CORTICOSTEROID-BINDING GLOBULIN (CBG): DEFICIENCIES AND THE ROLE OF CBG IN DISEASE PROCESSES

by

LESLEY ANN HILL

B.Sc. University of Western Ontario, 2011

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

July 2017

Abstract

Corticosteroid-binding globulin (CBG, *SERPINA6*) is a serine protease inhibitor family member produced by hepatocytes. Plasma CBG transports biologically active glucocorticoids, determines their bioavailability to target tissues and acts as an acute-phase negative protein with a role in the delivery of glucocorticoids to sites of inflammation. A few CBG-deficient individuals have been identified, yet the clinical significance of this remain unclear. In this thesis, I investigated 1) the biochemical consequences of naturally occurring single nucleotide polymorphisms in the *SERPINA6* gene, 2) the role of human CBG during infections and acute inflammation and 3) CBG as a biomarker of inflammation in rats. A comprehensive analysis of functionally relevant naturally occurring *SERPINA6* SNP revealed 11 CBG variants with abnormal production and/or function, diminished responses to proteolytic cleavage of the CBG reactive center loop (RCL) or altered recognition by monoclonal antibodies. In a genome-wide association study, plasma cortisol levels were most closely associated with *SERPINA6* SNPs and plasma CBG-cortisol binding capacity. These studies indicate that human CBG variants need to be considered in clinical evaluations of patients with abnormal cortisol levels. In addition, I obtained evidence that discrepancies in CBG values obtained by the 9G12 ELISA compared to CBG binding capacity and 12G2 ELISA are likely due to differential *N*-glycosylation rather than proteolysis, as recently reported. In relation to human inflammation, the bacterial protease *Pseudomonas aeruginosa* elastase was shown to cleave the RCL and α-2-macroglobulin specifically inhibited this. ICU patients with a variety of illnesses had significantly reduced plasma CBG levels, with the lowest levels in individuals with severe inflammation. Similar results were observed in a rodent model of inflammation, where significant reductions in plasma CBG levels were associated with CBG proteolysis and the down-regulation of hepatic *Serpina6*

expression. In addition, lower baseline plasma CBG levels in Harlan Sprague Dawley rats were linked with an increased susceptibility to inflammation. Together, the human and rodent studies highlight the importance of CBG in inflammatory reactions and suggest that CBG is a useful biomarker of inflammation onset and severity.

Lay summary

Corticosteroid-binding globulin (CBG) is a plasma protein that is known to bind and transport the steroid hormone cortisol within human blood. Cortisol controls the growth and development of our organs, helps us deal with stress, fights off infections, and assists in recovery after illness. In various disease states, CBG delivers and releases anti-inflammatory cortisol to sites of inflammation. A few individuals with DNA variations that cause deficits in CBG production and/or function have been identified, but the clinical significance is unclear. The objective of this research was to: (1) identify new CBG variants, and (2) investigate the role of CBG during inflammation. These studies have demonstrated how CBG variants influence the biological actions of cortisol and lay the foundation for further clinical investigations. Additionally, the results demonstrate that changes in CBG levels play a vital role in our bodies' response to inflammation.

Preface

A version of **chapter 3.2** has been published. **Hill LA**, Vassiliadi DA, Simard M, Pavlaki A, Perogamvros I, Hadjidakis D, Hammond GL. (2012). Two different corticosteroid-binding globulin variants that lack cortisol-binding activity in a Greek woman. Journal of Clinical Endocrinology and Metabolism. 97(11): 4260-7. I was responsible for experimental design, data acquisition and manuscript preparation. Dr. Vassiliadi was responsible for the acquisition of clinical endocrinology data (**section 3.2.1.1**). Dr. Simard produced the recombinant CBG variant proteins. All co-authors edited the manuscript.

A version of **chapter 3.3** has been published in an article of which I am a co-first author. Simard M*, **Hill LA***, Lewis JG, Hammond GL. (2014). Naturally Occurring Mutations of Human Corticosteroid-binding Globulin. Journal of Clinical Endocrinology and Metabolism. 100(1):E129-139. Together, Dr. Simard and I were responsible for the experimental design, data acquisition and manuscript preparation under the guidance of Dr. Hammond. I purified CBG variants using FPLC and biochemically characterized their steroid binding properties and measured CBG protein levels by ELISAs (**sections 3.3.1.1 and 3.3.1.3**). Dr. Simard produced the proteins in CHO cells, characterized their production and secretion (**section 3.3.1.2**) and completed the proteolytic digests (**section 3.3.1.4**). All co-authors edited the manuscript.

A portion of the work presented in **chapter 3.4** is used with permission from a published article of which I am an author. Bolton JL, Hayward C, Direk N, Lewis JG, Hammond GL, **Hill LA**, et al. (2014). Genome wide association identifies common variants at the *SERPINA6/SERPINA1* locus influencing plasma cortisol and corticosteroid binding globulin. PLoS Genetics. 10(7):

e1004474. For the manuscript, I completed CBG-cortisol binding capacity measurements described in **section 3.4.1.2**. Genetic investigations (**section 3.4.1.1**), plasma cortisol measurements (total and free) and CBG ELISA values (**section 3.4.1.2**) were completed by co-authors. Data presented in **section 3.4.1.3** is part of an ongoing research study that I am conducting under Dr. Hammond's guidance.

A portion of the work presented in **chapter 4.2** is from a published article of which I am an author. Simard M, **Hill LA**, Underhill CM, Keller BO, Villanueva I, Hancock RE, Hammond GL. (2014). Pseudomonas aeruginosa elastase disrupts the cortisol-binding activity of corticosteroid-binding globulin. Endocrinology. 155(8): 2900-2908. I completed the initial bacterial culture screen (**section 4.2.1.1**), time-course incubations (**section 4.2.1.2**), PAE characterization and Protease IV/TLCK inhibitor experiments (**section 4.2.1.4**). Dr. Simard completed the pH and protease inhibitor assays (**section 4.2.1.2**), as well as the PAE deficient strain experiments (**section 4.2.1.5**). Caroline Underhill was responsible for all FPLC purifications and Dr. Keller obtained and analyzed all mass spectrometry data (**section 4.2.1.3**). *Pseudomonas aeruginosa* media was kindly provided by Dr. Hancock. The manuscript was largely prepared by Dr. Simard. All co-authors edited the manuscript.

Work presented in **chapter 4.3** is part of an ongoing research study. Dr. Vassiliadi collected the ICU plasma samples and I conducted all experiments under Dr. Hammond's guidance.

A version of **chapter 5.2** is used with permission from a published article of which I am an author. Bodnar TS, **Hill LA**, Taves MD, Yu W, Soma KK, Hammond GL, Weinberg J. (2015).

Colony specific differences in endocrine and immune responses to an inflammatory challenge in female Sprague Dawley rats. Endocrinology. 156(12):4604-17. I was responsible for the acquisition and manuscript preparation of all CBG related data. Dr. Bodnar conducted the animal experiments (**section 5.2.1.1**), measured plasma corticosterone levels (**section 5.2.1.2**) and led the preparation of the manuscript.

A version of **chapter 5.3** has been published. **Hill LA**, Bodnar TS, Weinberg J, Hammond GL. (2016). Corticosteroid-Binding Globulin is a biomarker of inflammation onset and severity in female rats. Journal of Endocrinology. 230(2):215-225. Dr. Hammond and I were responsible for the experimental design. Animal experiments were completed by Dr. Bodnar and myself. Dr. Bodnar measured plasma cytokine levels (**section 5.3.1.5**) and I analyzed the data. I completed all of the remaining experiments and prepared the manuscript. All co-authors edited the manuscript.

Unless otherwise stated, all tables and figures consist of work completed exclusively by myself.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

11β-HSD	11-beta-hydroxysteroid dehydrogenase
α2M	Alpha 2-macroglobulin
AAT	Alpha-1-antitrypsin
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
CBG	Corticosteroid-binding globulin
cDNA	Complementary deoxyribonucleic acid
C/EBPβ	CAAT/enhancer binding protein beta
CFA	Complete Freund's adjuvant
CHO	Chinese hamster ovary
CRH	Corticotropin releasing hormone
CYP	Cytochrome P450
DCC	Dextran coated charcoal
DEX	Dexamethasone
DHEA-S	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERα	Estrogen receptor alpha
FPLC	Fast protein liquid chromatography
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GWAS	Genome-wide association study
HACA	11β-hydroxy-andros-4-en-3-oxo-17β-carboxylic acid
HepG2	Human hepatoblastoma

HNF	Hepatic nuclear factor
HPA	Hypothalamic pituitary adrenal
ICU	Intensive care cohort
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor
IgG	Immunoglobulin G
IL-4/6/10/13	Interleukin
Ka	Association constant
Kb	Kilo base
Kd	Dissociation constant
kDa	Kilo dalton
LPS	Lipopolysaccharide
MAF	Minor allele frequency
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NHLBI	National Heart, Lung, and Blood Institute
PAE	*Pseudomonas aeruginosa* elastase
P.aeruginosa	*Pseudomonas aeruginosa*
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RCL	Reactive center loop
RIA	Radioimmunoassay
SD	Sprague Dawley
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SERPIN	Serine protease inhibitor
SNP	Single nucleotide polymorphism

T4	Thyroxine
TLCK	N-α-tosyl-L-lysine chloromethyl ketone hydrochloride
TNF-α	Tumor necrosis factor-alpha
TPEN	N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine
TSH	Thyroid stimulating hormone
WT	Wild type (used to signify the common allele variant)

Acknowledgements

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

- Marie Curie

First, I would like to thank my supervisor, Dr. Geoffrey Hammond, whose guidance throughout my studies has equipped me with the skills and knowledge to be a successful researcher. I am truly appreciative of his constant support and encouragement over the years. It has been a privilege working with Geoff and I know that his teachings will continue to impact me throughout my scientific career.

I would also like to express my gratitude to my supervisory committee members, Dr. Angela Devlin, Dr. Tim Oberlander, Dr. Calvin Roskelley and Dr. Wendy Robinson. Your insight and perspective throughout my studies have helped me to be successful in my research.

I am particularly grateful to my lab members, past and present, who continually inspired and motivated me. Thank you for the intelligent discussions and for creating a wonderful working environment. A special thank you to Caroline Underhill for her technical assistance, advice and support over the years.

I must also thank and express my gratitude to the various collaborators I have worked with on a number of studies. My research would not be what it is without your advice and scientific perspective.

Finally, I would like to thank my friends and family, whose never-ending support I am forever grateful for. To my parents, no words can express how much I appreciate all of the love and encouragement you have given me while I pursue my goals. All of my success I owe to you.

Chapter 1: Introduction

1.1 Overview

In 1956, the first observations were made of a human plasma protein that was capable of binding glucocorticoids (1). Unlike albumin, which binds all classes of steroids with low affinity and high capacity (2), this protein, aptly named corticosteroid-binding globulin (CBG), but also referred to in the early literature as transcortin, binds glucocorticoids with a high affinity but a limited capacity. Glucocorticoid hormones are found in all vertebrate classes including fish, but CBG is only found in terrestrial vertebrates and aquatic mammals (3). Although the binding affinities of CBG for glucocorticoids vary widely between species, the main function of CBG is to regulate the levels of unbound or "free" glucocorticoids, with low levels (5-10%) of unbound cortisol or corticosterone circulating in a normal physiological state. Given that the CBG capacity in normal human plasma exceeds resting cortisol concentrations, CBG acts as a buffer to protect against large oscillations in cortisol levels caused by pulsatile circadian adrenal secretion (4, 5).

Surprisingly, when the human CBG cDNA was cloned in 1987, it was discovered that CBG is most closely related to several clade A serine protease inhibitors (SERPINAs) rather than other steroid-binding proteins, such as albumin, sex hormone binding globulin or vitamin D binding protein (6). The *SERPINA6* gene encodes human CBG, and its expression by hepatocytes is responsible for the production of plasma CBG. As a glycoprotein, CBG contains a high carbohydrate content of approximately 30% due to the presence of six consensus sites for *N*-glycosylation (6), which are variably utilized (7).

Unlike other SERPINA family members, CBG is non-inhibitory and undergoes an irreversible loss of high affinity cortisol binding upon extensive conformational changes that

follow cleavage of its surface exposed reactive center loop (RCL) by various proteases, such as neutrophil elastase (8, 9). This loss of CBG-steroid binding activity causes a substantial redistribution of plasma cortisol between albumin-bound and unbound or "free" fractions, thereby enhancing glucocorticoid bioavailability at sites of infection or inflammation (10). These findings provided evidence that CBG is not solely a steroid hormone transporter, but also plays an important role in acting as a reservoir, facilitating the localized delivery of glucocorticoids to sites of action.

The elucidation of CBG crystal structures explained how RCL proteolysis triggers a conformation rearrangement in the protein that causes a loss of steroid binding, as well as how naturally occurring mutations of critical residues alter the ability of CBG to bind ligands (11-13). Earlier studies identified individuals with different single nucleotide polymorphisms (SNPs) within their *SERPINA6* gene that alter either the steroid binding properties or production of CBG (14-16). Although the clinical consequences of these CBG variants are not uniformly consistent, associations with central obesity, fatigue and chronic pain have been noted (17).

