through the Clinical Trials Database, Department of Thoracic Medicine, and were mailed an invitation to participate. Control patients were recruited from the community and hospital outpatient clinics by advertising. Inclusion criteria: either no known AATD or previously diagnosed AATD of phenotypes ZZ, SZ or MZ, as confirmed by isoelectric focusing (Zerimech *et al.*, 2008), age ≥ 18 years. Exclusion criteria: Previous lung or liver transplant, known disease of the HPA axis, pregnancy, use of COCP/HRT, or active infective condition. Participants who responded to the invitation or advertising were screened and provided written informed consent prior to enrolment.

8.3.2 Assessments

Baseline measurements including height, weight, and BP were performed. Blood was collected and centrifuged with serum then aliquoted and stored at –20 ºC. Blood samples (median) were taken at 10:23 am in the control group and 11:37 am in the AATD group.

8.3.3 Laboratory measurements

Blood samples were collected for serum total and free cortisol as well as total CBG and haCBG. The intra-assay coefficients of variation for the total CBG and haCBG assays were 9.3% and 3.5% respectively. Hormone assays were described in Chapter 2.

Serum AAT levels were performed on patients with known AATD by immunoturbidity using a Tina-Quant α1 antitrypsin assay (Roche Diagnostics, Castle Hill, New South Wales) on a Thermo KoneLab 30 Analyser, calibrated with reference to CRM 470 protein standardisation materials (reference interval 0.9–2.0 g/L). Phenotyping was performed by isoelectric focusing using a Hydragel 18 α1AT isofocusing kit.

As NE is rapidly inhibited *in vivo*, direct measurement in circulating serum is of doubtful value. Furthermore, direct and indirect markers of elastin degradation can be measured, but have not been well validated and their role in clinical trials has been questioned (American Thoracic Society and European Respiratory Society, 2003). Hence we have not performed measures to confirm the widely accepted over-activity of NE in our AATD patients (American Thoracic Society and European Respiratory Society, 2003).

8.3.4 Statistical analyses

Data were analysed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc., San Diego, California). Results are presented as median (range) unless otherwise stated. Comparisons between groups were performed using the Mann-Whitney U test as data were not normally distributed. Chi-squared testing was used to determine differences between categorical variables. Comparison between three or more groups was performed by Kruskal-Wallis one-way analysis of variance of ranks. Correlations between continuous variables were assessed with Spearman's rank correlation coefficient. $P < 0.05$ was taken as statistically significant.

8.4 Results

Ten AATD patients responded to the invitation and were enrolled: see Table 8.2 for baseline characteristics. All seven women were post-menopausal. Two patients of ZZ genotype were receiving treatment with purified human AAT protein (Zemaira®, CSL Behring LLC; terminal half-life 5.1 days) and in these cases laboratory measures were taken at the dosing trough prior to the weekly infusion. Notably, the AAT levels of these patients did not differ from those AATD PiZZ patients not on AAT infusions ($P = 0.133$). Eight out of ten patients were taking long-term inhaled corticosteroids (ICS) for chronic obstructive pulmonary disease (COPD), one was taking long-term physiologic dose prednisolone (5 mg/day) and three had used a short course of high-dose oral corticosteroids within the past month. Four patients were non-smokers, four were ex-smokers and two were current smokers. As expected, patients with the ZZ genotype had significantly lower AAT levels than those with the SZ/MZ genotypes; 0.27 (0.02–0.4) *vs*. 0.43 (0.04–0.89) g/L, $P < 0.001$ (Table 8.3). Twenty-eight healthy controls were also analysed. They did not differ from AATD patients in gender, age or BMI, although none were taking long-term ICS. Three healthy controls were ex-smokers and there were no current smokers.

Table 8.2 Baseline characteristics in AATD patients and controls. Median (range).

	AATD *n* = 10	**Control** *n* = 28	***P* value**
Male:Female	3:7	12:16	0.48 [a]
Age (years)	63 (38−75)	62 (32−80)	0.74 [b]
Genotype			
• ZZ	60 %	NA	−
• MZ	30 %	NA	−
• SZ	10 %	NA	−
AAT (g/L) [c]	0.28 (0.02−0.89)	NA	−
BMI (kg/m^2)	25.3 (18.6−35.4)	26.0 (18.5−34.9)	0.83 [b]
Current smoker	20 %	0 %	0.06 [a]
Long-term ICS use	80 %	0%	*<0.0001* [a]

[a] Chi-squared test; [b] Mann-Whitney U test; [c] Reference interval 0.9–2.0 g/L; NA – not applicable.

Table 8.3 Serum AAT and cortisol concentrations in AATD patients and controls. Median (range).

	ZZ	MZ & SZ	Control	*P* value [b]
AAT (g/L) [a]	0.27 (0.02–0.4)	0.43 (0.04–0.89)	NA	*<0.001* [c]
Total cortisol (nmol/L)	291 (180–371)	296 (126–342)	350 (165–517)	0.19
Free cortisol (nmol/L)	17 (9.4–27.3)	17 (5.7–20.3)	23 (11–71)	0.14
Free cortisol (%)	5.5 (5.1–7.4)	5.3 (4.5–7.1)	6.5 (5.1–23.0)	0.17

[a] Reference interval 0.9–2.0 g/L; [b] ZZ vs. MZ & SZ vs. control; Kruskal-Wallis one-way ANOVA; [c] ZZ vs. MZ & SZ; Mann-Whitney U test.

Overall, total CBG and haCBG levels fell in a right-skewed distribution. CBG affinity form concentrations are compared in Figure 8.1. Total CBG concentrations did not vary between the patient groups. Patients with PiZZ AATD had higher haCBG (P = 0.0004), lower laCBG (P = 0.01) and a higher proportion of haCBG (P < 0.0001) than controls, with Pi MZ/SZ patients having intermediate values for all parameters (Figure 8.1). These analyses remained statistically significant when the two patients receiving infusion therapy were excluded (P = 0.006, 0.01 and < 0.0001 respectively). There was a trend toward higher total, free and percentage of free cortisol in the control group compared to AATD ZZ (P = 0.07, 0.05 and 0.07 respectively), but no difference between the three groups overall (Table 8.3). There was a significant negative correlation between AAT levels and percentage of haCBG:total CBG (Figure 8.2) but no correlation between AAT levels and free or total cortisol concentrations.

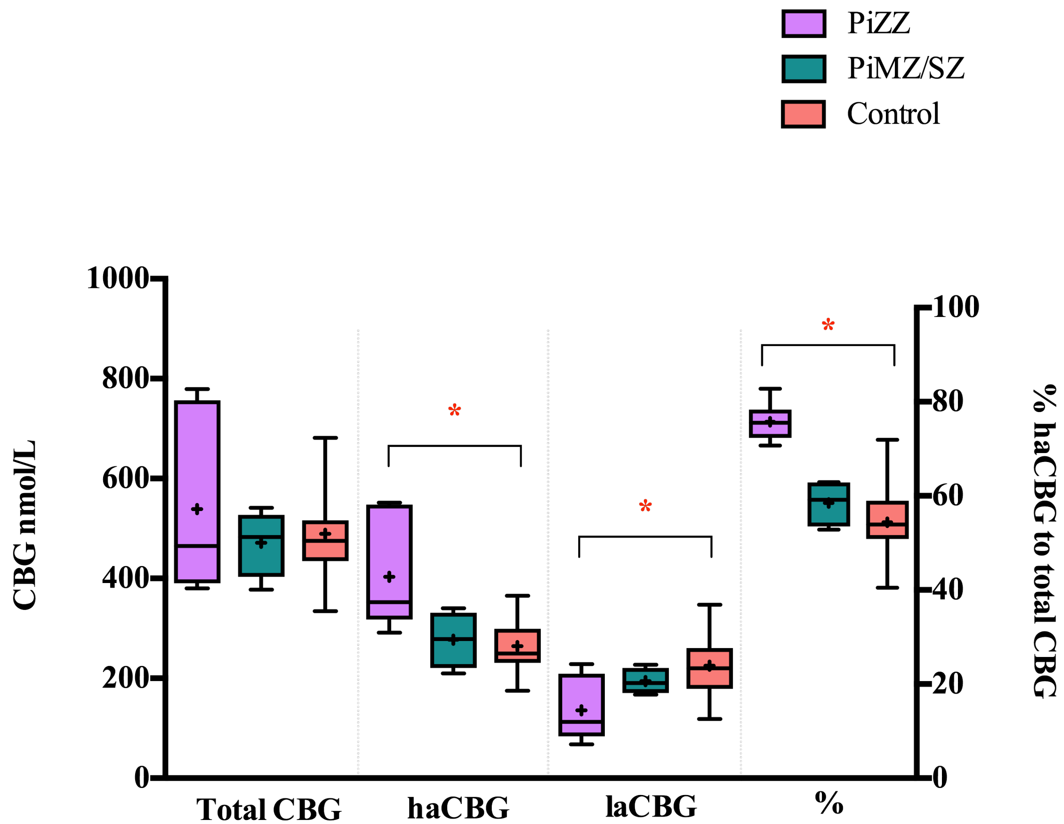


Figure 8.1 CBG affinity forms between AATD groups and controls. haCBG was highest and laCBG was lowest in the severe PiZZ groups (*P* = 0.005 and *P* = 0.036 respectively). The proportion of haCBG to total CBG was highest in the PiZZ group (*P* < 0.001). * *P* < 0.05. Box represents 25th to 75th percentiles with line at median and ✚ at mean; whiskers extend to minimum and maximum.