Immunoreactive CBG and CBG (*SERPINA6*) mRNA have both been found within some tissues, including the exocrine pancreas and kidney during mouse development (18), placental trophoblasts (19), and throughout the central nervous system (20), where CBG likely functions differently than in the blood (21). For example, within the developing mouse kidney, CBG is secreted into the renal tubules where it probably regulates the actions of corticosterone at times when glucocorticoids are known to influence renal morphogenesis (22). Moreover, within the intervillious space of the placenta, where the local concentrations of progesterone far exceed those of cortisol, CBG likely acts primarily as a progesterone binding protein.

Alterations in plasma CBG levels are observed in a number of physiological states, including pregnancy, development and inflammation. Dynamic changes in plasma CBG levels during fetal and neonatal development are believed to be important in modulating the effects of glucocorticoids on organ development and maturation. During inflammation, large reductions in plasma CBG levels occur in various pathological states, including following burn injury (23), cardiac surgery (24) and sepsis (25). Such changes in plasma CBG levels are important because they will influence the local bioavailability and action of glucocorticoids, which have diverse effects on biochemical processes throughout the body.

1.2 Glucocorticoid hormones

Glucocorticoids are steroid hormones produced and secreted by the adrenal glands in a circadian and stress-associated manner and are regulated by the hypothalamic-pituitary-adrenal (HPA) axis. In humans, the prevalent glucocorticoid is cortisol, while in rodents it is corticosterone because the *CYP17A1* gene that is required for cortisol production is not expressed in the rodent adrenal. The predominant means by which glucocorticoids exert their effects is by modulating gene expression through the glucocorticoid receptor (GR), although several rapid non-genomic actions have also been reported (26). Glucocorticoids regulate a number of physiological systems, and this is achieved by the widespread expression of GR throughout the body. Notably, glucocorticoids are vital to energy homeostasis, immunomodulation, stress and embryonic development, to name a few. Clinically, imbalances in plasma cortisol levels are associated with various pathological conditions, including Cushing's syndrome and Addison's disease, which are the result of chronic elevations or deficiencies, respectively (26). Synthetic glucocorticoids, such as prednisolone and dexamethasone (DEX),

have been utilized clinically for over 60 years and are commonly prescribed for chronic inflammation (rheumatoid arthritis) and immunosuppression (organ transplants) (26).

1.2.1 Production of glucocorticoids

Adrenal steroidogenesis is controlled by the HPA-axis, whereby the hypothalamus secretes corticotropin releasing hormone (CRH) that then acts on the anterior pituitary to stimulate the release of adrenocorticotropic hormone (ACTH) into the blood (Figure 1.1). Within the adrenal gland, ACTH stimulates the production and release of cortisol and corticosterone, from a specific region of the adrenal cortex, the *zona fasciculata*. To ensure the tight control of plasma glucocorticoid levels, feedback mechanisms exist at various points along the HPA-axis. For example, glucocorticoids exert negative feedback at both the hypothalamus and anterior pituitary to inhibit the release of CRH and ACTH, respectively, regulating the production and secretion of glucocorticoids from the adrenal glands.

Within the adrenal gland, the steroidogenic pathway leading to the production of glucocorticoids relies on the expression of specific enzymes within the mitochondria and smooth endoplasmic reticulum of *zona fasciculata* cells. As steroid hormones, glucocorticoids are derived from cholesterol through a series of enzymatic reactions. In the initial step of steroid biosynthesis, a 6-carbon residue is removed from the side chain of cholesterol, converting it into C21 pregnenolone. As seen in Figure 1.2, the synthesis of cortisol and corticosterone follow parallel pathways. However in order to produce cortisol, pregnenolone must first be converted to 17α-hydroxypregnenolone through the actions of the 17α-hydroxylase component of the P450 enzyme, CYP17A1. There is a lack of expression of *Cyp17a1* in the adrenals of some species, including rodents, and this results in the production of corticosterone only. In humans, while cortisol is the major glucocorticoid produced by the adrenal, corticosterone is also produced and

circulates in the blood. Once synthesized, these steroids are not stored within the adrenal glands, but are immediately released, by diffusion, into the blood.

1.2.2 Genomic and non-genomic actions of glucocorticoids

Based on the free hormone hypothesis, only the unbound or free fraction of steroids in plasma or the extracellular compartments is able to passively diffuse through the cell membrane and enter the cytoplasm of target tissues (27). Classically, steroid hormones are understood to exert their effects by binding to specific intracellular receptors located in either the cytoplasm or nucleus (28). Once within a target cell, glucocorticoids bind with cytoplasmic GR to induce conformational changes that cause GR to dissociate from chaperone proteins, such as heat shock proteins (29). Ligand-bound GR then translocates to the nucleus and dimerizes (30), where it acts as a transcription factor by binding to specific glucocorticoid-responsive elements (GREs) within regulatory regions of target genes, or to interact with other transcription factors to alter gene expression in highly regulated and cell specific manners (Figure 1.3) (31). Variability within the DNA sequence of a GRE dictates the transcriptional outcome (32). In addition, GR interacts with other multi-protein transcriptional complexes, such as co-activators, co-repressors and chromatin-remodeling complexes to mediate the desired effect (33). *In vivo*, the direct binding of GR to a GRE can be classified in two ways (Figure 1.3A, B); simple, in which GR alone binds to a GRE, or composite, where GR binds cooperatively with additional DNA-bound factors (34). In the absence of a DNA-binding site, an alternative, indirect way that GR mediates its genomic effects is by tethering (Figure 1.3C), which occurs when GR participates in protein-protein interactions with heterologous DNA-bound transcription factors that target the gene of interest (29, 34).

Further complexity of GR signaling comes from the fact that the GR gene (*NR3C1*) transcripts undergo extensive processing, including splicing, alternative translation initiation and post-translational modifications to yield multiple, functionally distinct GR isoforms (29, 35). In this classical, relatively slow acting (hours) mechanism, the fine-tuning of GR signaling is based on the availability of biochemically distinct GR isoforms within a given cell type.

In contrast to the classical genomic mechanism, glucocorticoids can also mediate their effects through rapid (minutes) non-genomic means that do not rely on the actions of GR within the nucleus (Figure 1.3D). Instead, glucocorticoids bind to membrane bound or cytoplasmic GR, activating intracellular signal transduction pathways, for example the mitogen-activated protein kinase pathway, and modifications to pathway proteins subsequently alters transcription (26). Additional rapid effects of glucocorticoids that are not specific to GR have been noted and are believed to be a result of physicochemical interactions of glucocorticoids with cell membranes of the cardiovascular, immune and neuroendocrine systems (26). Although classical and non-classical mechanisms are typically studied in isolation, *in vivo* they occur simultaneously, and therefore, the integrated effects of genomic and non-genomic actions need to be considered together.

1.2.3 Glucocorticoid bioavailability

Within the blood circulation, the non-specific carrier protein albumin binds all classes of steroids with a low affinity. Despite the low affinity of albumin for steroid ligands, its high plasma concentrations allow it to buffer fluctuations in steroid levels, aiding in the tight control of the free fraction of steroids in plasma. Although the concentration of plasma CBG is much lower than albumin, the high affinity and specificity of CBG for glucocorticoids gives it a vital role in determining the amount of free steroid. In line with the free hormone hypothesis that only

free steroids are biologically active (27), the plasma proteins albumin and CBG bind approximately 90-95% of circulating glucocorticoids, so that only 5-10% is free to enter cells and is considered "bioavailable" (36, 37).

Within target tissues, the inter-conversion of cortisol or corticosterone and their less active metabolites, cortisone and dehydrocorticosterone, respectively, provides further control over their bioavailability and action, and involves the actions of two 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes, 11β-HSD1 and 11β-HSD2 (Figure 1.3)**.** 11β-HSD1 primarily acts to convert cortisone and dehydrocorticosterone into the more active glucocorticoids cortisol and corticosterone, respectively, but its activity is reversible in some tissues. In contrast, 11β-HSD2 catalyzes the conversion of cortisol or corticosterone into their inactive metabolites cortisone and dehydocorticosterone, respectively.

Importantly, because cortisol has a greater affinity for the mineralocorticoid receptor than its main ligand, aldosterone, 11β-HSD2 is typically expressed in association with the mineralocorticoid receptor to protect it from being bound by cortisol. With respect to synthetic glucocorticoids, differences in their ability to bind plasma proteins and/or interact with 11β-HSD enzymes dictate their potency and metabolic clearance. For example, the high potency of DEX is attributed to the fact that it has a very low affinity for CBG and 11β-HSD2 is unable to inactivate it (26), and therefore, a majority of DEX is essentially free or bioavailable within the circulation.

1.3 Corticosteroid-binding globulin (CBG) is a SERPIN clade A family member

1.3.1 Structural organization and synteny of clade A *SERPIN* genes

SERPINs are believed to have undergone divergent evolution more than 600 million years ago (38) and can be found in all multicellular eukaryotes, with a majority, as the name

suggests, functioning to inhibit serine proteases. Inhibitory serpins are 'suicide' substrates that utilize a unique and extensive conformational change to irreversibly inhibit target proteases and are involved in various physiological processes, including phagocytosis, coagulation, complement activation, fibrinolysis and inflammation (39). Unlike most SERPIN family members, CBG (*SERPINA6*) and thyroxine-binding globulin (*SERPINA7*) act as non-inhibitory hormone-transport molecules. Angiotensinogen (*SERPINA8*) is also non-inhibitory and is the precursor of angiotensin I. Despite differences in function, SERPINs share a common three-dimensional protein structure that consists of three β-sheets and seven or more α-helices. Notably, a hallmark of the SERPIN structure is the RCL, an essential region of the protein that is responsible for interactions with target proteases.

It has been suggested that the multi-gene SERPIN family was established through inter- and intra- chromosomal gene duplication (40). Phylogenetic studies have divided eukaryotic SERPINs into 16 clades (A-P). Human clade A SERPINs comprise 13 members found on chromosomes 1, 14 and X. Of the 13 members, 11 of them, including *SERPINA6*, are found in a syntenic cluster on chromosome 14q32.1 (Figure 1.4), while *SERPINA7* is on chromosome X and *SERPINA8* is on chromosome 1. Despite diverging millions of years ago, *SERPINA* genes share a conserved structural organization, characterized by 5 exons, of which the first is non-coding and the last is partly non-coding (Figure 1.4). Further division of the human *SERPIN* gene locus reveals that a syntenic multigene locus of approximately 90 kb includes the genes encoding α-1 antitrypsin (*SERPINA1* or AAT), a SERPINA1-related pseudogene (*SERPINA2*), CBG (*SERPINA6)* and protein-Z-dependent protease inhibitor (*SERPINA10*) (41). The coordinated transcriptional activation of this sub-cluster of *SERPIN* genes appears to be regulated by a locus control region upstream of *SERPINA1* (42), which is especially relevant in

the context of their activities as acute phase proteins during inflammation (10). Specifically, it is of note that AAT and CBG are both targets for the serine proteinase, neutrophil elastase, and it has been suggested that CBG evolved to complement the activities of AAT in controlling inflammatory processes, as CBG transports anti-inflammatory glucocorticoids (10).

1.3.2 CBG primary structure

The molecular cloning of cDNAs from human liver and lung (6) and the human gene encoding CBG (43) allowed researchers to deduce the primary structure of the human CBG precursor polypeptide, and to demonstrate that the gene is comprised of five exons over approximately 19 kb, with exons 2 to 5 contributing to the coding sequence. Human CBG is produced as a 405 amino acid precursor, including a 22 amino acid signal polypeptide that is removed prior to secretion (6). The mature polypeptide of 383 amino acids contains six potential *N*-glycosylation sites that are differentially utilized (44). In comparison, rat CBG is produced as a 396 amino acid precursor, also with a 22 amino acid signal sequence, resulting in a mature polypeptide of 374 amino acids (45). Rat CBG also contains six potential *N*-glycosylation sites, all of which may not be utilized (10). The human and rat CBG sequences are compared for reference (Figure 1.5), where it can be seen that rat CBG has a shorter amino terminus, as well as areas where deletions of one or more amino acids have occurred, accounting for the difference in sequence length. Phylogenetic comparisons also reveal the presence of highly conserved amino acids that likely play important roles in the structure and functioning of CBG. For example, the tryptophan at position 371 of the mature human CBG polypeptide (Trp362 in rats) is highly conserved across all mammalian species and has been shown to directly participate in steroid binding (46, 47). Two cysteine residues are present within the human CBG primary structure, but studies have shown they do not disulfide bond (6). Moreover, only one cysteine residue is found

within the rat CBG primary structure, in a position corresponding to one of the cysteines within human CBG.

Alignments of CBG orthologs in current protein databases (NCBI Protein) shows a relatively low degree of sequence similarity between CBG from different mammalian species (ex. 60% between human and rat CBG) when compared to the glucocorticoid receptor (91% between human and rat GR). Importantly, it is remarkable that the most poorly conserved region (49%) of CBG corresponds to amino acids 330-360 in the human CBG mature polypeptide sequence that includes the RCL (10).

1.4 Transcriptional regulation of the *SERPINA6* gene encoding CBG

As in most mammalian species, the *SERPINA6* promoter contains TATA and CAAT-box motifs, as well as other sequence elements that appear to be responsible for its liver-specific expression (43). These were established in studies in rats, which identified five *cis*-acting sequence elements within the *Serpina6* proximal promoter that are also highly conserved in human *SERPINA6* (48). These protein-binding sites (P1-P5) resembled recognition sequences for known transcription factors, including hepatic nuclear factor 1-beta (HNF-1β), CCAAT-binding protein CP-2, D-site-binding protein, hepatic nuclear factor 3-alpha (HNF-3α), CAAT/enhancer binding protein beta (C/EBPβ), respectively. A summary of the various effects of hormones and drugs on CBG levels is presented in Table 1.1 with their relevant citations.

1.4.1 Proximal promoter

Reporter gene plasmids under the control of various rat *Serpina6* promoter fragments were introduced transiently into rat hepatoma cells (H4IIEC3) and utilized to further characterize regulatory elements that participate in *Serpina6* expression (48). The fragment containing *cis*-elements P1-P5, from -300 to +1 (transcription start site), produced maximal transcription

activity. A significant decline in transcription was observed when extending the promoter to -800, suggesting that repressor sequences are located within this region. Further characterization of P1 and P2 demonstrated that although HNF-1β interacts with P1, this site is not required for the basal activity of the rat *Serpina6* promoter (49). In comparison, deletion of the CCAAT-binding protein site, P2, abolished basal transcriptional activity, suggesting that this site is a key determinant of rat *Serpina6* expression (49).

Similar studies were completed to determine if SNP within the proximal human *SERPINA6* promoter alter basal transcriptional activity (50). Of the six *SERPINA6* SNP investigated, two increased basal promoter activity in human hepatoblastoma cells (HepG2), while three others that were located close to HNF-1 binding sites, differentially altered the transcriptional effect of HNF-1β and HNF-1α.

1.4.2 Effects of glucocorticoids

Over the years, it has been noted that glucocorticoids reduce plasma CBG levels. Clinically, low plasma CBG levels are seen in individuals with Cushing's syndrome, patients with excess endogenous glucocorticoid levels and patients receiving physiological and pharmacological doses of exogenous glucocorticoids (51). Studies in rats have further supported this, with decreased rat CBG levels observed following physiological and psychological stressors (52, 53). In addition, the treatment of rats and isolated rat livers with glucocorticoids resulted in the inhibition of hepatic CBG synthesis and secretion (54).

The synthetic glucocorticoid, DEX was shown to decrease plasma CBG levels in rats, even after hypophysectomy (55), suggesting that this effect was mediated by alterations in gene expression and not through the HPA axis. Further support for this was obtained when it was found that DEX administration decreased hepatic *Serpina6* mRNA abundance due to a decline in

gene transcription (56). In contrast to the inhibitory effect of DEX during adulthood, DEX increases ovine fetal hepatic mRNA and plasma CBG levels (57, 58), with similar increases in fetal plasma CBG levels observed in baboons treated with betamethasone (59). These findings suggest that differences exist between the regulation of plasma CBG during fetal life and adulthood.

Glucocorticoid response elements have never been identified within the proximal promoter regions of the genes encoding CBG in rats, mice or humans. Therefore, it has been suggested that glucocorticoids mediate their effects on *SERPINA6* expression via the glucocorticoid receptor (GR) acting in concert with other transcription factors, rather than through direct GR-mediated actions (48). Building on reports that C/EBPβ (60) and HNF3α (61) are important for GR-mediated signaling, a recent study investigated the GR responsiveness of the proximal rat *Serpina6* promoter by introducing mutations into these transcription factor-binding sites (62). The study indicated that C/EBPβ, and not HNF3α, mediates the DEX induced inhibition of the *Serpina6* promoter via the co-recruitment of ligand activated GR (62), thus suggesting a tethering mechanism for the glucocorticoid mediated inhibition of *Serpina6* expression. Interestingly, in the same study, when stressors of variable intensity were compared, a graded level of inhibition was observed in line with the severity of stressor, suggesting that stressor severity influences the degree of *Serpina6* gene inhibition (62).

1.4.3 Estrogen and growth hormone effects:

In humans, plasma CBG levels do not differ between males and females in the normal, healthy state (63). Although there is little variation in human plasma CBG levels throughout adulthood, two-fold increases are observed during pregnancy and in women using oral contraceptives (17, 64-66). These increases in CBG have been attributed to an estrogen receptor

alpha (ERα) dependent mechanism (67), although the molecular mechanism for this has not been established. However, in support of this, mitotane, an antineoplastic drug used to treat adrenocortical carcinomas, has been shown to increase plasma CBG levels through an ERα dependent mechanism (67).

In comparison to humans, female rats have approximately twice the levels of plasma CBG when compared to males (68). This sex difference has been indirectly attributed to sex differences in the pulsatility of plasma growth hormone (GH) levels (69-71). In rats, GH stimulates the hepatic expression of *Serpins*, including the *Serpina6* gene (72), and continuous infusion of human or bovine GH increased serum CBG levels, where as intermittent GH replacement at 12-hour intervals had no influence (69). The latter findings hint to a possible underlying mechanism related to observations that females have a more continuous release of GH, in comparison to the more pulsatile release of GH in males (73). Moreover, the sex differences in adult plasma GH levels appear to be influenced by neonatal androgen imprinting on the pulsatile release of GH from the pituitary (71).

In contrast to the stimulatory effect of GH observed in rats, humans receiving growth hormone treatment have significant reductions in plasma CBG levels (74). Treatment of human HepG2 cells with insulin-like growth factor 1 (IGF-1) also results in decreased CBG protein levels through reductions in mRNA levels (75). The pulsatile secretion of GH is known to stimulate the hepatic production and secretion of IGF-1 (76) and this GH/IGF-1 relationship may somehow account for the GH mediated decreases in plasma CBG observed *in vivo* (74).

1.4.4 Effects of cytokines

Various cytokines regulate human and rat CBG levels. For instance, studies using HepG2 cells established an inverse relationship between IL-6 and CBG production (77). Although the

underlying mechanism for this relationship has not been fully elucidated, evidence exists that IL-6 acts at the transcriptional and/or posttranscriptional level. At the posttranscriptional level, reductions in CBG production by HepG2 cells have been attributed to decreases in *SERPINA6* mRNA stability (78). The regulation of IL-6 on CBG production is also predicted at the transcriptional level, given the presence of an IL-6 regulatory element within the rat *Serpina6* proximal promoter (48), which is conserved within the human *SERPINA6* gene. Building on these earlier *in vitro* studies, an inverse relationship has been observed between IL-6 and plasma CBG levels in humans (23, 79). Similarly, tumor necrosis factor-alpha (TNF-α) has also been shown to decrease rat serum CBG levels (80). In contrast, IL-1β was shown to stimulate CBG secretion from HepG2 cells, without any change in *SERPINA6* mRNA levels, suggesting that this effect was due to alterations in post-translational processing and/or secretion (77).

1.5 Hepatic and extra-hepatic expression

1.5.1 Hepatic expression

The first hint that plasma CBG is synthesized by hepatocytes came from Weiser *et al.* (81), who demonstrated that rat hepatic slices synthesized a protein with similar steroid-binding and electrophoretic properties as CBG. The presence of CBG mRNA and protein in the liver was subsequently demonstrated in various species, with the earliest observations made in guinea pigs (82, 83) and rats (84). Evidence for the production of human plasma CBG by hepatocytes followed when it was shown that human CBG could be synthesized from the cell-free translation of hepatic mRNA (85), in addition to the secretion of CBG by HepG2 cells (86).

1.5.2 Extra-hepatic expression

It is now established that hepatocytes are solely responsible for the production of plasma CBG (21), but CBG is also produced in several extra-hepatic tissues. CBG mRNA has been

detected in myometrium of the near term pregnant hamster (87), mouse ovaries (88), rabbit lung, spleen, and ovaries, and fetal rabbit kidneys (89). In mice, the transient production of *Serpina6* mRNA and protein in fetal and postnatal kidneys (18, 22) occurs at a time when glucocorticoids play important developmental roles in the renal tubules (22). Mouse *Serpina6* mRNA has also been detected in exocrine cells of the fetal pancreas around days 15 and 16 of gestation (18).

In humans, the extra-hepatic expression of *SERPINA6* has been noted at low levels in the lung (6) and heart (90, 91). Within the heart, *SERPINA6* mRNA has been detected within cardiomyocytes co-localizing with mineralocorticoid receptors (90). Interestingly, neutrophil elastase, a protease capable of cleaving the RCL of CBG, has also been found near and within the plasma membrane of cardiomyocytes (90). In women, evidence of *SERPINA6* expression has been found within the uterine endometrium, corpus luteum and placenta (92, 93).

Several immunohistochemical studies have localized CBG within human lymphocytes (94), rat adipocytes (95) and various regions of the central nervous system of guinea pigs (96), humans (20) and rats (97, 98), including cerebrospinal fluid (99). Notably, CBG has been localized within various regions of the hypothalamus, including axonal projections of the lateral hypothalamus, median eminence and magnocellular neurons (98). Within magnocellular neurons of the hypothalamus, CBG is colocalized with vasopressin (20, 98), a hormone that is known to act synergistically with CRH to stimulate ACTH release from the anterior pituitary (100). Moving further down the HPA-axis, CBG has also been localized within guinea pig (96) and rat (97) anterior pituitary cells that secrete ACTH. Although caution needs to be exercised when interpreting immunohistochemical observations, especially from studies that employ antiserum that has not been rigorously tested for specificity, these data have been interpreted to suggest that

CBG may play a role in HPA-axis mediated stress reactions, perhaps by regulating the negative feedback of glucocorticoids on CRH and ACTH neurons (98).

It also remains to be determined whether the localization of CBG in many of these extra-hepatic tissues is due to *de novo* synthesis or simple sequestration from circulation, but the presence of CBG within them is not surprising, given that many are the target of CBGs main steroid hormone ligands, glucocorticoids and progesterone. Moreover, although the biological significance of CBG production by extrahepatic tissues remains obscure, it is predicted to control the tissue availability of its steroid ligands locally by regulating the access of glucocorticoids to intracellular glucocorticoid receptors.

1.6 Beyond plasma transport – CBG function

The primary role of CBG is to transport and regulate the plasma distribution and levels of free cortisol or corticosterone, depending on the species (37, 64). Under normal conditions, plasma CBG binds 80-90% of circulating cortisol in humans, leaving 10-15% albumin-bound and 5-10% free (Figure 1.6A) (36, 37). Evidence for a key role of CBG in determining the free fraction of glucocorticoids within the circulation comes from human (16, 101) and rodent (102) studies. Perturbations in the plasma distribution of cortisol, with an enhanced percent-free fraction, have been observed in patients with CBG variants that have a reduced affinity for cortisol (16, 101). Further, in *Cbg*$^{-/-}$ mice, free corticosterone levels have been reported to be 10-times higher than in their wild-type counterparts (102). Therefore, both the concentration of plasma CBG and the affinity (Kd) of its steroid binding site play roles in determining the ratio of free and bound steroid, with alterations modifying the amount of free glucocorticoid available to target tissues (64). In this way, CBG plays critical roles in regulating glucocorticoid

bioavailability during development and in maintaining physiological homeostasis in health and disease, facilitating glucocorticoid transport to target cells.

The steroid-binding properties of CBG have been extensively studied (3) and some of the more common steroid ligands of human CBG and their corresponding binding affinities (Kd), ranging from 13 nM for cortisol to greater than 100 μM for dexamethasone, are provided in Figure 1.6B (2, 3). In comparison to human CBG, which binds cortisol with a dissociation constant of 0.72 nM (103), rat CBG has about 4-fold lower affinity (Kd 2.86 nM) for corticosterone (104). Of note, these affinity measurements were made using the same assay protocol at 0°C. This is important because when comparing affinity constants obtained in different laboratories caution needs to be taken due to differences in methodology, sample dilution and temperature, all of which have an effect on the resulting value. As shown in Figure 1.6C, temperature is a particularly important parameter, with dramatic, 40-fold, changes in association constants (Ka) observed from 4°C to 37°C (105).

In addition to its primary role as a carrier protein, it has been proposed that CBG mediates the targeted delivery of glucocorticoids to sites of inflammation (9, 10). Evidence suggests that the targeted delivery of anti-inflammatory glucocorticoids is accomplished through the cleavage of the CBG RCL by proteases present at sites of inflammation, for example by elastase (8, 9) released by activated neutrophils. In addition, the steroid binding affinity of CBG decreases at elevated body temperatures (5, 105) that might occur during acute inflammation and this is expected to increase the free or "bioavailable" fraction of glucocorticoids in the blood. In this way, CBG acts as a reservoir for glucocorticoids that can be directly transported to and released at target tissues during inflammation (5, 64, 106, 107).

1.7 Protein structure

1.7.1 Binding-site topography

Based on the extensive sequence similarities between CBG and other SERPINA family members, crystal structures of AAT (108) and alpha 1-antichymotrypsin (109) allowed for early predictions of the tertiary structure of CBG. Structurally and functionally important elements of CBG were later delineated within crystal structures of both rat (11, 13) and human (12, 13) CBG in complex with steroid ligands. These CBG crystal structures revealed the presence of a single high-affinity steroid-binding site close to the surface of the protein, three β-sheets (sA-sC), ten intervening α-helices (hA-hJ), six *N*-glycosylation sites and a poorly structured RCL domain (11-13). Of particular importance are β-sheet B and α-helices A and H, which contain residues that hydrogen bond with steroid ligands (11, 37).