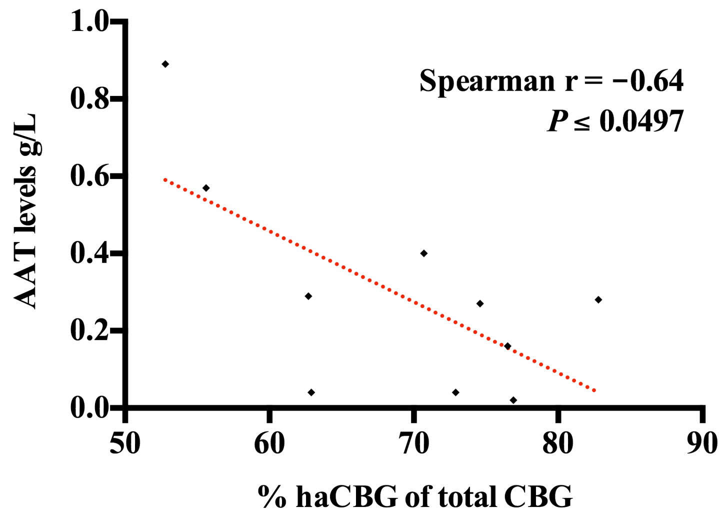


Figure 8.2 Relationship between AATD levels and proportion of haCBG. There was a significant negative correlation between the proportion of haCBG:total CBG and AAT levels in patients with AATD. ——— Line of best fit

8.5 Discussion

We have shown for the first time that despite low circulating levels of AAT, CBG cleavage is paradoxically reduced in patients with AATD compared with healthy controls. This is contrary to our hypothesis as AATD patients should have increased uninhibited NE activity (Weitz *et al.*, 1992; Campbell *et al.*, 1999; American Thoracic Society and European Respiratory Society, 2003; Carter *et al.*, 2011), thereby resulting in increased CBG cleavage. A "dose-response" relation was seen in that those with more severe genotypes/lower AAT levels had higher haCBG than milder genotype states. The reduction in CBG cleavage is seen more clearly in patients with the severe PiZZ genotypes than in those with the less severe PiSZ or MZ genotypes, who have higher levels of AAT.

Similar to the increased proteolytic damage seen in AATD-related emphysema, we hypothesised that the proteinase-antiproteinase imbalance caused by the lack of AATs inhibitory influence over NE in AATD may result in increased cleavage of CBG. In support of this hypothesis, there is direct evidence for increased catalytic activity of NE in AATD (Weitz *et al.*, 1992) as well as diffusion-based mathematical and *in vitro* modelling that predicts that the duration in which the molar concentration of NE exceeds AAT in the microenvironment of activated neutrophils, and is therefore enzymatically active, increases from 20 seconds in normal patients to 80 seconds in those with PiZZ AATD (Liou and Campbell, 1996; Campbell *et al.*, 1999), theoretically providing NE more opportunity to cleave CBG. Additionally, replacement of purified AAT in patients with the PiZZ phenotype restores the anti-elastase activity in alveolar tissue as determined by bronchoalveolar lavage (Gadek *et al.*, 1981). Furthermore, we have shown that in septic shock, a condition associated with high levels of NE (Endo *et al.*, 1995), increasing illness severity is strongly correlated with depletion of haCBG levels through CBG cleavage (Nenke *et al.*, 2015). If excessive CBG cleavage also occurred in AATD, this could have had significant implications for the targeted delivery of cortisol, via haCBG, to inflammatory sites (Nenke *et al.*, 2015), potentially contributing to the chronic destruction characteristic of AATD and moreover, resulting in increased free cortisol which may inhibit stimulation of cortisol via the HPA axis. However, we did not find depletion of haCBG in AATD.

There could be a number of explanations for our findings. Firstly, physiologic CBG cleavage, both systemically and locally, may be regulated by a range of alternative proteinases to allow other important tissues access to less tightly bound cortisol, while NE-mediated cleavage may be limited to providing delivery of cortisol exclusively to inflamed areas. We have reported that up to 30–40 % of circulating CBG in apparently healthy volunteers may be laCBG

(Lewis and Elder, 2013; Nenke *et al.*, 2016c) and thus CBG cleavage may be occurring outside areas of inflammation. Cortisol is required by a large range of cells, and has metabolic, cardiovascular and neurocognitive effects in addition to its anti-inflammatory and immunoregulatory functions (Chrousos, 1995). Chymotrypsin has been found to cleave CBG at one of two Leucine residues located within its RCL (Leucine^{346} or Leucine^{348}) (Lewis and Elder, 2014), and the metalloprotease LasB, a virulence factor secreted by *P. aeruginosa*, preferentially cleaves CBG *in vitro* between residues Asparagine^{347} and Leucine^{348} (Figure 5.1) (Simard *et al.*, 2014), both with resultant loss of steroid-binding affinity. Furthermore, when the crystal structure of CBG was recently solved, it was in the absence of a protease but was cleaved at a site separate from the NE cleavage point, at either Threonine^{349} or Serine^{350} (Gardill *et al.*, 2012). However, Hammond *et al.*, incubated human CBG with plasmin, thrombin and cathepsin G *in vitro*, and found no evidence of cleavage (Hammond *et al.*, 1990). The possibility remains that other yet unrecognised endogenous or exogenous proteinases could be implicated in systemic or localised tissue cleavage (Lin *et al.*, 2010). This mechanism for cleavage would not be increased in the absence of AAT, as in our study. It should be noted that the monoclonal antibody used in our ELISA directed against the RCL of CBG targets the epitope STGVTLNL corresponding to amino acids 341–348 at positions P9 to P2 of the RCL (Chapter 1) (Lewis and Elder, 2011), thus our assay would detect laCBG if cleaved by chymotrypsin, NE, or LasB, but not if cleaved by the unknown factor suggested by Gardill *et al.* (Gardill *et al.*, 2012) as that cleavage site is beyond our epitope and does not result in transition to the relaxed conformer of CBG.

AAT is made in excess by the liver and therefore rapidly neutralises the low levels of circulating NE and other proteinases (Henriksen, 2014). It is possible that the decreased levels of AAT are still sufficient to inhibit proteinases in the general circulation of relatively stable

patients (Weitz *et al.*, 1992; Campbell *et al.*, 1999) and therefore protect CBG from systemic cleavage. AAT is an acute phase reactant and systemic levels, which are already 1–2 magnitudes greater than that of CBG, may increase up to 3–4 fold in response to inflammation or tissue injury (Carrell *et al.*, 1982; Janciauskiene *et al.*, 2011), although this rise is minimal in patients with AATD (Hill *et al.*, 1999). The patients in our study did not report acute exacerbations of their COPD at the time of sampling, although three had experienced an exacerbation in the preceding month. In acute exacerbations of COPD in AATD, concentrations of the pre-inhibition NE activity marker Aα-Val 360 increase then rapidly return to baseline, in support of increased catalytic activity during exacerbations (Carter *et al.*, 2011). Had we examined patients during infective respiratory exacerbations, greater CBG cleavage might have been evident.

In addition, ancillary proteinase inhibitors may prevent excess cleavage of CBG by NE in the inflamed lungs of patients with AATD. The smaller, supplementary ‘alarm’ antiproteinases elafin and secretory leukocyte protease inhibitor (SLPI) are secreted mainly by mucosal epithelial cells, particularly type II pneumocytes, and by macrophages and neutrophils in the presence of inflammatory cytokines, and usually work in concert with AAT to protect epithelial tissues from excessive proteolysis (Knight *et al.*, 1997; Williams *et al.*, 2006; Henriksen, 2014). We speculate that up-regulation of these proteinase inhibitors in inflammation (Betsuyaku *et al.*, 2002; Williams *et al.*, 2006), despite the absence of AAT, may be sufficient to limit proteolytic cleavage of CBG, although the literature is inconsistent in this regard (Hollander *et al.*, 2007; Demkow and van Overveld, 2010).

Finally, important post-translational modifications to CBG, for example glycosylation or inactivation by oxidation, might alter the way the RCL of CBG is recognised or cleaved, or

the rate of laCBG or haCBG clearance in AATD as discussed in previous chapters (Hossner and Billiar, 1981; Gardill *et al.*, 2012; Simard *et al.*, 2014).

The outcomes of this study have potential relevance to the role of cortisol an its tissue delivery/immunomodulatory function in AATD patients. Our study showed reduced levels of laCBG, consistent with impaired haCBG cleavage. Concomitant low free cortisol levels are consistent with this interpretation. Given that studies of CBG-deficient patients suggest that it is free, rather than total, cortisol that is regulated by the HPA axis (Lewis *et al.*, 2005) an associated defect in central drive to the HPA axis is implied. In addition, depletion of laCBG may limit the ability to buffer free cortisol at inflammatory sites in patients with AATD and deplete the laCBG-cortisol reservoir. Normally, laCBG would be around 4% saturated with cortisol in the circulation, but this would rise to 16% in enclosed inflammatory spaces following CBG cleavage (Chan *et al.*, 2013). Low levels of laCBG may also result in decreased cortisol half-life, as unbound cortisol is known to be cleared more quickly (Bright, 1995).

We have shown in Chapters 6 and 7 that impaired CBG cleavage is related to markers of disease severity in metabolic syndrome and in rheumatoid arthritis. Thus impaired cleavage of CBG may be a potential mechanism by which chronic inflammation is perpetuated. This hypothesis is supported by the findings in the current chapter, where CBG cleavage is paradoxically limited despite ongoing inflammatory damage. Impaired cleavage in chronic inflammatory disorders may provide a mechanism to preserve haCBG-cortisol for acute, life-threatening insults, but the cost incurred is an increased risk of chronic inflammation.

To our knowledge, assessment of the HPA axis, with its potent anti-inflammatory effects, has not previously been performed in AATD. In patients without AATD, levels of AAT are negatively correlated with total and calculated free cortisol levels, with no correlation to haCBG or laCBG (Bolton *et al.*, 2014). ICS suppress bronchial inflammation in asthma, but have little anti-inflammatory effect in COPD (Keatings *et al.*, 1997). In addition, it is unclear whether ICS have an effect on the proteinase-antiproteinase imbalance *in vitro* or *in vivo*. In the most relevant study, two-weeks of inhaled fluticasone propionate (500 μg BD) did not alter AAT or SLPI levels nor NE inhibitory capacity in healthy lungs *in vivo*, despite significantly depressed serum cortisol levels (Kamal *et al.*, 2002). In contrast, high doses of oral prednisolone (40 mg/day) do appear to increase SLPI levels and increase NE inhibitory capacity (Stockley *et al.*, 1986), and this might be predicted to protect CBG from excessive cleavage. However, as none of our patients were taking long-term supraphysiological anti-inflammatory doses of exogenous corticosteroids and their measured cortisol levels were normal, we do not anticipate that ICS use had an impact on the proteinase-antiproteinase balance in these patients and is therefore not sufficient to explain the changes in haCBG and laCBG seen in our study. Exclusion of patients taking ICS was not practical given that they comprise standard therapy in this population. Nonetheless, studies examining the effect of systemic or inhaled corticosteroids on haCBG and laCBG levels are needed.

The limitations of the study include the unavoidable use of ICS by our patients, and the modest number of AATD participants, although we were still able to see significant changes in haCBG and laCBG levels despite the small cohort. None of our patients had the exceedingly rare null variants (conferring undetectable AAT levels), and thus we could not assess cleavage in the complete absence of AAT. Future studies might wish to examine bronchoalveolar lavage fluid for haCBG and laCBG forms to reveal whether localised

cleavage is occurring, given that CBG has been found in such secretions, although it is unclear whether this is due to local synthesis (Hammond *et al.*, 1987) or passive diffusion (Loric *et al.*, 1989; Stockley, 2014).

8.6 Conclusion

In conclusion, proteolytic cleavage of CBG is paradoxically decreased in patients with a deficiency of the proteinase inhibitor AAT, despite known excessive NE activity. These results suggest a hitherto unappreciated effect of AATD on CBG cleavage, with implications for cortisol transport and delivery from high affinity and reduced low affinity CBG. Using AATD as a model, we have shown that NE may not be the primary mechanism for systemic CBG cleavage under low-inflammatory conditions. Impaired cleavage may be a potential mechanism in chronic inflammation pathogenesis.

Chapter 9

Differential effect of oestrogen on CBG affinity forms; pregnancy and the oral contraceptive pill

9.1 Introduction

Pregnancy is associated with profound immune and endocrine changes necessary for allograft tolerance and foetal maturation, including activation of the HPA axis which results in sustained physiological hypercortisolism (Figure 9.1). Total serum cortisol, either bound to CBG or albumin, as well as free cortisol levels, are elevated 2–3 fold as the result of placentally-derived CRH (pCRH) which is under positive feedback from maternal cortisol, and hyper-responsiveness of the adrenal cortex to ACTH stimulation (Rosenthal *et al.*, 1969; Demey-Ponsart *et al.*, 1982; Potter *et al.*, 1987; Dorr *et al.*, 1989; Scott *et al.*, 1990; Ho *et al.*, 2007; Jung *et al.*, 2011). Total serum CBG levels in pregnancy are also elevated 2–3 fold (Rosenthal *et al.*, 1969; Moore *et al.*, 1978a; Demey-Ponsart *et al.*, 1982; Potter *et al.*, 1987; Scott *et al.*, 1990; Ho *et al.*, 2007; Jung *et al.*, 2011), as they are in women taking the COCP, which results in supraphysiologic oestrogen levels (Musa *et al.*, 1967; Moore *et al.*, 1978b; Ho *et al.*, 2007; Jung *et al.*, 2011).

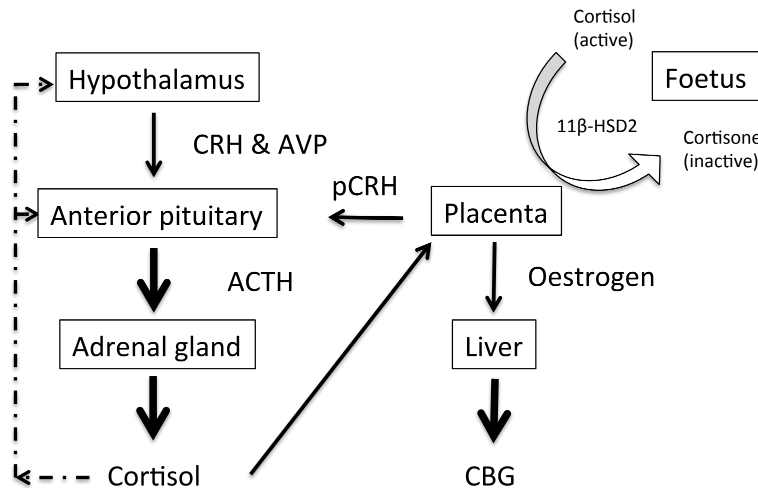


Figure 9.1 The HPA axis in pregnancy. Circulating cortisol and CBG levels increase 2–3 fold. The foetus is protected from hypercortisolaemia, to a degree dependent on gestational age, by increased 11β-HSD2 activity in the placenta Solid line represents stimulation, dashed line represents inhibition.

The typical glycosylation profile of CBG (Chapter 5, Figure 5.2) is altered significantly during pregnancy with enhanced oligosaccharide branching (Akhrem *et al.*, 1982; Strel'Chyonok *et al.*, 1982). A pregnancy-specific form of CBG (preg-CBG) consisting of only tri-antennary oligosaccharides with increased sialic acid content appears, accounting for 10% of maternal CBG (Strel'chyonok *et al.*, 1984; Avvakumov and Strel'chyonok, 1987; Avvakumov and Hammond, 1994; Mitchell *et al.*, 2004). Preg-CBG may be important in maternal-foetal cortisol transfer (Ho *et al.*, 2007).

The effect of pregnancy on CBG affinity forms is unknown. Hence, we studied CBG affinity form levels in a group of pregnant and post-partum women and compared these to women taking oral oestrogen-containing contraceptives and healthy controls. Increased haCBG levels may serve a teleological purpose, including preparation for potential puerperal sepsis, a

leading cause of maternal and foetal morbidity and mortality (Chebbo *et al.*, 2016), or allow for CBG binding competition from the high progesterone concentrations during pregnancy.

9.2 Hypothesis and aims

Our aim was to describe the changes in CBG affinity forms under the hyper-oestrogenaemic states of pregnancy and COCP use. We hypothesised that elevated haCBG levels may be present in pregnancy, providing an expanded reservoir of conditionally available cortisol.

9.3 Research design and methods

9.3.1 Study Participants

We performed a prospective observational study in pregnant women, women taking the COCP and healthy controls at two tertiary centres in Adelaide, Australia, between 2013 and 2015. The protocol was approved by the Women's & Children's Health Network and the RAH HREC. Pregnant women were recruited from outpatient clinics or inpatient wards; women taking the COCP and healthy controls were recruited by local advertising. All provided written informed consent. Inclusion criteria: pregnant women. Exclusion criteria: active labour, concurrent infection or other acute illness and use of immunosuppressive drugs including betamethasone to improve foetal lung maturity. A single blood sample was collected at the time of enrolment. For women on the COCP, samples were taken during the active phase of their pill cycle. Where possible, a post-partum sample was collected from the same patient, although some post-partum patients did not have a pregnancy sample taken. Medical and demographic data were also recorded.

9.3.2 Laboratory methods

Blood samples were analysed for serum total and free cortisol as well as total CBG and haCBG. Hormone assays were described in Chapter 2. The intra-assay coefficients of variation were 5.0 % and 2.7 % for total and haCBG respectively.

Owing to manufacturer updates, a conversion factor derived from internal validation analysis was applied to COCP cortisol results using a Passing and Bablock fit equation ($0.9 + 0.78x$). The purpose of total and free cortisol measurement was to assess a change in the free fraction in relation to changes in haCBG concentration, not to provide an assessment of overall cortisolaemia.

9.3.3 Statistical analyses

Data were analysed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc., San Diego, California). Results are presented as mean ± SEM unless otherwise stated. Comparison between three or more groups was performed by one-way ANOVA with *post hoc* analysis carried out by Tukey's multiple-comparison test with multiplicity adjusted P values reported. Correlations between continuous variables were assessed with Pearson's correlation coefficient. $P < 0.05$ was taken as statistically significant. In women taking the COCP, afternoon total cortisol values above 650 nmol/L ($n = 9$ out of 27) were considered aberrations due to venipuncture stress and excluded from the main analysis; however, a separate column including these is shown in Table 9.2.

9.4 Results

Thirty pregnant women, 7 post-partum women, 27 women taking the COCP and 23 healthy controls were enrolled; their baseline characteristics are detailed in Table 9.1. Women taking

the COCP were younger than the women in other groups ($P < 0.05$). Four pregnant women were in the second trimester while 26 were in the third trimester. Post-partum samples were collected from seven women, at a mean 10.7 weeks (range 5.4–20.6) following delivery. Twenty-one women (78%) taking the COCP were receiving 25–35 µg of ethinyloestradiol. Of the remaining six women, four received 20 µg and two received 50 µg ethinyloestradiol per day.

Table 9.1 Baseline characteristics of pregnant and post-partum women, women on the COCP and healthy controls. Mean (± SD).

	Pregnancy (*n* = 30)	Post-partum (*n* = 7)	COCP (*n* = 27)	Healthy controls (*n* = 23)
Age (years)	31.9 (4.9)	33.2 (4.3)	24.0 (6.0)*	36.0 (8.8)
Gravity	2.4 (1.5)			
Parity	1.2 (1.2)			
Mode of Delivery [a]				
• **NVD**	12			
• **LSCS**	9			
Gestational age at enrolment (weeks)	34.2 (4.5)			
Weight at birth (g) [b]	3329 (488.4)			

Data missing for [a]9 and [b]11 patients. NVD – normal vaginal delivery; LSCS – lower segment caesarean section. * P <0.05 when compared with controls.