Early fluorescence quenching experiments predicted that a tryptophan residue within human CBG was located within the steroid-binding site (110), with site-directed mutagenesis confirming that Trp371 is required for steroid binding (46). The CBG crystal structures (11-13) confirmed the integral role of this residue in steroid binding, as it makes several points of contact with the steroid-ligand backbone. Crystal structures also revealed the importance of a stacking interaction between Trp371 in β-strand s5B and Arg15 in helix A. Through this pi-stacking interaction, the highly conserved residue Arg15 (Arg10 in rats) positions Trp371 (Trp362 in rats) within the steroid-binding site, ensuring that Trp371 is in the correct orientation to achieve direct points of contact with the steroid ligand (11).

The crystal structure of rat CBG provided unprecedented insight into the topography of the steroid-binding site, identifying the residues that either directly or indirectly influence steroid binding (11). In addition to Trp362, 11 other residues can make contacts with cortisol via polar

or hydrophobic interactions (Figure 1.5), and all of the key residues that interact with steroids in the binding site are strictly conserved when mammalian CBG sequences are compared. Subtle differences in the amino acid composition of the CBG binding site, as well as hydrogen bonding capabilities of the steroid backbone, account for species-specific differences in the preferred steroid ligand for CBG. For example, when comparing rat and human CBG, an extra hydrogen bond is formed between cortisol and His368 of human CBG, which is substituted with a lysine in rat CBG (12).

Similarly, in reference to the CBG steroid-binding pocket, differences in the hydrogen bonding capabilities of steroid ligands dictate their binding affinity. For example, when comparing cortisol and progesterone (see Figure 1.2 for structures), the structural basis for the increased binding affinity of cortisol for CBG becomes apparent. When progesterone binds to CBG, only a single direct hydrogen bond is formed via the carbonyl oxygen at C20 (13). In comparison, the presence of three hydroxyl groups on cortisol (C11, C17, C21) allow for additional polar interactions between the steroid and CBG residues, accounting for the increased affinity of cortisol for CBG (13).

Further, in terrestrial vertebrates other than mammals, the amino acid composition of the CBG binding site is quite different; in avian CBG only five of the residues that are involved in mammalian CBG-steroid interactions are conserved (111). Most notably, the tryptophan residue that is vital to steroid binding in mammalian CBGs is replaced by an asparagine in zebra finch CBG, which is also a site for *N*-linked glycosylation (111). Other amino acids that interact to position the tryptophan in the steroid-binding site of mammalian CBGs are also not conserved in other vertebrates, and this alters their steroid-binding properties. For example, in birds, CBG has a high affinity for cortisol, corticosterone, progesterone and the androgens, testosterone and 5α-

dihydrotestosterone, and the nanomolar affinities of androgens are believed to compensate for a lack of sex-hormone binding globulin (112).

1.7.2 Allosteric mechanisms controlling CBG-ligand interactions

Analyses of crystal structures also provided insights into the mechanisms controlling CBG-ligand interactions. In the cortisol-bound state, the CBG RCL is exposed and displays a typical "stressed" (S) serpin conformation, whereas upon cleavage, the CBG RCL fully inserts into the protein core and displays a "relaxed" (R) conformation. The S-conformation of cortisol-bound rat CBG (11) is structurally similar to ligand bound antithrombin (113) and heparin cofactor II (114), as the RCL is fully ejected and helix-D is over-wound by two helical turns. Based on similarities to antithrombin, an allosteric coupling mechanism has been proposed for CBG, where in the ligand-free state, the RCL is partially inserted into the central β-sheet (11).

In this scenario, the partial insertion of the RCL is accompanied by the slight unwinding of helix D and this reversible process is thought to facilitate steroid binding and dissociation under normal physiological conditions. Thus, small movements of the RCL and helix D are coupled to the steroid-binding pocket, influencing its shape and flexibility. Occupancy of the steroid-binding site orients the RCL in a way that transitions the steroid-binding site from a moderate to a high-affinity state. Support for this allosteric mechanism is provided by CBG variants, where increased cortisol-binding affinities were observed for variant proteins in which the CBG RCL appears to be constitutively expelled (115). The partial insertion of the CBG RCL in the "ligand free" state may also be biologically significant, in that it might limit protease recognition and cleavage (11), while in the ligand-bound state full exposure of the RCL would make it more vulnerable to protease attack. At this time, further research comparing the crystal structures of "ligand free" and ligand-bound CBG are required to test these hypotheses.

1.7.3 Reactive center loop recognition by proteases

In the native state, CBG exists in the S-conformation with its RCL fully expelled and exposed for targeting by proteases. Upon cleavage, the CBG RCL fully inserts into β-sheet A as a novel strand (s4A), unwinding helix D and irreversibly disrupting the CBG steroid-binding site (12, 13) in what is referred to as the "stressed to relaxed" or S-to-R transition (116). This conformational change results in a significant (~10-fold) decrease in the CBG-cortisol binding affinity (8, 9) and a concomitant increase in thermo-stability (12). Helix D has been shown to contact residues within loop segments interconnecting strands of β-sheet B (11), and these strands contain residues that directly interact with steroid ligands. After RCL cleavage, helix D unwinds and this leads to changes in the positioning of residues within the inter-strand loops of β-sheet B, thereby altering the positioning of key residues involved in steroid binding. Given the extensive conformational changes that occur during CBG RCL insertion, it becomes clear why this leads to functionally significant reductions in CBG-steroid binding affinity.

Within *SERPIN* gene sequences, the RCL is the region that exhibits the least sequence similarity (117) and both the loop length and amino acid composition are believed to have evolved to accommodate optimal interactions with target proteases. Even within a given SERPIN family member, considerable interspecies variability occurs in the RCL (118) and this has been suggested to be due to evolutionary pressures to adapt to external forces like exposure to species-specific pathogens (119). Within the CBG RCL, residues 333-349 are denoted P17-P1 (Figure 1.7) based on their proximity to the relative position of the neutrophil elastase cleavage site (P1-P1') of the structurally related SERPINA1, AAT (115). Two endogenous proteases that specifically cleave the human CBG RCL have been identified; neutrophil elastase (8, 9) and chymotrypsin (120). Neutrophil elastase cleaves the CBG RCL between P6 (Val344) and P5

(Thr345) (Figure 1.7), mediating the local delivery of cortisol to sites of inflammation (9, 10). Two major cleavage sites in the CBG RCL were identified after incubation with chymotrypsin, one after P4 (Leu346) and another after P2 (Leu348) (Figure 1.7), but the physiological significance of CBG cleavage by chymotrypsin remains unclear (120). In addition, a recent crystal structure of human CBG obtained in complex with progesterone displayed the R-conformation associated with RCL cleavage, and this was unexpected because the protein had not been treated with a protease prior to crystallization (13). Using electron density maps, the RCL cleavage site identified in the human CBG crystal structure was narrowed down to two possibilities; after P1 (Thr349) or P1' (Ser350) (Figure 1.7) (13).

Even though there is limited sequence similarity within the RCL of CBGs and closely related SERPINA members, such as AAT, a few residues are highly conserved, hinting to their importance: position P17 is always glutamic acid, P16 is glutamic acid/lysine/arginine, P15 is typically glycine and P12 to P9 are small residues, such as alanine, valine, serine, threonine or glycine (Figure 1.7). The highly conserved glutamic acid residue at P17 has been suggested to have an important stabilizing function for the RCL (116). Within the RCL, the P15-P9 "hinge" region provides the mobility required for RCL insertion after cleavage (121). A detailed mutagenic study of the human CBG RCL revealed the vital role that P15, P14, P12, P10 and P8 play in RCL insertion after cleavage by neutrophil elastase (115). A lack of CBG RCL insertion after cleavage was predicted for the CBG RCL variant proteins G335P (P15), V336R (P14) and T338P (P12), based on the finding that CBG-steroid binding was largely unaffected after cleavage of their RCL (115). In contrast, substitutions G340P (P10) and T342P (P8) resulted in a partial loss in CBG-cortisol binding after proteolysis, and this was attributed to the RCL only partially inserting (115).

1.8 Importance of carbohydrate additions

Within the endoplasmic reticulum and Golgi apparatus, many secreted proteins are post-translationally modified by the actions of multiple glycosidases and glycosyltransferases, which may influence the folding and conformation of proteins through interactions between the glycan and specific amino acid residues (122-124). The importance of *N*-glycans in post-translational quality-control mechanisms is well appreciated and includes roles in intracellular trafficking that influences secretion, protein stability, plasma half-life and interactions with plasma membrane receptors and proteases, to name a few (122-124).

Early studies established that CBG is extensively glycosylated (Figure 1.8) and that *N*-glycosylation influences the plasma half-life of CBG (125), which is estimated to be around 10-13 hours (126, 127). The examination of human and rat CBG primary structures revealed the presence of six consensus sites for *N*-glycosylation (6, 45), with five of six sites utilized per CBG molecule, resulting in *N*-linked oligosaccharides making up close to 30% of its molecular mass (128). When comparing the positions of *N*-glycosylation sites in human and rat CBG, all but Asn154 in human CBG and Asn194 in rat CBG are conserved (Figure 1.5). Further biochemical analyses of CBG glycosylation demonstrated that individual CBG molecules vary considerably in the type and utility of *N*-linked oligosaccharides, providing an explanation for the substantial micro-heterogeneity observed after gel electrophoresis (128-132). The detailed analysis of human CBG's *N*-linked oligosaccharides confirmed the differential utilization of each glycosylation site and provided a site-specific characterization of monosaccharide composition and relative abundance (133).

The importance of CBG glycosylation in the acquisition of a functional steroid-binding site is highlighted by the fact that *E.coli* produced recombinant CBGs have ~10-fold lower

steroid-binding affinities than natural CBG proteins, and this is attributed to a lack of *N*-glycosylation (37, 111). In terms of specific residues, the glycosylation of Asn238 of human CBG has been shown to be required for high-affinity steroid binding (44) and a lack of an *N*-glycan at this position is predicted to negatively effect the folding and/or stability of the steroid-binding site (7). In addition, among CBG glycosylation sites, Asn238 has the lowest degree of branching, as it contains only non-fucosylated bi- and tri-antennary N-acetyl-glucosamine chains, and it has been suggested that this site facilitates the accessibility of steroids to the binding site (133).

In addition to influencing the steroid-binding properties of CBG, the glycosylation of CBG may also serve a protective role, restricting the interaction of proteases with cleavages sites within the RCL. Interestingly, there is a glycosylation site within the RCL of human CBG at Asn347, which is positioned N-terminal to the neutrophil elastase cleavage site (8, 9), and immediately in between (Figure 1.7) the two main chymotrypsin cleavage sites (120). The presence of this *N*-glycan in the human CBG RCL may not hinder insertion of the RCL following neutrophil elastase cleavage because an oligosaccharide would not be attached to it, whereas cleavage of the RCL by chymotrypsin at a position C-terminal of Asn347 might impede RCL insertion and limit the effects on steroid-binding site disruption. It is also possible that the accessibility of proteases to their cleavage sites within the human CBG RCL may be influenced by both the extent of glycosylation at Asn347 and the types of *N*-glycans attached to it (134).

There is evidence that the composition of CBG carbohydrate chains may vary under different physiological conditions and after hormone treatments. For instance, alterations in the glycan composition of plasma CBG have been observed during pregnancy (135), as well as in culture medium from HepG2 cells following treatment with several hormones (136). It has been

suggested that these differences in the glycosylation of CBG may alter its plasma clearance (125). In addition, during pregnancy, the oligosaccharides associated with human CBG have a higher degree of branching, i.e. predominantely tri-antennary glycoforms, sialylation and occupancy (135), as well as an increased binding affinity to synctiotrophoblast cell membranes (137). Electrophoretic profiles of maternal CBG in rabbits (126) and sheep (58) are also consistent with pregnancy-specific alterations in the carbohydrate composition of serum CBG.

Although typically not taken into consideration, *N*-glycans extensively decorate the surface of CBG (Figure 1.8), and the differential utilization of *N*-glycosylation sites and the types of oligosaccharides attached to them may influence the immunological recognition of surface epitopes on CBG. This is likely to be of particular importance for monoclonal antibodies raised against unglycosylated synthetic peptide antigens, where variations in *N*-glycosylation at specific sites of CBG may alter its recognition in immunoassays.

1.9 Naturally-occurring mutations effecting CBG production and function

1.9.1 Humans

Before DNA sequencing was commonplace, the existence of CBG variants was suspected based on reduced plasma CBG-steroid binding affinities (103, 138, 139). The first non-synonymous substitution identified in the human CBG coding sequence associated with abnormalities in cortisol binding was L93H (14, 140). Until recently, genetic variations in *SERPINA6* were considered rare, but several non-synonymous SNP have been reported, which alter the production or function of plasma CBG. These *SERPINA6* SNP were identified in patients presenting with low total plasma cortisol levels in association with a variety of clinical conditions, including chronic pain, fatigue, depression, hypotension, and excess body weight

(14-16, 141, 142). However, not all individuals with CBG variants that are produced or function abnormally display these clinical features (101, 141, 142).