9.4.1 CBG levels in pregnant and post-partum women

During pregnancy, total CBG and haCBG levels (nmol/L) were significantly greater than those of healthy controls (877 ± 27 *vs.* 466 ± 13 nmol/L, $P < 0.0001$ and 727 ± 27 *vs*. 262 ± 13 nmol/L, $P < 0.0001$ respectively; Figure 9.2). laCBG levels (151 ± 22 nmol/L) were no different from healthy controls (203 ± 14 nmol/L, $P = 0.35$) while %haCBG:total CBG was significantly higher in pregnancy (83.3 ± 2.3 *vs*. 56.7 ± 2.7%, $P < 0.0001$). Hence, the elevation of CBG in pregnancy is due to higher levels of haCBG. There was no difference in

total CBG, haCBG, laCBG or %haCBG:total CBG levels between post-partum women and healthy controls.

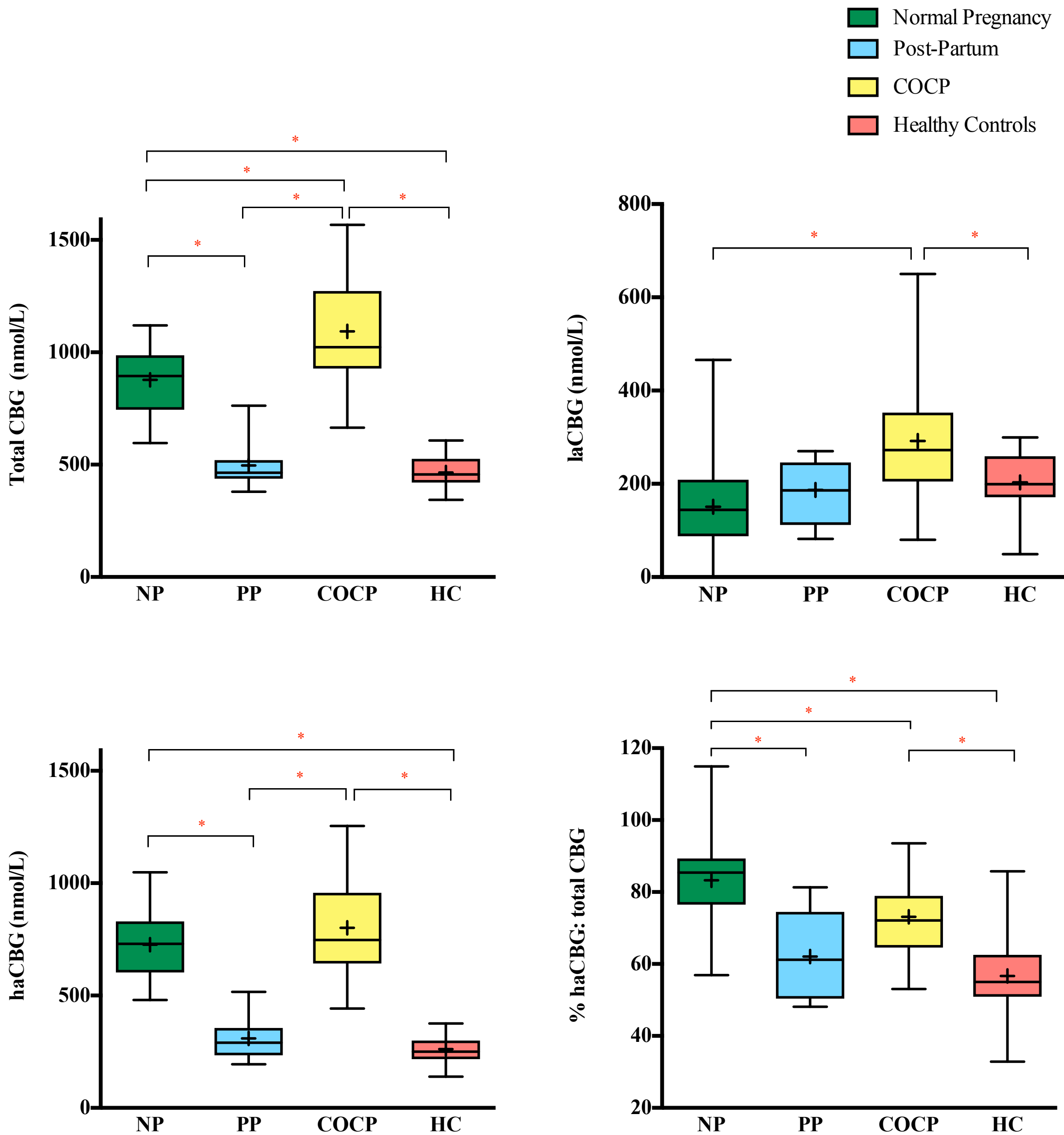


Figure 9.2 CBG affinity forms in oestrogenic states. Total CBG, haCBG and % haCBG:total CBG were higher in pregnancy than post partum and healthy controls. Women on the COCP had the highest total CBG, haCBG and laCBG. * $P < 0.05$ between two groups; Line at median; + at mean; box extends from 25th to 75th percentiles; whiskers from maximum to minimum.

9.4.2 CBG levels and the COCP

Women taking the COCP had the highest total CBG levels (1093 ± 44 nmol/L, $P < 0.0001$ *vs*. pregnant, post-partum and control groups; Figure 9.2). haCBG levels in women on the COCP were higher than healthy controls (802 ± 41 nmol/L, $P < 0.0001$), but were no different to pregnant women ($P = 0.27$). The COCP group also had higher levels of laCBG than both pregnant women and healthy controls (292 ± 27 *vs*. 151 ± 22; $P < 0.0001$ and 203 ± 14 nmol/L; $P = 0.04$ respectively). % haCBG:total CBG was higher than healthy controls ($P < 0.0001$), but lower than pregnant women ($P = 0.001$). Thus in contrast to pregnancy, COCP use was associated with elevation of both ha and laCBG.

9.4.3 Cortisol levels in pregnant and post-partum women

Total and free cortisol levels were increased in pregnancy compared with healthy controls ($P < 0.0001$ and $P = 0.035$ respectively; Table 9.2). The fraction of free cortisol was also increased in pregnant women ($P = 0.0024$). Total cortisol levels were higher in pregnant than post-partum women ($P = 0.02$) but free cortisol and fraction of free cortisol were no different ($P = 0.14$ and 0.93).

Table 9.2 Serum cortisol levels in pregnant and post-partum women, women on the COCP and healthy controls. Mean (± SEM).

	Pregnancy	**Post-partum**	**COCP**	**COCP [a]**	**Healthy controls**
Total cortisol (nmol/L)	656 (58)*	334 (64)	494 (39)	621 (51)*	319 (34)
Free cortisol (nmol/L)	33.8 (3.0)*	17.8 (5.1)	39.8 (3.7)*	49.7 (4.1)*	20.0 (2.9)
Ratio of free:total cortisol	5.2 (0.1)*	4.9 (0.4)*	8.0 (0.2)*	8.0 (0.2)*	6.0 (0.0)

* $P < 0.05$ when compared to controls; [a] Includes outliers (see Methods section 9.3.3).

There was no correlation between CBG affinity form levels and maternal age or gestational age. There was a trend towards higher total and free cortisol with later gestational age (P = 0.052, r = 0.36 and P = 0.071, r = 0.33 respectively).

9.4.4 Cortisol levels and the COCP

Compared to controls, women on the COCP had higher free cortisol and fraction of free cortisol ($P = 0.003$ and $P < 0.0001$; Table 9.2). Total and free cortisol levels were no different compared with pregnant women, but the fraction of free cortisol was higher in women on the COCP ($P < 0.0001$).

9.5 Discussion

The oestrogenic states of pregnancy and COCP use are associated with an equivalent increase in haCBG concentrations; however, COCP use is also associated with a rise in laCBG. These results are consistent with an oestrogen-mediated increase in CBG synthesis in both pregnancy and with the COCP, but with reduced CBG cleavage in pregnancy relative to the COCP, most likely due to pregnancy-induced CBG glycosylation. Increased serum haCBG levels in pregnancy may provide an increased reservoir of CBG-bound cortisol for immunomodulatory purposes in puerperal infection, or allow for cortisol binding in the face of competition from high circulating progesterone concentrations.

The elevated haCBG of pregnancy would increase the reservoir of readily available CBG-bound cortisol by three-fold. Speculatively, this may be advantageous in the case of sepsis, particularly puerperal infection since we have shown that high haCBG levels are associated with better prognosis in the cascade of sepsis-septic shock-death in humans (Nenke *et al.*, 2015). CBG also binds progesterone, which increases 200–300 fold during pregnancy and can

displace cortisol increasing absolute and relative CBG-bound progesterone (Doe *et al.*, 1969; Rosenthal *et al.*, 1969; Abou-Samra *et al.*, 1984; Dorr *et al.*, 1989). Thus higher levels of haCBG may be necessary to overcome this binding competition. However, given that the binding affinity of progesterone for CBG is 35% lower than cortisol for CBG ($K_a = 0.26 \times 10^{-8}$ *vs.* $K_a = 0.4 \times 10^{-8}$) (Stroupe *et al.*, 1978), and that the concentration of cortisol is still 2.7 times greater than progesterone in the maternal circulation (Benassayag *et al.*, 2001), binding competition from progesterone alone does not fully explain the need for such a substantial rise in haCBG. The cortisol-progesterone CBG interaction is particularly important at the materno-foetal interface. Here the progesterone:cortisol ratio is 75 times higher than in the maternal circulation, thus intervillous CBG is heavily progesterone-laden (Benassayag *et al.*, 2001). The lower binding affinity of CBG for cortisol in the intervillous space (Benassayag *et al.*, 2001) suggests that there may be an increase in laCBG at that site, however, neither haCBG/laCBG concentrations in the intervillous space nor their binding affinities for progesterone have been reported. Elevated haCBG may also provide a buffer against the increased cortisol levels in pregnancy, protecting the tissues from excessive free cortisol exposure.