Of the previously identified CBG variants, CBG Leuven (L93H), CBG Lyon (D367N) and CBG G237V result in decreased or undetectable cortisol-binding activity (14, 16, 101). A decreased capacity for cortisol was reported for the CBG E102G variant, and this has been attributed to a conformational/folding defect (143). Crystal structures of rat (11, 13) and human (12, 13) CBG have provided insight into the consequences of naturally occurring CBG variants. For instance, Leu93 is located within helix D, close to the loop that connects β-strands, s4B and s5B, that contain residues found within the steroid-binding pocket. Located on the rim of β-strand s4B pointing towards helix D, Asp367 is not a key residue for steroid binding, but it may participate in allosteric coupling of the steroid-binding site with RCL insertion. Residue Gly237 is on the surface of CBG, within the loop connecting β-strands s2B and s3B, and likely alters the relative position of β-strands that contain residues that either directly or indirectly participate in steroid binding through hydrogen bonding networks. Also of note is the fact that Gly237 is located next to Asn238, the *N*-glycosylation of which appears to influence the formation of a high affinity CBG-steroid binding site (44).

Two rare single nucleotide deletions in codons for residues (Leu5 and Trp11) within the secretion signal polypeptide result in premature termination of CBG synthesis (15, 141) and a complete lack of CBG production. More recently, CBG A51V, with reduced CBG secretion and low plasma levels, was identified at a relatively high frequency of ~1:30 in Han Chinese (143). The importance of this residue is highlighted by the fact that an alanine is highly conserved in this position across mammalian CBG molecules and other clade A SERPINs. When CBG is intact, Ala51 is directed between β-strands s3A and s5A, whereas when the CBG RCL is

cleaved, the side chain points directly towards s4A, which is the additional β-strand formed by the inserted RCL (143). It is predicted that during the processing of CBG for secretion, Ala51 influences folding events that may be critical for protein stability. In contrast, a common CBG variant (A224S) and two rare CBG variants (R64Q and R64W) do not alter the production or function of CBG (143, 144).

1.9.2 Rodents

Several CBG SNP resulting in non-synonymous substitutions have also been identified in different rodent species and strains. When compared to Wistar rats, a methionine to isoleucine (M276I) substitution in Biobreeding rats results in a 50% decrease in CBG corticosterone-binding affinity (104). Residue Met276 is on the outer β-strand of sheet A (s6A) and its side chain points towards β-sheet B, which contributes to the interior hydrophobic core of the protein and is thought to be important in protein stability (11). A reduced steroid-binding affinity was surprising given that both methionine and isoleucine are nonpolar, hydrophobic amino acids. Interestingly, this substitution only alters the binding affinity of rat CBG for corticosterone, and does not affect the binding properties of cortisol or other natural steroid ligands. Notably, a methionine at residue 276 is conserved in rat and mice, but isoleucine is present at this position in rabbit and human, both of which have a high affinity for cortisol. Strain specific variations in the rat CBG coding sequence have been previously characterized in eight different rat strains (Table 1.2), with Biobreeding and Long Evans rats demonstrating decreased CBG-corticosterone binding affinities, compared to other strains tested (145). Interestingly, similar to Biobreeding rats, three other strains, including Lewis rats, have the M276I substitution, but a decrease in corticosterone-binding affinity is not observed, and an additional valine to methionine (V285M) substitution is also found in these rat strains. Although Met276 and Val285 are not in close

proximity and do not directly interact, this suggests that differences in these residues act together somehow to alter the steroid-binding affinity of CBG, with the combination of Ile276 and Val285 having detrimental effects.

A similar observation was made in mice, where the CBG variant K201E reduces corticosterone-binding affinity (146). A lysine residue at this position is highly conserved amongst CBG sequences, including rat CBG. Based on the rat CBG crystal structure (11), this lysine residue (Lys200 in rat) is located on the protein surface within β-strand s2C, where it contributes to protein stability by forming salt bridges with Glu188 and Asp190.

1.9.3 Pigs

Abnormal cortisol levels and obesity in a pig intercross have been linked to four CBG variants: G307R and I265V appear to reduce CBG-cortisol binding affinity, whereas S15I and T257M did not have any effects on the steroid-binding affinity (147). Using structural homology modeling, the locations of the mutations were identified, with the function altering Gly307 and Ile265 located on s6A and s3B, respectively. The structural rationale for the decreased steroid-binding affinity observed for these two variants is not immediately obvious. Yet, it has been hypothesized that Gly307 may stabilize the correct folding of CBG or indirectly influence the binding pocket, and Ile265, as part of a highly conserved sequence of amino acids, may indirectly modulate steroid binding through long range interactions with ligand-binding residues (37).

1.10 Roles during pregnancy, normal development and diseases

A summary of the associations between clinical conditions and plasma CBG levels is presented in Table 1.1, with their relevant citations.

1.10.1 Pregnancy and development

1.10.1.1 Pregnancy

In humans, maternal plasma CBG levels increase 2- to 3-fold during the second and third trimesters of pregnancy, in concert with increased concentrations of total and free cortisol (148). Increased plasma CBG levels during pregnancy have been attributed to estrogen-stimulated increases in hepatic CBG synthesis (149), as well as a prolonged half-life of a pregnancy-specific CBG glycoform (150). During the final weeks of human pregnancy, maternal plasma CBG levels begin to decrease, with corresponding increases in free cortisol levels (149). Similar fluctuations in maternal plasma CBG levels are also found in rats (151), mice (18), and rabbits (89) prior to delivery, with levels decreasing towards term, normalizing to non-pregnant levels shortly after delivery.

Even greater (12-fold) increases in maternal progesterone levels occur as human pregnancy progresses towards term (152). As a result of local progesterone production by placental trophoblasts, the steroid environment in the intervillious space also differs greatly from peripheral maternal and fetal circulations, with a massively increased progesterone-to-cortisol molar ratio. Since human CBG binds progesterone almost as well as cortisol (Figure 1.6B) (2), these enormous increases in progesterone are expected to displace cortisol from the CBG-steroid binding site and alter the local concentration and bioavailability of CBG's steroid ligands at the maternal-fetal interface (19). In support of CBG acting as a regulator of progesterone during human pregnancy, plasma CBG levels are correlated with circulating progesterone during the first two trimesters, as well as with the amount of progesterone in the intervillous blood (153). Of note, the regulation of progesterone and glucocorticoids at the maternal-fetal interface likely varies considerably between species, because the corpus luteum rather than the placenta is

responsible for a majority of progesterone production in some species, including rodents (21). This, and the remarkable differences in placentation between species should be considered when comparing the possible roles of CBG-progesterone interactions during reproduction across species.

When examining the pregnancy outcomes and health of neonates of CBG-deficient mothers, no obvious effects were observed (153). Interestingly, the offspring born to CBG-deficient women demonstrated a female skewed sex ratio (153). Similar female skewing of the sex ratio has been observed after significant stress events during pregnancy in humans (154) and rodents (155). Although the biological basis for female skewing of the sex ratio is unknown, it has been suggested that CBG deficiency might contribute to inappropriate exposure to cortisol or progesterone during gestation and this may lead to male fetal demise (153).

Decreased plasma CBG levels are associated with pregnancy associated disease states, including preeclampsia and gestational diabetes (149). In mothers with these conditions, significant reductions in plasma CBG, as well as in total and free cortisol levels were observed during the third trimester. In addition, in women with preeclampsia, the typical late pregnancy fall in CBG and concomitant rise in cortisol is diminished. In gamete recipient pregnancies, reductions in plasma CBG, total and free cortisol levels were also observed as early as 20 weeks gestation (149).

1.10.1.2 Fetal and developmental CBG levels

In the mammalian fetus, plasma CBG levels generally begin to rise at the mid-point of pregnancy, reaching a maximum in late gestation, followed by a gradual decline to term in mice (18), rabbits (89), rats (151), and baboons (4). Postpartum, neonatal CBG levels remain low during the first week of life in humans, and gradually rise thereafter to adult levels (156), which

are attained by 12 months of age (157). In rodents, adult plasma CBG levels are reached around postnatal weeks 6-8 in rats (151), and by approximately 4 weeks postnatally in mice (18). A sex difference in plasma CBG levels exists in rodents, with females having almost twice the CBG levels found in males (18, 68, 151), and studies have attributed this difference to neonatal androgen imprinting in males (70, 158, 159). In mice, this sex difference becomes apparent by 4 weeks of age (18), where as in rats, a sex difference in plasma CBG levels is not apparent until 7-8 weeks of age (151, 158).

In terms of development, the ontogeny of kidney *Serpina6* expression in rodents is of interest because it occurs during periods of active tissue remodeling when substantial proteolytic activity takes place, and it is suspected that CBG may act as a substrate for proteinases during this time (18, 22). Reductions in plasma CBG levels occur as term approaches in fetuses, and in neonates, will increase free glucocorticoid levels that are required for the maturation of organs, such as the lungs (10). In mice, the postnatal synthesis of CBG by the kidneys starts at birth, prior to the onset of CBG synthesis by the liver, peaks at 2-3 weeks and disappears by 4 weeks (22). CBG produced by the developing kidney is secreted luminally into the proximal convoluted tubules and is thought to regulate the activity of free corticosterone excreted into the developing renal tubules of the immature rodent kidneys (22, 160). Other species including rabbits (89) and sheep (161) express CBG in the kidneys during fetal life, and the delayed production of CBG in the postnatal rodent kidney may reflect the fact that renal morphogenesis occurs at a later developmental stage in rodents in comparison to many other species. However, while abnormal renal development or function has not been observed in CBG deficient humans (15) or mice (102), some humans with CBG deficiencies present with hypotension (15, 16).

1.10.2 Inflammation

There is significant interplay between the immune and endocrine systems during inflammation, with shared receptors, ligands, and regulatory feedback mechanisms (162). Following an immune challenge, cytokines stimulate the HPA axis, altering hormone release at the level of the hypothalamus, pituitary and adrenal glands, and this in turn has downstream effects on immune function and the resolution of inflammation (163). As a result, changes in communication between cytokines and the HPA axis can impair recovery and exacerbate underlying pathological conditions.

During inflammation, CBG responds as an acute phase "negative" protein, and reductions in its plasma levels can be attributed to proteolytic cleavage (9) as well as a down-regulation of its production by the liver (Figure 1.9) (23, 56, 77). In humans, proteolysis of the RCL of CBG by neutrophil elastase appears to be an early event during inflammatory reactions, rendering CBG non-functional and promoting the localized release of CBG-bound glucocorticoids at sites of inflammation (64, 106). Enhanced exposure of tissues to anti-inflammatory glucocorticoids represses cytokine production and activity (164), thereby limiting cytokine-mediated tissue damage (165). At the same time, plasma glucocorticoid concentrations increase acutely as a result of HPA activation in response to stress (166), and likely act in synergy with elevations in inflammatory cytokines, such as interleukin-6 (IL-6), to further reduce plasma CBG production, and amplifying free glucocorticoid exposures (23, 77, 78). During recovery from inflammation, normalization of CBG levels likely plays a role in determining when, and to what extent, glucocorticoids act to restore the normal homeostatic balance (Figure 1.9).

The significance of CBG RCL cleavage by the pancreatic digestive enzyme chymotrypsin (120) remains to be determined, but neutrophil elastase, which is present at sites of

inflammation is expected to cleave CBG at these locations. The decrease in CBG steroid-binding activity associated with RCL cleavage causes a substantial redistribution of plasma cortisol between the albumin-bound and unbound or "free" fractions, thereby enhancing glucocorticoid bioavailability at sites of infection or inflammation (10). As a result, the local bioavailable concentration of glucocorticoids may increase by 3-4-fold (167). Systemically, in response to an inflammatory stress, increases in ACTH-driven glucocorticoid production far exceeds the reduced steroid binding capacity of CBG, thereby increasing plasma free cortisol levels. In addition, the steroid-binding affinity of CBG is also reduced in response to increased temperature through undefined mechanisms, and may accentuate the actions of glucocorticoids under pathological conditions (105, 168).

Significant decreases in plasma CBG levels have been observed in human patients suffering from a variety of acute inflammatory conditions, such as severe burn injury (23), open-heart surgery (24), multi-trauma (169), acute pancreatitis (170), sepsis (25) and septic shock (25, 171, 172). Differentiating based on the cause of inflammation, decreased plasma CBG levels were found in patients with septic shock due to bacteria (171, 173), fungi (174) and parasites (175). In addition, rapid decreases in plasma CBG levels have been reported in rodents after thermal injury (176, 177) and in pigs treated with lipopolysaccharide (178).

Associations between plasma CBG levels and inflammatory outcomes have been observed in rodents and support the idea that CBG plays a physiological role during the inflammatory process. For instance, Fisher rats, which have higher plasma CBG and corticosterone levels than Lewis rats (179), generally fail to develop inflammation, and this is linked to appropriate HPA axis stimulation by inflammatory mediators and subsequent feedback between HPA and immune systems (180). In addition, Harlan SD rats are more sensitive to

inflammatory challenges after treatment with lipopolysaccharide, when compared to Charles River SD rats (181). Studies in CBG deficient mice further support an important role for CBG during inflammatory processes. For instance, a genetic study revealed that C57BL/6 mice are much more sensitive to an acute challenge with TNF-α than DBA/2J mice, and this trait was mapped to the *Serpina6* locus (182). Notably, C57BL/6 mice have been reported to have 50% lower plasma CBG levels than DBA/2J mice(183). A key role for CBG in these differential responses is further supported by studies in $Cbg^{-/-}$ mice, in which survival rates after an acute inflammatory challenge are compromised (102).

1.10.3 Obesity and metabolic disorders

Glucocorticoids play roles in controlling appetite, glucose metabolism, and fatty acid metabolism and storage (184). In obesity, although cortisol secretion increases, circulating cortisol levels are maintained at relatively normal values due to a simultaneous increase in metabolism (185). In individuals with obesity and insulin resistance, conflicting results in terms of CBG levels have been observed; some studies found reductions in plasma CBG levels in these pathological states (186-188), while others did not (189, 190). Despite conflicting results *in vivo*, dose-dependent decreases in CBG mRNA levels and protein secretion from HepG2 cells occur *in* vitro following insulin treatment (75). Evidence for a relationship between CBG and insulin is provided by clinical studies that found CBG to be a marker of insulin secretion (186, 187, 191).

Negative correlations have been reported between plasma CBG concentrations and obesity parameters, including body mass index, waist circumference and waist-to-hip ratio (192). Notably, a high prevalence of obesity has been reported amongst individuals with CBG deficiencies (16, 139, 141). Increased *in* vitro preadipocyte proliferation and enhanced differentiation of subcutaneous adipose has also been reported in tissue samples from a human

CBG-null homozygote (193). On the other hand, significant reductions in CBG levels have been observed after severe weight loss due to profound caloric restriction (194, 195). Overall, although seemingly subtle, CBG may play a role in glucocorticoid-dependent metabolic disorders (196).

1.10.4 *Cbg*$^{-/-}$ mouse model

Genetically deficient *Cbg*$^{-/-}$ mice have been generated to investigate the role of CBG in mediating glucocorticoid action and related physiological responses (102, 197). As expected, a complete loss of corticosterone-binding activity is observed in these animals, in addition to decreased total corticosterone in comparison to wild type (WT) mice (102, 197). An increased corticosterone turn over rate, as indicated by a decrease in half-life, was also observed (102). Together, these results confirm the primary role of CBG in regulating plasma glucocorticoid levels. A number of contrasting results have also been observed when comparing the two separately generated *Cbg*$^{-/-}$ mouse lines. For example, one study reported a 300% increase in ACTH levels (102), while the other study found no differences (197). Despite elevated free corticosteroid levels in one of the *Cbg*$^{-/-}$ lines, decreased transcription of hepatic corticosterone target genes were detected, suggesting an inability to appropriately respond to excess free glucocorticoid levels (102). In contrast, no significant differences in glucocorticoid target gene expression were found in the other model (197). In line with reports of a fatigue phenotype in human individuals with CBG variants (15, 16), *Cbg*$^{-/-}$ mice display reduced activity levels (102).

Biochemical and behavioural responses to stress have also been investigated in *Cbg*$^{-/-}$ mice, with a diminished stress induced rise in corticosterone noted (197). In terms of altered behavioural responses to stress, *Cbg*$^{-/-}$ mice subjected to prolonged uncontrollable stress displayed despair-like behaviours, including learned helplessness (197). Stress-induced

impairments in memory retrieval have also been observed, and this finding was reversed with administration of corticosterone into the hippocampus (198).

1.11 Hypotheses and objectives

I sought to investigate the role of CBG in health and disease. My central hypothesis was that CBG plays a critical role in the delivery of glucocorticoids to sites of inflammation and tissues during dynamic, protease-rich, remodeling processes.

Given that only a few individuals have been reported with *SERPINA6* SNP and the clinical consequences remain unknown, we set out to biochemically characterize previously unknown non-synonymous *SERPINA6* SNP reported in SNP database releases. We hypothesized that additional CBG deficiencies exist that alter functional characteristics of CBG. The goal was to create a comprehensive list of *SERPINA6* SNP that could be utilized in future clinical studies investigating the consequences of CBG deficits on human health. In addition, I contributed to an international genome wide-association study to identify the primary determinant of plasma cortisol levels. Specifically, studies were carried out to characterize naturally occurring *SERPINA6* genetic variants (**chapter 3**), addressing the following objectives:

- Comprehensive screen for functionally relevant variants
- Investigate clinical associations of CBG variants

In relation to disease, decreases in plasma CBG levels have been noted in various inflammatory conditions, yet the exact timing and mechanisms of these changes are unknown. My work addressed the hypothesized that changes in plasma CBG are mediated by reductions in *SERPINA6* expression and/or proteolytic cleavage during acute inflammation (Figure 1.9), and I sought to further elucidate the timing of plasma CBG changes during inflammation and the mechanisms responsible, utilizing both human samples and a rodent model of acute

inflammation. Studies aiming to further define the role of CBG in acute inflammation were carried out to address the following specific objectives:

- Characterize bacterial proteases capable of cleaving the human CBG RCL (**chapter 4.2**)
- Analyze plasma CBG levels in an ICU patient cohort (**chapter 4.3**)
- Follow time course changes of plasma CBG in a rat model of acute inflammation and elucidate the mechanisms responsible for these alterations (**chapter 5**)

Table 1.1 Hormones, drugs and clinical conditions that alter plasma CBG levels.

	Hormones and Drugs	Conditions
Increase	Estrogens‡ (65, 66) Interleukin 1β (IL-1β) (77) Glucocorticoids (fetus) (57, 58) Mitotane (66, 67)	Pregnancy (135, 148, 150)
Decrease	Glucocorticoids‡ (51, 54, 55) Insulin (75) Growth Hormone (GH) (74) Interleukin-6 (IL-6) (77, 79) Tumor necrosis factor alpha (TNF-α) (80)	Cushing's syndrome (51) Obesity (186-188) Severe caloric restriction (194, 195) Polycystic ovary syndrome (199) Pre-eclampsia (149) Gestational diabetes (149) Gamete recipient pregnancy (149) Cirrhosis (200) Burn injuries (23) Acute pancreatitis (170) Sepsis (25) Septic Shock (25, 171, 172) Multi-trauma (169) Open-heart surgery (24)

‡Natural and synthetic

Table 1.2 Strain variations in the rat coding sequence and the effect on CBG-corticosterone binding affinity (adapted from (145)).

Rat Strain	Substitutions	Kd (nM)
Fischer Sprague Dawley Wistar	Met^{276} Val^{285}	2.86
Biobreeding Long Evans	Ile^{276} Val^{285}	4.74
Brattleboro Brown Norways Lewis	Ile^{276} Met^{285}	2.72

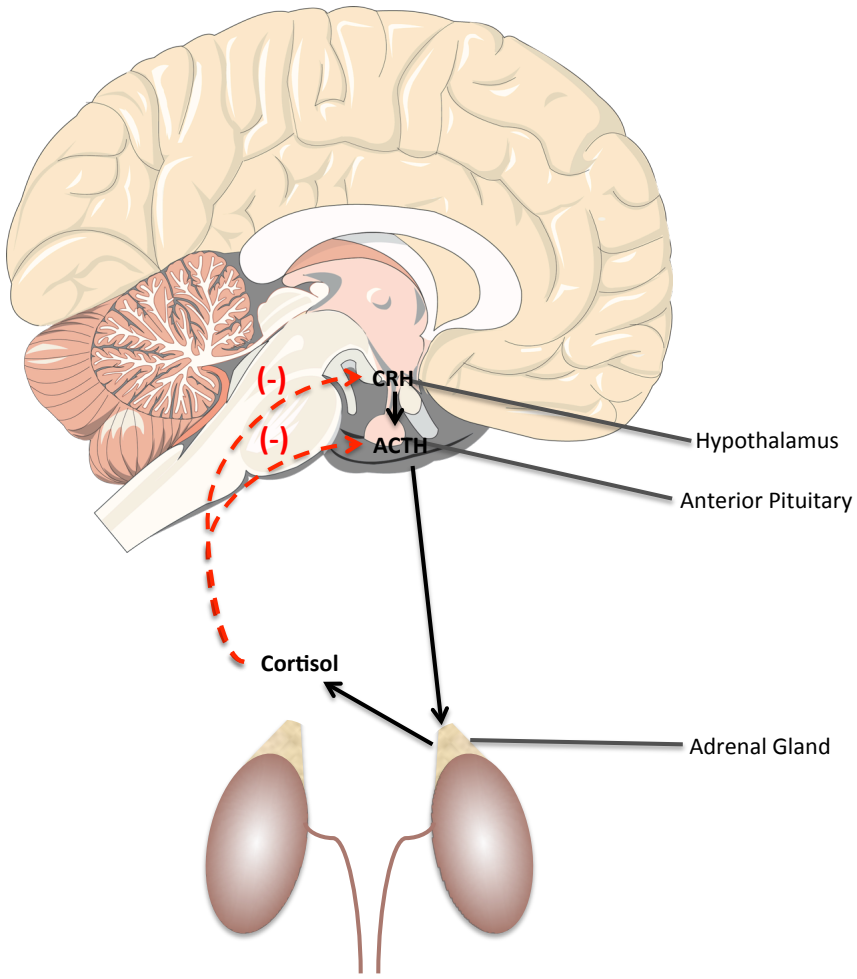


Figure 1.1 The hypothalamic-pituitary-adrenal (HPA) axis. In response to stress, nerve cells in the paraventricular nucleus of the hypothalamus produce and release corticotropin-releasing hormone (CRH). From there, CRH is transported to the anterior pituitary gland, where it acts on corticotropes to stimulate the production of proopiomelanocortin and the release of adrenocorticotropic releasing hormone (ACTH) into the blood circulation. ACTH then acts on cells within the zona fasiculata of the adrenal cortex to stimulate the production and secretion of glucocorticoids, cortisol and corticosterone. When glucocorticoid levels become high, they act through negative feedback (*red arrows*) on the hypothalamus and anterior pituitary to inhibit the release of CRH and ACTH.

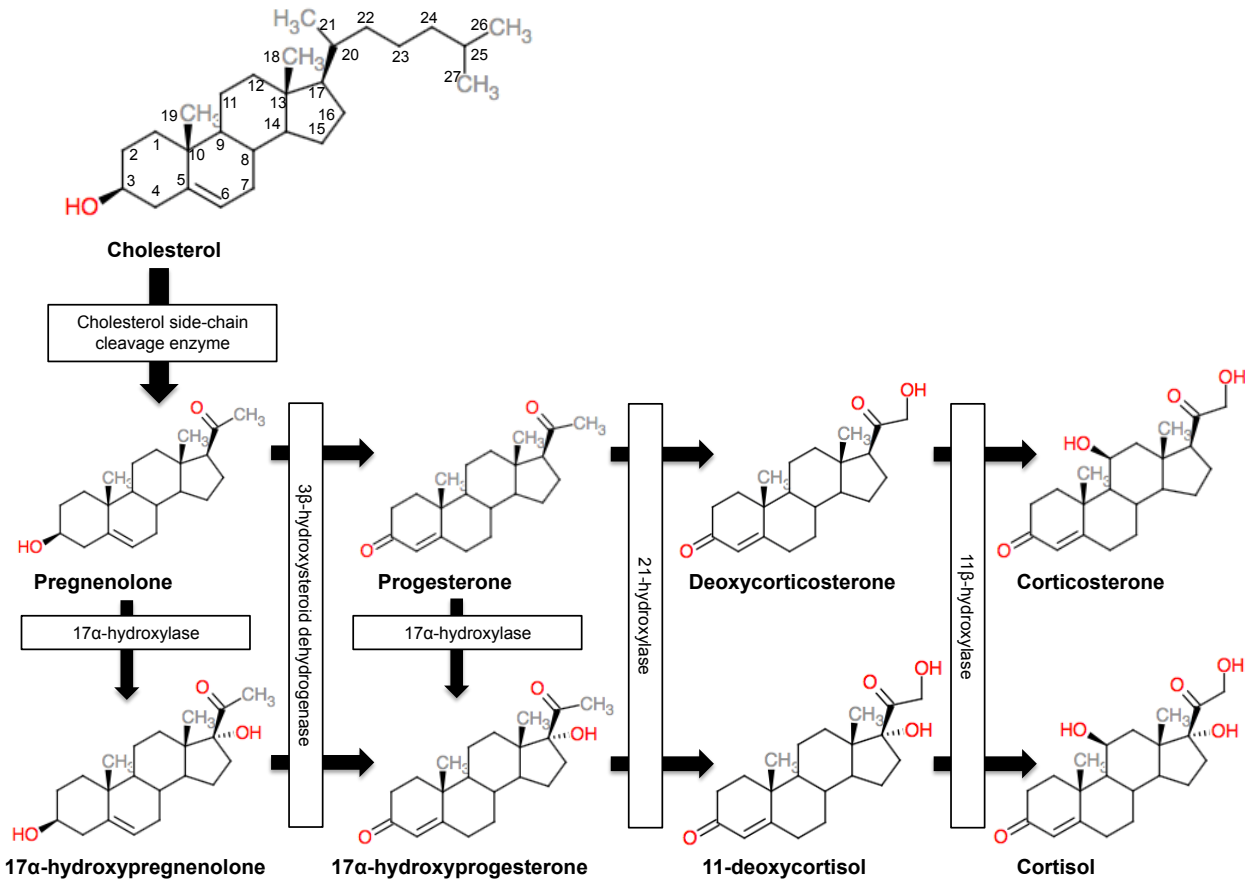


Figure 1.2 Simplified representation of the glucocorticoid steroidogenic pathway. The enzyme-mediated biosynthetic pathways that convert cholesterol to the glucocorticoids, corticosterone and cortisol, are shown. In the first step, the side chain of cholesterol is cleaved by the cholesterol side-chain cleavage enzyme (*CYP11A1*) to generate pregnenolone. The synthesis of cortisol and corticosterone follow parallel pathways, however, for cortisol production, pregnenolone is first converted to 17α-hydroxypregnenolone through the actions of the 17α-hydroxylase (*CYP17*) enzyme. Further steroid conversions are performed by 3β-hydroxysteroid dehydrogenase (*HSD3B1*), 21-hydroxylase (*CYP21*) and 11β-hydroxylase (*CYP11B1*), to yield cortisol or corticosterone. The basic cyclopentanoperhydrophenanthrene ring structure and carbon numbering system of steroid hormones is depicted on cholesterol for reference. Steroid names are in *bold* and enzyme names are *boxed*.