Complex immunological changes occur throughout gestation, facilitating seemingly disparate processes including trophoblastic invasion, foetal recognition, semi-allograft tolerance and parturition while still maintaining the mothers protective defences (Szekeres-Bartho, 2002; Yeh *et al.*, 2013). We know that total CBG levels are already increased as early as 11–16 weeks gestation (Demey-Ponsart *et al.*, 1982; Scott *et al.*, 1990) but as we had mainly late gestation pregnancies in our current study, it is unclear what the pattern of haCBG changes are culminating in the elevated levels seen here. Cortisol inhibits the production of pro-inflammatory, Th1-type cytokines while stimulating anti-inflammatory Th2 cytokines. This

may contribute to the shift in immunological phenotype seen during pregnancy, and potentially the expression of associated autoimmune diseases (Elenkov *et al.*, 2001). Speculatively, haCBG levels may be important in cortisol-mediated immune moderation of pregnancy. haCBG changes may also be different at the immunologically privileged materno-foetal interface compared with the maternal periphery. Further research involving serial sampling starting in early pregnancy is required to understand the changes in haCBG over the course of gestation.

Elevated circulating levels of haCBG in pregnancy may be due to increased production and/or decreased clearance. CBG is mainly produced by hepatocytes (Hammond *et al.*, 1987), but local expression has also been shown in human reproductive tissues including endometrium, ovary, fallopian tube, and placenta (Misao *et al.*, 1994; Misao *et al.*, 1999a; Misao *et al.*, 1999b; Miska *et al.*, 2004) and may contribute to increased production in pregnancy (Misao *et al.*, 1999a; Mihrshahi *et al.*, 2006). Foetal CBG is unlikely to contribute to the rise we observed given that the concentration of CBG in the maternal circulation is ten times greater than in the foetal circulation, and that immunologically CBG in the intervillous space matches maternal CBG rather than foetal CBG (Benassayag *et al.*, 2001). Exogenous oestrogens cause a similar rise in total CBG levels *in vivo* (Ho *et al.*, 2007; Jung *et al.*, 2011) while *in vitro* stimulation of Hep G2 cells with oestradiol causes an increase in acidic glycoforms of CBG, approximating the changes seen in pregnancy (Mihrshahi *et al.*, 2006). This study shows that the rise in total CBG following exogenous oestrogen administration is due to a similar increase in haCBG, albeit with an additional rise in laCBG. Furthermore, sex differences in CBG levels with females having approximately 10% higher total CBG levels than men (Fernandez-Real *et al.*, 2002; Lewis *et al.*, 2004; Lewis *et al.*, 2010; Nenke *et al.*, 2016c) have traditionally been attributed to higher oestrogen levels in women. These data suggest that

oestrogen has a stimulating effect on CBG production, although the mechanism for this is unknown.

CBG, an α_1-glycoprotein, displays significant carbohydrate microheterogeneity at its six consensus sites (Figure 5.2) (Hammond *et al.*, 1987). We propose the differential findings in CBG affinity forms between pregnant and COCP-taking women are due to altered glycosylation profiles. During pregnancy, additional maternal glycoforms with enhanced glycosylation (shifting from bi-antennary to tri-antennary chains), increased sialic acid content and higher molecular weight are well recognised (Mitchell *et al.*, 2004) with the classically described preg-CBG variant containing exclusively tri-antennary oligosaccharide chains and constituting around 7–14% of circulating CBG in pregnancy (Reviewed in Ref. Strel'chyonok and Avvakumov, 1990). Altered CBG glycosylation is thought to affect the interaction of CBG with specific-membrane receptors (Avvakumov and Strel'chyonok, 1988; Strel'chyonok and Avvakumov, 1991; Sumer-Bayraktar *et al.*, 2011). Specifically, preg-CBG has a significantly higher binding affinity for syncytiotrophoblast cell membranes than non-pregnant CBG, perhaps facilitating the delivery of maternal cortisol to the foetus or participating in the regulation of placental CRH production (Strel'chyonok and Avvakumov, 1990; Mitchell *et al.*, 2004). The heavy glycosylation of CBG in pregnancy may alter its clearance rate (Hossner and Billiar, 1981), contributing to increased levels of circulating haCBG. Examination of glycoforms present in women exposed to exogenous oestrogen using modern techniques (Sumer-Bayraktar *et al.*, 2011) to determine whether a similar pattern of increased branching also occurs is yet to be undertaken.

Evidence of increased CBG cleavage, such as elevated laCBG levels, may have been expected in pregnancy, given that elevated CRP levels and total white cell and neutrophil counts suggest a mild pro-inflammatory state (Belo *et al.*, 2005; Larsson *et al.*, 2008). Data,

however, are inconsistent as to whether absolute levels of NE are increased in pregnancy (Greer *et al.*, 1989; Rebelo *et al.*, 1995; Belo *et al.*, 2005), although the elastase-to-neutrophil ratio is reduced in all trimesters (Belo *et al.*, 2005). Normal laCBG levels and contrastingly elevated total and haCBG levels, as reported here, suggest that CBG cleavage is not increased in pregnancy. This implies that cleavage under physiological conditions is not substrate-driven, but is regulated, by unknown mechanisms. Women taking the COCP, however, had similar haCBG levels but also had elevated laCBG, which points towards an increase in cleavage in women exposed to pharmacologic doses of exogenous oestrogen. An alternative view is that glycosylation on Asparagine^{347} in the RCL of CBG could compromise antibody recognition and thus be interpreted as CBG cleavage. If this were the case, compromised antibody recognition would also occur in pregnancy, where enhanced glycosylation is a renowned feature. The elevated absolute and relative haCBG levels in pregnancy, however, refute glycosylative interference as an explanation for the elevated laCBG seen with COCP use.

The maternal HPA axis has a significant impact on the HPA axis of the offspring both *in utero* and in later life (Sandman *et al.*, 2011; Henley *et al.*, 2016a), although this is generally attributed to abnormal cortisol levels. As mentioned above, the implication for the foetus of elevated haCBG in pregnancy is adequate cortisol delivery, while abnormal haCBG/laCBG levels may alter this transfer. A common mutation, CBG A51V, resulting in a 30% reduction in circulating total CBG levels during pregnancy is associated with a female-skewed sex ratio in offspring (Lei *et al.*, 2015). Levels of haCBG and laCBG are not known in those with the CBG A51V mutation. However, assuming haCBG and laCBG are present in the same proportions as seen in our study, these women would have a substantially lower haCBG-cortisol pool. This could contribute to the apparently disproportionate number of stillbirths (2

out of 48) observed in the CBG-deficient women, and may be associated with early miscarriage of male foetuses, contributing to the skewed offspring ratio.

Free cortisol was found to be elevated in both the pregnant and COCP groups. However, this is at odds with previous studies that have found no increase in free cortisol levels in women on the COCP using the gold standard of 24-hour urine free cortisol excretion (Jung *et al.*, 2011). The single cortisol measurement used in our study is not recommended for, nor intended to provide, a measurement of overall cortisolaemia in these patients, but to determine whether a change in haCBG levels related to a change in the free cortisol fraction, which it did not. We believe the elevated free cortisol levels and free cortisol fractions seen in young females on the COCP seen here are an artificial stress effect of venipuncture rather than a reflection of true hypercortisolaemia, hence their segregation during the analysis.

9.6 Conclusion

In conclusion, the rise in total CBG during pregnancy is due to increased haCBG. The COCP was associated with a rise in haCBG comparable to pregnancy; however, laCBG was also increased. These results are consistent with an oestrogen-mediated increase in CBG synthesis in both pregnancy and with the COCP, but with reduced CBG cleavage in pregnancy relative to the COCP, due to pregnancy-induced CBG glycosylation. Increased serum haCBG levels in pregnancy may provide an increased reservoir of CBG-bound cortisol for immunomodulatory purposes in puerperal infection, or allow for cortisol binding in the face of competition from high circulating progesterone concentrations.

Chapter 10

Discussion and future directions

CBG is a highly adapted cortisol transport protein, with a major influence over free cortisol availability. The permanent transition from high-affinity CBG to low-affinity CBG that occurs following enzymatic proteolysis of the proteins exposed reactive centre liberates bound cortisol by virtue of the inherent plasticity of the steroid binding site. Given the diverse and potent effects of glucocorticoids on inflammation (Figure 10.1), the phenomenon of NE-mediated cleavage targeting the delivery of cortisol to inflamed sites has impelled more dedicated investigation into the relevance of these two affinity forms to HPA axis function and dysfunction.