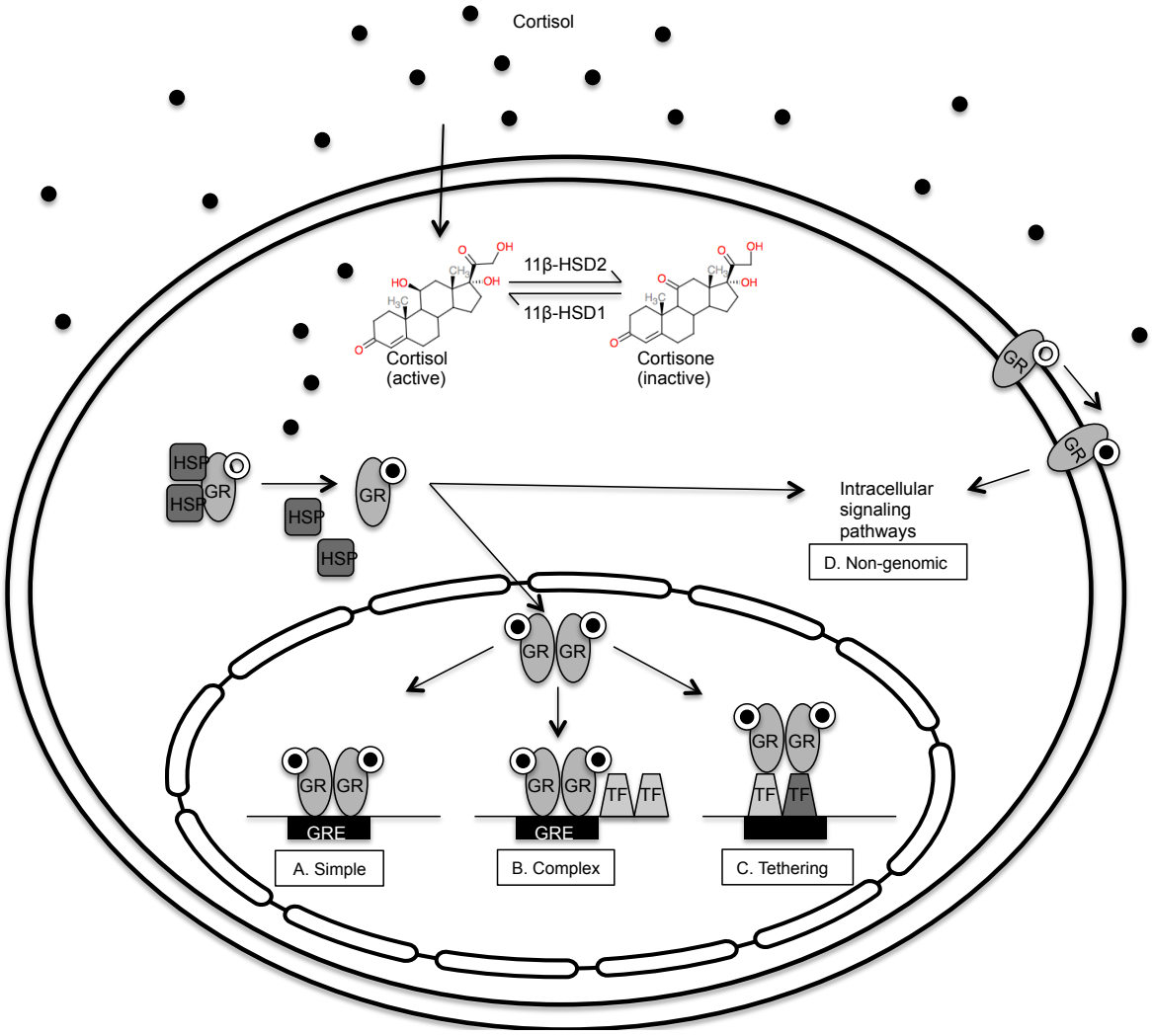

Cortisol
11β-HSD2
11β-HSD1
Cortisol
(active)
Cortisone
(inactive)
HSP
GR
Intracellular
signaling
pathways
D. Non-genomic
TF
GRE
A. Simple
B. Complex
C. Tethering

Figure 1.3 Mechanisms of glucocorticoid action. At target tissues, glucocorticoids are regulated through the action of 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes, of which 11β-HSD2 converts cortisol to the inactive metabolite cortisone, and 11β-HSD1 converts cortisone to cortisol. Classically, unbound glucocorticoids passively diffuse across the cell membrane where they bind with glucocorticoid receptors (GR) in the cytoplasm of target tissues. The binding of glucocorticoids to GR induces conformational changes that cause GR to be released from chaperone proteins, such as heat shock proteins (HSP). (A-C) GR then diffuses into the nucleus where it dimerizes and exerts its transcriptional effects by binding to glucocorticoid response elements (GREs), either through direct or indirect mechanisms. Direct binding of GR to a GRE is classified in two ways: (A) simple, where GR binds alone or (B) composite, in which GR cooperatively binds with additional DNA-bound transcription factors (TF). (C) In the absence of a GRE, GR exerts its effects by tethering to heterologous DNA-bound transcription factors on the gene of interest. (D) Glucocorticoids also mediate their actions through non-genomic mechanisms, whereby membrane bound or cytoplasmic GR activate intracellular signal transduction pathways.

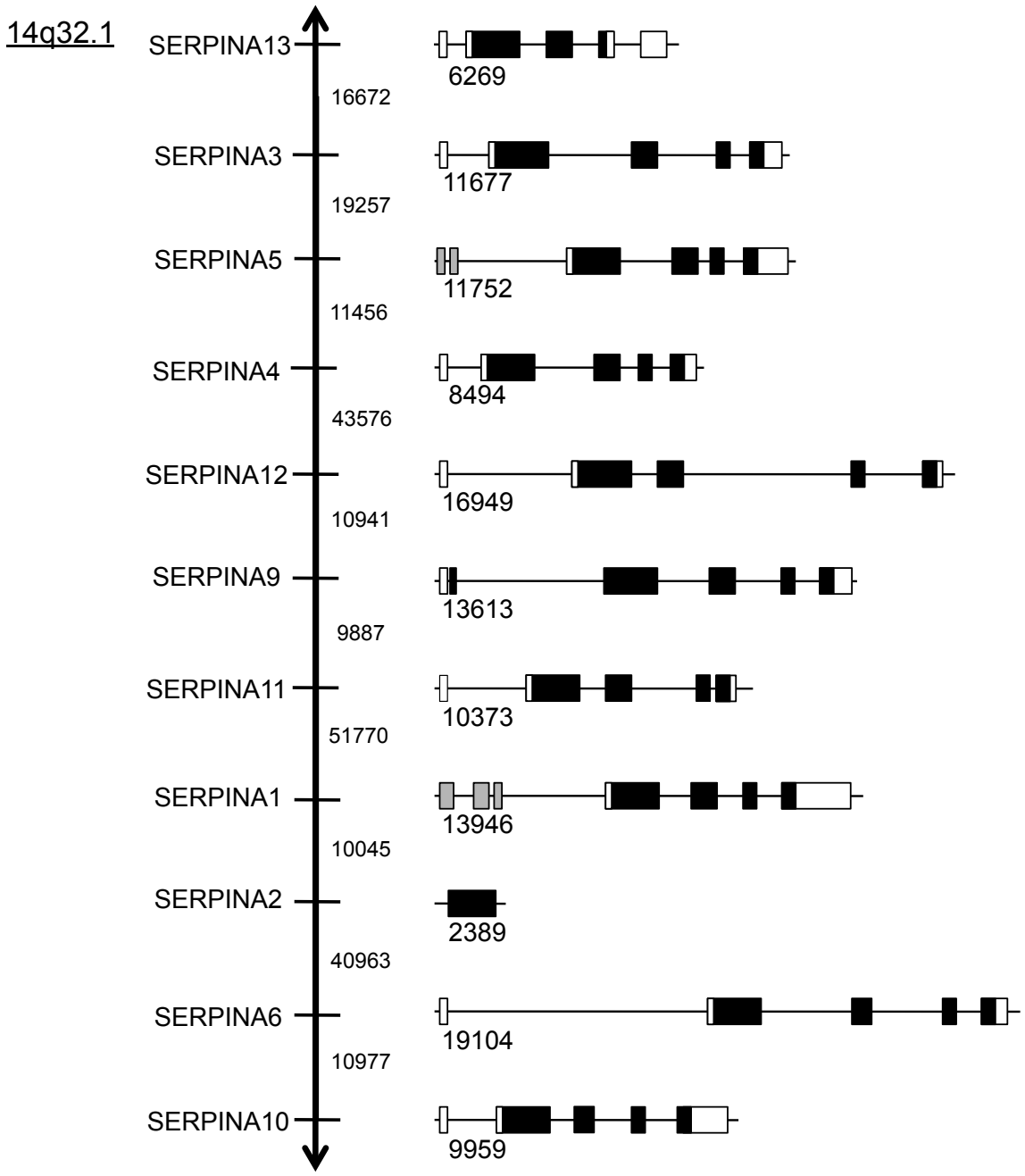
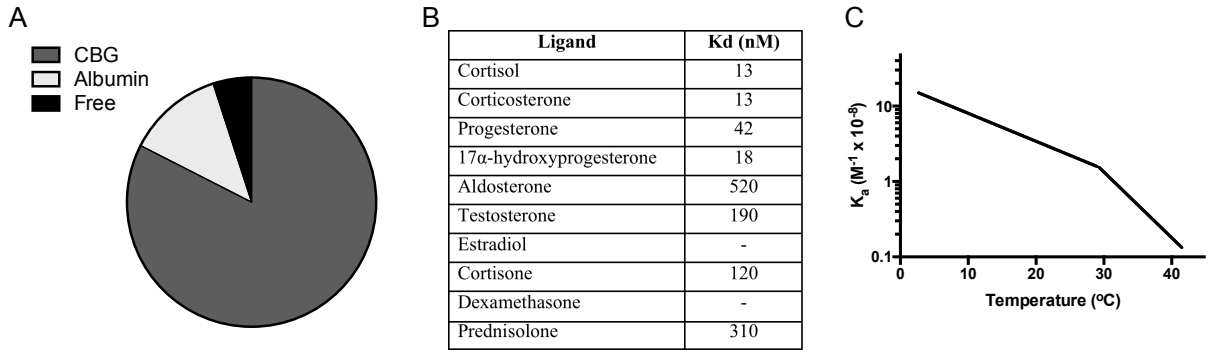

14q32.1
SERPINA13
6269
16672
SERPINA3
11677
19257
SERPINA5
11752
11456
SERPINA4
8494
43576
SERPINA12
16949
10941
SERPINA9
13613
9887
SERPINA11
10373
51770
SERPINA1
13946
10045
SERPINA2
2389
40963
SERPINA6
19104
10977
SERPINA10
9959

Figure 1.4 The chromosomal localization and gene structure of *SERPINA* members on Chromosome 14. As shown here, of the 13 human clade A SERPINs, 11 are found within a syntenic cluster on chromosome 14q32.1. The arrangement of each *SERPINA* gene along the chromosome is provided, with the number of base pairs separating neighbouring genes given between the hashmarks. With the exception of *SERPINA2* and *SERPINA13*, *SERPINA* members share a conserved gene structure, characterized by 5 exons, of which the first is non-coding and the last is partly non-coding. *SERPINA2* and *SERPINA13* are pseudo-genes. *SERPINA13* is distinguished from the other SERPINA members by a partially non-coding exon 4 and a completely non-coding exon 5. The number of base pairs comprising each *SERPINA* member is shown under each gene structure. It should also be noted that *SERPINA1* and *SERPINA5* contain alternative first exons, which are highlighted in *grey*.

Figure 1.5 Sequence alignment of human and rat CBGs. Secretion signal polypeptide residues are included and are numbered negatively with respect to residue 1 of the mature CBG protein, which is marked by *black boxes*. Important residues involved in steroid interactions and *N*-linked glycosylation are shown in *boldface type*; amino acids with side chains that interact with cortisol are highlighted in *grey*, and consensus sequences for *N*-linked glycosylation are *underlined*. Structural elements, such as helices and beta-sheets are also marked for reference.