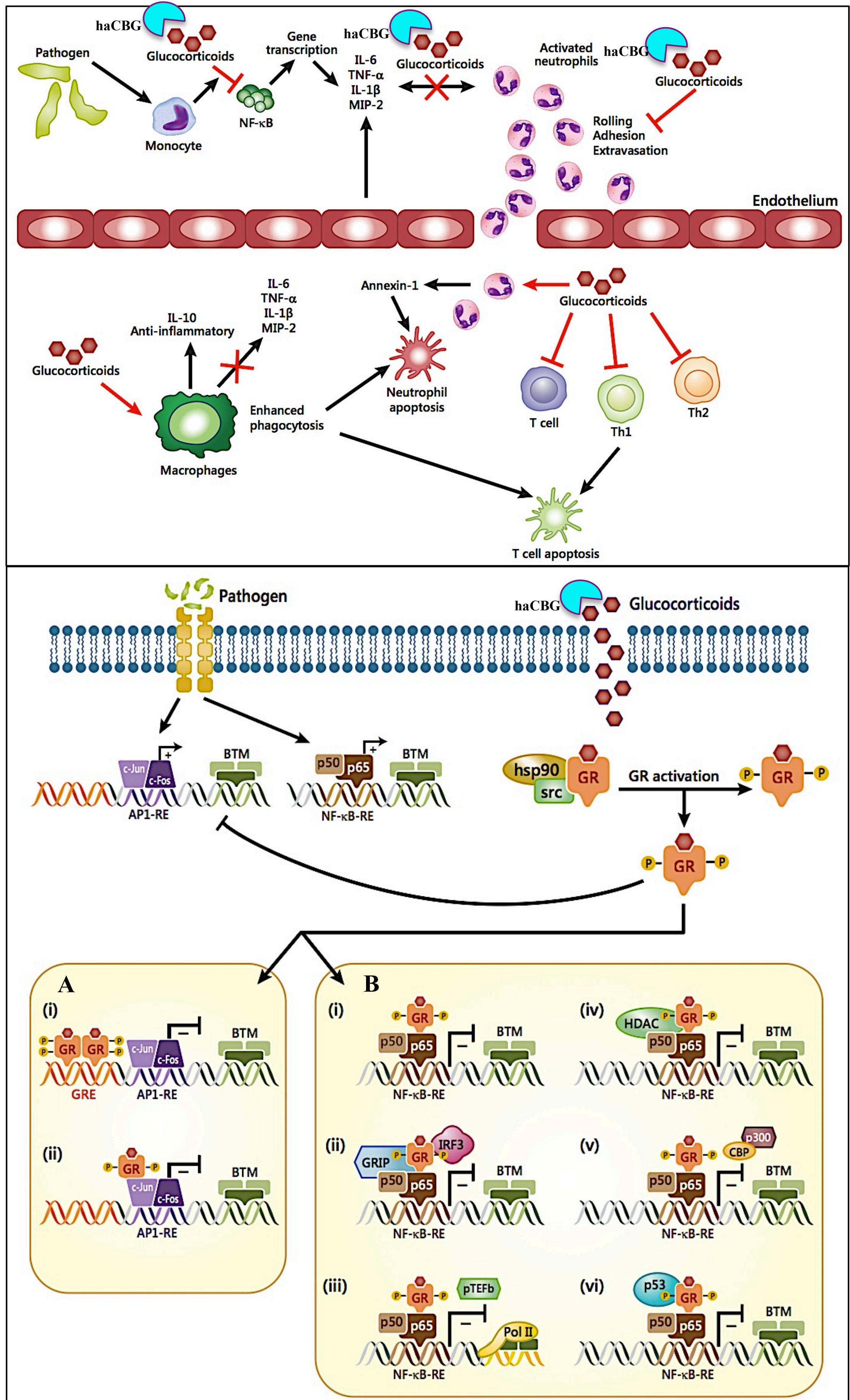

haCBG
Pathogen
Glucocorticoids
Monocyte
NF-κB
Gene transcription
IL-6
TNF-α
IL-1β
MIP-2
Activated neutrophils
Rolling
Adhesion
Extravasation
Endothelium
Annexin-1
IL-10
Anti-inflammatory
Macrophages
Enhanced phagocytosis
Neutrophil apoptosis
T cell
Th1
Th2
T cell apoptosis
hsp90
src
GR
GR activation
AP1-RE
NF-κB-RE
BTM
c-Jun
c-Fos
p50
p65
A
B
(i)
(ii)
(iii)
(iv)
(v)
(vi)
GRE
HDAC
GRIP
IRF3
pTEFb
Pol II
CBP
p300
p53

Figure 10.1 (Previous page) Extracellular and intracellular effects of glucocorticoids on inflammation. Top panel; Glucocorticoids affect nearly every cell type by virtue of almost ubiquitous expression of the glucocorticoid receptor (GR). Exposure to pathogens leads to a fast activation of the immune response. Glucocorticoids are able to promote resolution of inflammation by repressing the expression of adhesion molecules, preventing rolling adhesion and extravasation of neutrophils. Glucocorticoids also induce the expression and secretion of Annexin-1, which is able to induce apoptosis of neutrophils at the site of inflammation. Prolonged glucocorticoid exposure induces tissue resident macrophages to undergo a phenotypic change to become M2-like These macrophages no longer produce pro-inflammatory cytokines but instead produce anti-inflammatory IL-10, have enhanced phagocytic activity to remove apoptotic cells, and promote tissue healing. Glucocorticoids also act on naïve and differentiated T cells that have been recruited to the inflammatory site by blocking T helper 1 (Th1)- and Th2-derived cytokine production and inducing apoptosis. Bottom panel; 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. A) Effect of glucocorticoid-GR complex on AP-1 activity: (i) at some promoters, GR is able to simultaneously bind to a GRE (glucocorticoid-responsive element) and tether to c-Jun to repress the transcriptional activity of AP-1; (ii) 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. B) Effect of glucocorticoid-GR complex on NF-κB activity: (i) GR can physically interact with p65, which represses the activity of NF-κB; (ii) GR can recruit GRIP (GR interacting protein) which blocks the formation of the NF-κB/IRF3 heterodimer; (iii) 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; (iv) GR is able to repress NF-κB activity by recruiting a HDAC (histone deacetylase); (v) GR prevents NF-κB from interacting with p300 and CPB; and (vi) 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.

Release of cortisol from haCBG (cyan) promotes inflammation resolution by facilitating localised access of cortisol to its receptor, thus CBG is the "primary gatekeeper of steroid action". Dysfunction of haCBG activity impairs cortisol delivery in acute and chronic inflammation. In acute systemic inflammation, depletion of haCBG results in local deprivation of cortisol. In chronic inflammation, impaired ability to cleave CBG also deprives local sites of anti-inflammatory cortisol. In both situations, inflammation may be perpetuated. Adapted from Cruz-Topete and Cidlowski (2015).

This thesis examined the importance of haCBG in cortisol delivery in the settings of acute and chronic inflammation. Using measurement of total CBG, haCBG and laCBG in serum I also studied the dynamics of CBG cleavage in these states and in pregnancy.

The major findings of this thesis include:

- ❖ Acute systemic inflammation, including infection and septic shock, is associated with haCBG depletion which limits the targeted delivery of anti-inflammatory cortisol.
- ❖ In chronic inflammation, including metabolic syndrome and rheumatoid arthritis, an inability to appropriately cleave CBG may contribute to the pathogenesis of chronic inflammation by impairing adequate cortisol delivery.
- ❖ Elevation of CBG in pregnancy involves the high-affinity form exclusively, whereas both forms are increased in women taking the COCP.

Other noteworthy findings include:

- ❖ CBG cleavage does not occur within 6 hours of an inflammatory insult.
- ❖ Proteases other than neutrophil elastase are likely to mediate CBG cleavage in the basal state.
- ❖ The important thermocouple effect of CBG on cortisol release, particularly in early inflammation is supported *in vivo*.
- ❖ Two dynamic pools of CBG-cortisol exist to buffer free cortisol levels.

These findings add significantly to the current literature and the implications are substantial. First and foremost, the breakthrough findings in septic shock (Chapter 3) translated the previous basic, scientific data on CBG cleavage into clinically significant, *in vivo* evidence of the CBG-cortisol delivery phenomenon. This transformed our understanding of the role of haCBG. We showed for the first time that severity of inflammatory illness is associated with plummeting haCBG levels, indicating increased CBG cleavage. Furthermore, the decline in haCBG is clearly better correlated with severity of disease than is free or total cortisol, the controversial current standard for decision making regarding exogenous glucocorticoid

administration, and may be related to risk of death. This is plausible biologically, as the generally elevated free cortisol levels are distributed widely amongst tissues, but not targeted specifically to the areas of greatest need, a process afforded by sufficient haCBG. We propose that there is an absolute level of haCBG below which cortisol is unable to adequately execute its acute counter-inflammatory and cardiovascular functions and therefore haCBG levels may be of utility in predicting benefit from exogenous glucocorticoids in septic shock, a contentious area in which such a reliable biomarker is urgently required. Furthermore, preservation of free cortisol levels by both declining CBG and reduced cortisol metabolism (Boonen *et al.*, 2013) may inhibit central control of the HPA axis, producing lower HPA axis drive, thus, both local cortisol delivery and drive to cortisol production may be impaired, contributing to uncontrolled inflammation. The potential for us to administer CBG therapeutically, to assist in cortisol delivery and quell the inflammation that is currently insuppressible, would be revolutionary.

In Chapter 4 we demonstrated that within the first six hours following administration of pro-inflammatory TNF-α, there was no evidence of CBG cleavage or change in production rates. This is understandable given cleavage would require neutrophil activation which takes longer than 6 hours (Michie *et al.*, 1988a) and a change in production rates which would require nuclear genomic change. Instead, any decline in CBG is likely due to dilution or sequestration, consistent with the pathology of acute inflammation with small vessel dilatation and increased vascular permeability (oedema) preceding leukocyte emigration and activation (Kumar *et al.*). There was, however, an early elevation in the free:total cortisol ratio, prior to HPA axis activation, providing preliminary support for the *in vitro* evidence of CBG as a protein thermocouple, whereby pyrexia in inflammation results in decreased cortisol-binding affinity which automatically increases the local availability of cortisol

dramatically. This reaffirms that CBG is a highly adapted cortisol modulator, being able to liberate increasing amounts of free cortisol to protect the body during the hyper-acute stages of stress, while other regulatory processes and defence systems are still being instituted. In Chapter 5 we also found a positive correlation between body temperature and free cortisol levels, again supporting the thermocouple mechanism. Of interest are the similarities between CBG and thyroxine-binding globulin (TBG), another serpin protein, which is also able to release its hormone ligand, thyroxine, via a temperature-sensitive mechanism. Mutations of TBG have been identified in aboriginal Australians which affect the ability of the RCL to initiate perturbation of the thyroxine/triiodothyronine-binding site and thus respond to increasing temperatures (Qi *et al.*, 2014). Speculatively, similar mutations could exist for CBG, impeding the temperature-dependent release of free cortisol, which could have significant clinical consequences, particularly given that we have shown that this is the primary mechanism modulating immediate (<6 h) cortisol release under pro-inflammatory cytokine stress.

The later chapters of this thesis examined chronic inflammation, where we found that impaired CBG cleavage was associated with disease severity. A large study showed that anthropometric measures, including waist circumference, weight, and body fat, correlated positively with haCBG levels and % haCBG:total CBG, and correlated negatively with total CBG and laCBG. These data are consistent with obesity as a chronic inflammatory state, but suggest that impaired CBG cleavage is also associated with obesity. Most importantly the findings were strongest in patients with metabolic syndrome. These results are paralleled in the rheumatoid arthritis cohort, who had lower total CBG and laCBG than healthy controls, indicating impaired CBG cleavage, which related to disease activity within the RA group. Impaired cleavage occurred despite an adequate inflammatory stimulus and relative

hypocortisolaemia. Overall, an impaired ability to cleave haCBG would deprive inflamed tissues of cortisol delivery, perpetuating chronic inflammation. We hypothesise that glycan alteration and/or genetic variation may contribute to impaired cleavage, by hindering access of proteases to the RCL or other mechanisms.

In septic shock, we found a positive correlation between the percentage of circulating neutrophils and the relative and absolute levels of laCBG. We attributed this to increased neutrophil activation leading to increased CBG cleavage. This association was not seen, however, with our studies of bacteraemia and AATD. In patients with severe infection, we showed that CBG cleavage continued to occur even when neutrophil counts were $< 0.5 \times 10^9$/L, while in patients with AATD, we showed that excess NE does not equate to excess CBG cleavage. This implies that the widely acknowledged premise of NE–mediated cleavage is more complex than currently recognised. Evidently, other factors including alternate proteases, glycosylation modifications and protease inhibitors contribute to the haCBG → laCBG + cortisol equation. This is reaffirmed by the finding that Pseudomonal LasB is able to cleave CBG (Simard *et al.*, 2014), dependent upon glycosylation (Sumer-Bayraktar *et al.*, 2016), and may enhance NE-mediated CBG cleavage *in vivo* (Nenke *et al.*, 2017). The clinical importance of chymotrypsin's ability to cleave CBG remains unknown (Lewis and Elder, 2014). These influences need to be more fully explored before CBG could be considered as a drug delivery (Chan *et al.*, 2014) or steroid-sparing agent.

Following the realisation that haCBG and laCBG coexist in the circulation (Lewis and Elder, 2013) and have similar half-lives (Lewis *et al.*, 2015), and knowing that laCBG retains a cortisol-binding capacity at least three times greater than albumin, interest has been drawn to the presence of two circulating pools of cortisol — haCBG-cortisol and laCBG-cortisol.

Under basal conditions, laCBG may be 4% saturated with cortisol; re-equilibration in states of significant localised cleavage would result in 16% saturation, adding significant buffering capabilities (Chan *et al.*, 2013) at enclosed inflammatory sites. The relevance of laCBG-cortisol buffering in systemic inflammation is unknown, but may not be as significant. Presumably, association and dissociation of cortisol from laCBG is not protease dependent, but would be responsive to temperature changes. Results from our studies show that absolute levels of laCBG are increased following COCP use, and the concentration of laCBG relative to haCBG is increased in septic shock compared with controls. In chronic inflammation (RA, MetS, AATD), the concentration of laCBG relative to haCBG is greatly decreased. The buffering potential of laCBG clearly varies across disease states and may impact upon HPA axis feedback. Modelling studies have produced formulae that depict the effect of increasing laCBG on free cortisol levels (Nguyen *et al.*, 2014).

Finally, in pregnancy we have shown that the known rise in CBG is due to increased haCBG with no change in laCBG. This may have important implications for foetal cortisol transfer, immunomodulation towards an immune tolerant phenotype, and for survival in the event of life-threatening puerperal infection, which again comes back to the findings of increased illness severity/mortality in association with depleted haCBG in acute systemic inflammation. We found a similar elevation in haCBG but in contrast there was also an increase in laCBG. We propose pregnancy-induced CBG glycosylation changes are responsible for the differences in these two hyper-oestrogenaemic states.

Future directions

Each of the studies presented in this thesis produced novel results and lay a strong foundation for further development.

The septic shock study provided evidence that haCBG becomes depleted with life-threatening illness, in relation to illness severity. An inverse relationship of haCBG to mortality was suggested by the study. A larger study, powered to determine whether low levels of haCBG are associated with increased mortality is recommended. As a result of the septic shock study, we hypothesised that haCBG depletion may impair the delivery of cortisol to the vasculature, resulting in circulatory collapse. A large, prospective study to evaluate whether haCBG levels can predict the pressor response to exogenous hydrocortisone administration could address this hypothesis. If affirmative, haCBG levels could be used to individualise exogenous glucocorticoid administration. Finally, the administration of synthetic haCBG may ultimately be used to improve outcomes in septic shock, and circumvent the potential harms inherent to therapeutic glucocorticoid use. The benefits of haCBG are likely to extend beyond the critical care setting and could theoretically be applied to any of the numerous inflammatory conditions that display variable steroid-responsiveness with no biomarker or predictor of success.

With regard to chronic inflammation, we showed that CBG cleavage is reduced in RA compared with controls, with increased CBG cleavage rates and enhanced delivery of cortisol being associated with remission. A longitudinal study looking at changes in CBG cleavage in individuals over an extended period of time in relation to disease improvements and flares is needed to confirm these findings. Again, cleavage characteristics but could be useful in directing therapy in these patients, by predicting who will benefit from low-dose exogenous glucocorticoid administration.

The data presented here provide a significant advance on what was previously known about the functions of haCBG and laCBG, although many questions still remain. In particular, the binding affinity of these two forms for endogenous and synthetic glucocorticoids other than cortisol need to be explored, particularly if consideration was to be given to the therapeutic administration of haCBG.

Some of the major findings of this thesis are potentially due to altered glycosylation profiles of CBG leading to variable CBG RCL exposure and susceptibility to cleavage. Glycan analysis is performed with liquid chromatography–mass spectrometry, however a comprehensive interpretation of the functionality of glycans includes time-based digestion assays and molecular dynamics simulation. Glycoform profiles have been performed in only a limited number of clinical scenarios including healthy subjects, pregnant women, and following pseudomonal LasB exposure. Both in pregnant women and following LasB, the findings have been highly valuable, showing significant glycoform variations with clearly important implications. Notably, altered glycosylation of other acute phase proteins is well recognised (McCarthy *et al.*, 2014; Mastrangelo *et al.*, 2015). We advocate examination of glycan profiles in patients with acute and chronic inflammatory conditions to determine whether this post-translational modification is responsible for some of the impaired CBG cleavage seen in chronic inflammation. Comparison of the glycan profiles of pregnant women and those taking the COCP will expand our findings from Chapter 9 and potentially confirm the mechanism for the reduced cleavage seen in pregnancy compared to women on the COCP.

Another contributing factor that needs to be explored is genetic variations. Mutations of *SERPINA6* were previously considered rare, however, polymorphisms are becoming

increasingly recognised with 1:36 Han Chinese harbouring a mutation (Lin *et al.*, 2012). Furthermore, mutations with potential significance have been identified, but have not yet been characterised in humans (Appendix B; Table B2). It would be pertinent for further CBG studies to consider storing samples suitable for DNA analysis. During my research I encountered a number of opportunities where examining for mutations may have proven fruitful. These included samples that had almost 100% of total CBG being intact haCBG; which may represent an inherent resistance to cleavage, and healthy controls with total CBG levels more than 2.5 standard deviations below the mean; which may relate to a mutation that decreases synthesis or secretion of CBG. Previously, the CBG Leuven (L93H; Appendix B) variant was identified from a group of 22 patients with septic shock (Smith *et al.*, 1992). Thus DNA analysis of groups of patients with acute or chronic inflammation, or at least subsets with identifying features, would be worthwhile.

One limitation of the studies presented in this thesis is that there is no measure of NE activity to relate to CBG cleavage. As mentioned in Chapter 8 (Section 8.3.3), NE is rapidly degraded *in vivo* and systemic measurement of free NE is usually not possible. Some studies use the circulating NE-AAT complex as a measure of NE activity, but this does not reflect the destructive potential of NE within localised inflammatory sites, and can be affected by situations that increase or decrease AAT levels. Other studies use direct or indirect markers of elastase degradation, although these tests have not been well validated and their role in clinical trials has been questioned (American Thoracic Society and European Respiratory Society, 2003). For these reasons, we have no measure of NE activity. To strengthen further research in this field, it would be pertinent to somehow assess NE activity in patients where CBG cleavage is increased as opposed to where cleavage is decreased. This will help to confirm whether other proteases are involved in cleavage regulation.

A second drawback to these studies was the lack of data regarding the effect of renal and hepatic disease on CBG levels. Given that CBG is primarily synthesised and cleared by hepatocytes (Hossner and Billiar, 1981), an assessment of the variation in ha/laCBG production occurring in acute hepatitis, and along the spectrum of chronic liver damage would be important. Additionally, CBG may be produced and cleared via receptors in the kidney (Maitra *et al.*, 1993; Scrocchi *et al.*, 1993) and ha/laCBG loss may occur during dialysis (Zager *et al.*, 1988) depending on the size of the filtration membrane, which would impact steroid-binding and CBG-cortisol pools in some patients. Clarification of these issues will strengthen future research.

CBG plays a dynamic role in regulating levels of free cortisol and has aptly been termed the "primary gatekeeper of steroid action" (Hammond, 2016). haCBG appears to play an important, but differential role in acute and chronic inflammation, where the concentration of haCBG and its susceptibility to cleavage impact the delivery of cortisol and the resolution of inflammation. CBG concentration, binding affinity and cleavage susceptibility are subject to modification by external factors including temperature, glycosylation and genetic variability that require consideration in future studies. The potential to exploit the cortisol delivery capabilities of haCBG therapeutically, to control cortisol distribution, maximise bioavailability and limit toxicity in inflammatory states is compelling and offers important opportunities for glucocorticoid biology.

Appendix A: Changes made to haCBG assay procedure

Table A1 Changes to the preparation of materials.

	Original preparation	**Modified preparation**
PBS	11.5 g Disodium hydrogen orthophosphate anhydrous 2.96 g Sodium dihydrogen orthophosphate 5.84 g NaCl Dilute to 1000 mL distilled water Adjust pH to 7.2	Obtain PBS; 20x, 500 mL from Media Production Unit. Add 50 mL to 950 mL distilled H_2O
Assay Buffer	PBS (as above) + 0.1% gelatin (1 g) + 0.1% Tween 20 (1 mL)	PBS (as above) + 0.1% gelatin (1 g) + 0.1% Tween 20 (1 mL)
TMB substrate	8.2 g sodium acetate anhydrous 3.6 g citric acid Dissolve in 600 mL water Add 270 mg TMB dissolved in 400 mL methanol. For extended use add 250 µL 30% H_2O_2 to 500 mL substrate & mix. Store in dark labelled bottle.	No change
Wash solution	8 g NaCl 1 L distilled water 1 mL Tween 20	1 L PBS (as above) + 1 mL Tween 20
0.9 M HCL	5 mL 36M HCL to 195 mL distilled water = 0.9M HCL	No change

Table A2 Changes to reagents.

	Original reagents	**Modified reagents**
Coating antibody	In-house CBG polyclonal antibody (IgG fraction)	No change
Total CBG signal antibody	12G2 monoclonal antibody	No change
haCBG signal antibody	9G12 monoclonal antibody	No change
Total CBG label antibody	Pharmingen rat anti-mouse IgG_1-HRP	No change
haCBG label antibody	Nordic anti-mouse IgG_{2a}-HRP	No change
Standard	SHBG/pooled third trimester pregnancy plasma	Human SerpinA6 / CBG Protein (His Tag)[a]
Plates	Microtest III, Falcon, 3912 rigid flat bottom plate	Costar COR3590: Corning 96 well EIA/RIA clear flat bottom polystyrene high bind microplate

[a] Preparation of recombinant CBG for standard: molecular mass is 55-60 kDa therefore, the expected concentration when reconstituted is between 8333 and 9090 nmol/L, averaged to 8700 nmol/L (conversion factor to mg/L is 17.4). To get the top standard representing 2560 nmol/L, dilute this to 1:3.4 (so that is 10 µL in 24 µL). Then dilute 1:1000 to make the top standard (10 →990 and 100 → 900) if the other sample dilutions are also 1:1000.

Table A3 Changes to the method.

	Original method	**Modified method**
Plate Coating	CBG polyclonal antibody 100 µL in 20 mL PBS (2 plates) 100 µL/well, cover and store overnight at room temperature or at 4 ^{o}C x 3 or more days	No change
Plate set up	Wash plates × 3 using automated washer	Hand wash plates × 4, invert plates on absorbent tissue and allow to completely drain, tapping forcefully if necessary to remove any residual fluid
	Block × 10 mins with 200 µL/well of AB Empty AB, blot on towel	No change
Standards	1:500 dilution of SHBG (10 µL to 490 µL AB) Then 1:10 dilution (100 µL to 900 µL AB) – top standard (2560 nmol/L) in dilution block	Recombinant CBG as in Table A2.
	Serially dilute 1:2 (500 µL in 500 µL AB) – 1280, 640, 320, 160, 80 & 40. 0 = AB	No change
	Mix by pipetting	Mix by vortex
Sample preparation	1: 1000 dilution; - 1:100 – 10 µL serum to 990 µl AB - Then 1: 10 – 100 µL above solution to 900 µL AB Using dilution block, mix by pipetting	No change to dilution. Do not use dilution block, use individual Eppendorf tubes, mix by vortex at each step.
Plating	100 µL/well of standard or sample in duplicate	No change
	Incubate 30 min at room temperature	Incubate 60 min at room temperature
Signal antibody	Wash × 3	Hand wash × 4
	Dilute 12G2 or 9G12 (1:20) – 500 µL in 11 mL AB.	No change
	100 µL/well × 30 min	100 µL/well × 90 mins
Label antibody	Wash × 3	Hand wash × 4
	Add anti-mouse IgG_1-HRP (1:2000) or anti-mouse IgG_{2a}-HRP (1:1000), 100 µL/well	No change
	Incubate × 15 min	Incubate × 30 mins
Colour development	Wash × 3	Hand wash × 4
	100 µL TMB, stop with 100 µL 0.9M HCL Read absorbency @ 450 nm	No change

Appendix B: Identified CBG variants

Table B1 CBG variants detected in humans and associated clinical outcomes.

CBG variant	**Nucleotide change**	**Discovery**	**CBG affect**	**Biochemical findings**	**Clinical features**
CBG Leuven L93H (Van Baelen *et al.*, 1982; Smith *et al.*, 1992; Van Baelen *et al.*, 1993)	T>A	Isolated in 3 unrelated individuals from a population study; subsequently detected in one out of 22 patients from a septic cohort	3 fold reduction in CBG-cortisol binding affinity	• Normal CBG levels	Not described
CBG Lyon D367N (Emptoz-Bonneton *et al.*, 2000; Torpy *et al.*, 2001; Brunner *et al.*, 2003; Buss *et al.*, 2007; Cizza *et al.*, 2011; Hill *et al.*, 2012)	G>A	Isolated from at least 5 pedigrees and an isolated (de novo) case.	4 fold reduction in CBG-cortisol binding affinity	• ↓ TC • Normal FC • ↑ %FC • Normal ACTH and 24 hour UFC • ↓ CBG	• Chronic fatigue • Weakness • Depression • Hypotension • Muscle fatigue
CBG Null/Adelaide Trp11Stop (Torpy *et al.*, 2001; Cizza *et al.*, 2011)	G>A Premature stop codon in pro-CBG molecule	Isolated from a large Italian-Australian kindred and in pedigrees from the Italian village of origin. Some also carried CBG Lyon D37N.	Complete loss of CBG synthesis	• Normal 24 hour UFC • ↓TC • ↑FC • ↑%FC • 50% ↓ in CBG in heterozygotes • Undetectable CBG in homozygotes	• Hypotension • Chronic fatigue • Chronic pain • Obesity
CBG A224S (Smith *et al.*, 1992; Torpy *et al.*, 2001; Torpy *et al.*, 2004; Perogamvros *et al.*, 2010; Lin *et al.*, 2012)	G>T	Found with increased frequency from a candidate gene study in an Australian chronic fatigue cohort; also seen in conjunction with other CBG mutations	No apparent affect on binding affinity or production	• ↑plasma CBG • Trend to ↓TC and FC	• Chronic fatigue
CBG G237V	G>T	Isolated from a single kindred	Complete loss of	• ↓↓TC	• Hypotension

(Perogamvros *et al.*, 2010)			CBG-cortisol binding affinity	• Normal FC • ↓CBG • ↑% FC • ↑cortisol pulsatility	• Fatigue
CBG Santiago Leu5CysfsX26 (Torpy *et al.*, 2012)	13delC. Frameshift → Premature stop codon in pro-CBG	Isolated from a 9 year old Spanish male, also heterozygous for A224S	Decreased CBG synthesis	• ↓TC • 50% ↓in CBG • Normal ACTH	• Chronic fatigue • Weakness • Headaches
CBG A51V (Lin *et al.*, 2012; Lei *et al.*, 2015)	C>T	CBG polymorphism screening study in Han Chinese, frequency 1:35	Decreased synthesis and/or secretion of CBG *in vitro* in CHO cells	• CBG 30-50% ↓ in heterozygotes • Higher female-to-male live birth rate	Not described
CBG E102G (Lin *et al.*, 2012)	A>G	CBG polymorphism screening study in Han Chinese	Reduced CBG-cortisol binding capacity *in vitro* in CHO cells	No apparent change to CBG	Not described
CBG Athens W371S (Hill *et al.*, 2012)	G>C	Isolated from a single Greek kindred, also heterozygous for CBG Lyon D367N and CBG A224S	Complete loss of CBG-cortisol binding affinity	• Normal CBG levels • ↓TC • Normal FC • ↑%FC • Normal 24 hour UFC	• Obesity

T – thymine; A – adenine; C – cytosine; G – guanine; TC – total cortisol; FC – free cortisol; UFC – urine free cortisol; CHO – Chinese hamster ovary. Adapted from (Meyer *et al.*, 2016).

Table B2 Variants identified from SNP databases or mutagenesis studies: clinical implications unknown.

CBG variant	**Nucleotide change**	***In vitro* CBG abnormality**
CBG H14Q (Simard *et al.*, 2015)	C>A	Reduced CBG-cortisol binding affinity
CBG H89Y (Simard *et al.*, 2015)	C>T	Reduced CBG-cortisol binding affinity
CBG H14R (Simard *et al.*, 2015)	A>G	Reduced CBG-cortisol binding capacity
CBG R260L (Simard *et al.*, 2015)	G>T	No binding activity
CBG I48N (Simard *et al.*, 2015)	T>A	Decreased CBG synthesis and/or secretion
CBG P246Q (Simard *et al.*, 2015)	C>A	Decreased CBG synthesis and/or secretion
CBG I179V (Simard *et al.*, 2015)	A>G	Resistance to protease cleavage and residual binding affinity after RCL cleavage
CBG I279F (Simard *et al.*, 2015)	A>T	Resistance to protease cleavage and residual binding affinity after RCL cleavage, reduced CBG-cortisol binding affinity
CBG A256T (Simard *et al.*, 2015)	G>A	Loss of antibody recognition
CBG T349A (Simard *et al.*, 2015)	A>G	Increased antibody recognition
CBG T342P (Lin *et al.*, 2009)	NA	Resistant to proteolytic cleavage
CBG G335P (Lin *et al.*, 2009) CBG V336R CBG T338P	NA NA NA	Residual binding affinity after RCL cleavage
CBG Q232R (Braun *et al.*, 2010)	NA	Decreased cortisol-binding affinity and higher sensitivity for heat inactivation
CBG G335V (Braun *et al.*, 2010)	NA	Higher sensitivity for heat inactivation and to elastase cleavage
CBG T342A (Braun *et al.*, 2010)	NA	Resistance to elastase cleavage

SNP – single-nucleotide polymorphism; T – thymine; A – adenine; C – cytosine; G – guanine; NA – not available. (Meyer *et al.*, 2016).

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