Ligand	Kd (nM)
Cortisol	13
Corticosterone	13
Progesterone	42
17α-hydroxyprogesterone	18
Aldosterone	520
Testosterone	190
Estradiol	-
Cortisone	120
Dexamethasone	-
Prednisolone	310

Figure 1.6 Steroid-binding properties of human CBG. (A) Under normal conditions, CBG binds 80-90% of circulating cortisol, with the remainder either bound to albumin or freely circulating. (B) Common endogenous and exogenous steroid ligands of CBG and their binding affinity constants (dissociation constant, Kd), as measured at 37°C (adapted from (2, 3)). (C) Significant decreases in the CBG-cortisol binding affinity (association constant, Ka) are observed with increases in temperature (adapted from (105)).

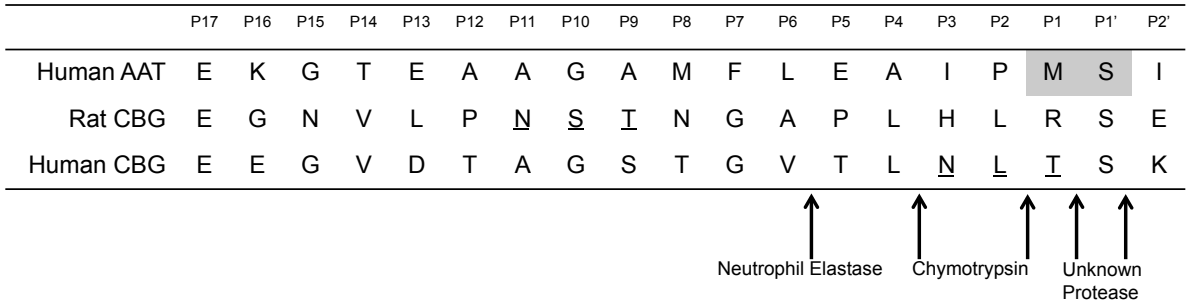


Figure 1.7 SERPINA1 and SERPINA6 reactive center loop (RCL) alignment and cleavage site identification. As seen here, considerable variability exists in the RCL amino acid sequence of SERPINs. Residues 333-351 of the human CBG RCL are shown and denoted P17-P2', with P1-P1' representing the relative position of the neutrophil elastase cleavage site of alpha-1-antitrypsin (AAT, *SERPINA1*), highlighted in *grey*. Notably, a consensus sequence for *N*-linked glycosylation (*underlined*) exists within the rat and human CBG RCLs.

Two endogenous proteases have been shown to cleave the human CBG RCL: neutrophil elastase cleaves between P6 (Val344) and P5 (Thr345) (9)and chymotrypsin cleaves after leucines present at P4 (Leu346) and P2 (Leu348) (120)In a recent human CBG crystal structure, the RCL was cleaved by an unknown protease after P1 (Thr349) or P1' (Ser350) (13).

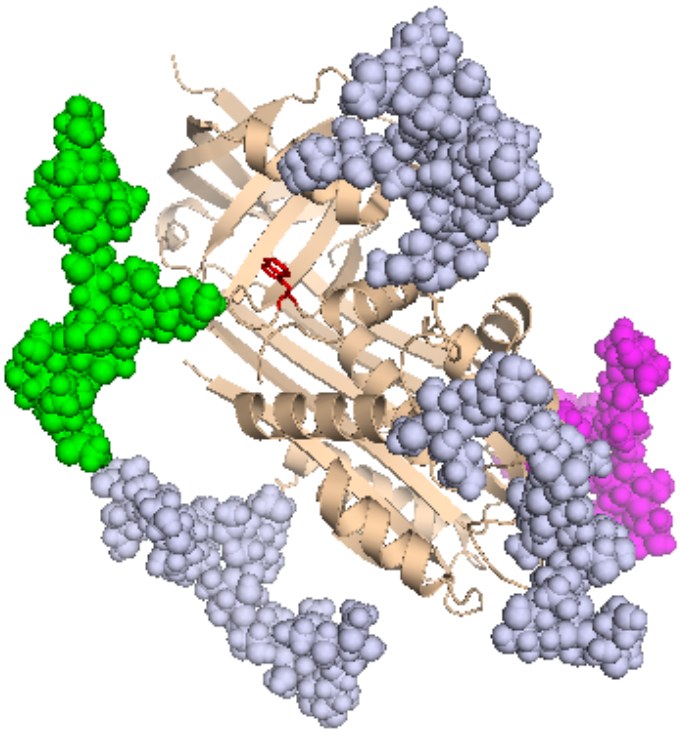


Figure 1.8 Structural model demonstrating the extent of *N*-linked glycosylation of human CBG in its relaxed (R) conformation. In the R conformation, the human CBG RCL has been cleaved and inserted into the core of the protein. The *N*-glycans shown are based on their reported most frequent compositions (133). The predicted *N*-glycan at Asn9 could not be added because this position is lacking in the crystal structure that the model is based on. The CBG protein is coloured *beige* and glycans are coloured *grey*, with the exception of Asn238 and Asn347, which are coloured *green* and *pink*, respectively. Glycolysation of Asn238 is required for the acquisition of a high affinity steroid-binding site (44) and Asn347 is located within the CBG RCL. To highlight the location of the steroid-binding site Trp373 that partakes in vital π-stacking interactions with cortisol is shown in *red*. In this representation, RCL-cleaved human CBG (PDB ID 4BB2) was utilized for *in silico* modeling of *N*-linked glycans, using the program GlyProt (http://glycosciences.de). Illustrations were created using the program PyMol (http://pymol.org).

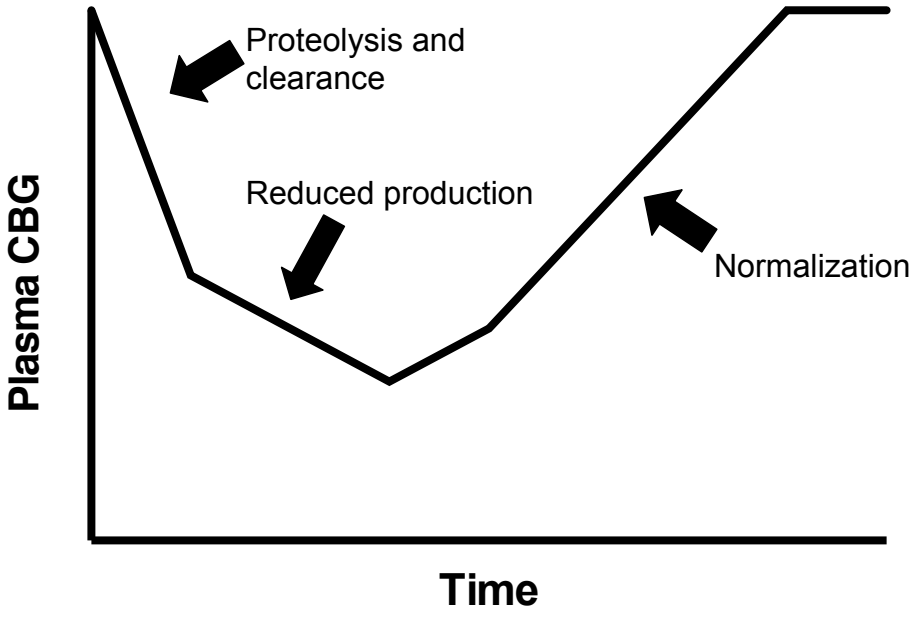


Figure 1.9 Plasma CBG levels over the course of inflammation. During inflammation, CBG acts an acute phase "negative" protein. Proteolysis of the CBG RCL appears to be an early event, rendering RCL-cleaved CBG non-functional (8, 9). This is followed by reductions in plasma CBG protein production, likely as a result of increased glucocorticoid and cytokine levels, which have been shown to repress CBG production (23, 56, 77). Such decreases in CBG-steroid binding affinity and plasma CBG production lead to increases in the amount of free or bioavailable anti-inflammatory glucocorticoids available to act at sites of inflammation. At some point during inflammation, plasma CBG levels begin to normalize, and this is expected to be important during recovery, in determining the ability of glucocorticoids to act and restore the normal homeostatic balance.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Reagents

Labeled steroids, [^{3}H]-cortisol and [^{3}H]-corticosterone, were purchased from PerkinElmer (Waltham, MA) and unlabelled steroids were obtained from Steraloids (Newport, RI, USA). Restriction enzymes (*Xho*I and *Hpy*188I) were purchased from New England Biolabs (Whitby, ON).

Neutrophil elastase and *Pseudomonas aeruginosa* elastase were obtained from Elastin products Co., Inc (Owensville, Missouri USA). Neutrophil elastase was reconstituted in 0.05 M NaAc, pH 5.0, 0.1 M NaCl, and LasB was reconstituted in 50 mM Tris-HCl, pH 7.5, 10 mM CaCl2. Bovine α-chymotrypsin (type II from pancreas) and human α-chymotrypsin (from pancreas) were reconstituted in 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl at 1 μg/μl and 0.2 μg/μl, respectively (Sigma Aldrich). Protease inhibitors, including phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), and N-α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), were from Sigma Aldrich.

2.1.2 Mammalian cells

Chinese hamster ovary (CHO) cells were utilized for the production of recombinant human CBG proteins. Cells were maintained in α-minimum essential medium, containing 10% fetal bovine serum and 1% penicillin-streptomyocin, at 37°C in the presence of oxygen and 5% CO2. Cell culture reagents, including Lipofectamine 2000®, were purchased from Life Technologies (Burlington, ON).

2.2 Animal experiments

2.2.1 Animals and housing

For experiments in **chapter 5.2**, adult female Sprague Dawley rats were obtained from Charles River Laboratories International, Inc. (St. Constant, QC, Canada) and Harlan Laboratories, Inc. (Hsd: Sprague Dawley SD, Frederick, MD) (n = 29/vendor). Female Sprague Dawley rats (n=24, 51-52 days old) were received from Charles River Laboratories International, Inc. (St. Constant, QC, Canada) for experiments in **chapter 5.3**.

At The University of British Columbia, rats were maintained at the Center for Disease Modeling or the Modified Barrier Facility on a 12:12 hour light/dark cycle, with controlled temperature (21-22°C) and *ad libitum* access to standard laboratory chow (Purina Laboratory Rodent Diet #5001, Delta, BC, Canada) and water.

In addition, throughout all adjuvant-induced arthritis experiments (**chapter 5**), rats were housed with CareFRESH® (Healthy Pet, Ferndale, WA) bedding to minimize discomfort, and monitored for signs of pain, discomfort or infection, and for general signs of health including activity, coat quality, and ability to rear.

All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the University of British Columbia Animal Care Committee.

2.2.2 Adjuvant-induced inflammation model

2.2.2.1 Treatment

On postnatal day 55-60, rats were anesthetized with isoflurane, and each received two 0.05 mL intradermal injections at the base of the tail. Rats were injected with complete Freund's adjuvant or physiological saline. Complete Freund's adjuvant (CFA) was prepared by grinding

Mycobacterium tuberculosis H37 RA (Difco laboratory, Detroit, MI) and dissolving the powder in incomplete Freund's adjuvant (201).

For experiments in **chapter 5.2**, two doses of CFA were prepared initially: 12 mg/mL and 3 mg/mL, resulting in a dose of 1.2 or 0.3 mg/rat. These doses were chosen with the aim of identifying a dose that results in mild-moderate arthritis in ~50% of rats. At 0.3 mg, none of the Charles River rats showed signs of inflammation on day 15 post-injection; however, ~40% of Harlan rats displayed signs of severe inflammation. As a result, two CFA doses were added, 0.6 mg and 0.2 mg/rat, designed to induce mild-moderate inflammation in Charles River and Harlan rats, respectively. Following injections, rats were single housed, weighed and paw volume was measured using a plethysmometer (IITC Life Science Inc., Woodlan Hills, CA).

In the adjuvant time course study (**chapter 5.3**), rats were injected with 0.6 mg CFA (n=20), prepared as above, or with physiological saline (control; n=4), three days after baseline blood sampling.

2.2.2.2 Clinical scoring

To calculate clinical scores, each of the four paws was given a score of 0-4, where 0 = no signs of inflammation, 1 = single focus of redness or swelling, 2 = two or more foci of redness or swelling, 3 = confluent but not global swelling, 4 = severe global swelling (201). Rats achieving an overall clinical score ≥ 8 (out of a possible 16) at any point during the study were classified as developing severe inflammation, whereas rats with a clinical score < 8 were classified as developing mild-moderate inflammation.

2.2.2.3 Blood sampling

Following an acclimatization period of 5 days, pre-treatment (baseline) blood samples were taken from the tail vein, under light isofluorane anesthesia, between 11 am and 1 pm for the preparation of serum, which was stored at -80°C until analyzed.

In the time course study (**chapter 5.3**), post-treatment, rats were split into two groups (n=12 per group; 2 controls and 10 adjuvant-treated rats). Blood samples (~50 μL) from the two groups were alternatively collected between 11 am and 1 pm under light isoflurane anesthesia from the tail vein over a 16-day experimental period. Serum was prepared and stored at -80°C until assayed. At the time of blood sampling, rats were weighed and clinical signs of inflammation were scored and recorded.

2.2.2.4 Termination and tissue collection

On day 16 post-injection, rats were decapitated between 08:00 and 10:30 hr and trunk blood was collected in tubes containing EDTA. In the time course study, one animal with severe inflammation was sacrificed on experimental day 14 for humane reasons. Blood was centrifuged and plasma was collected and stored at -80°C. In addition, livers and spleens were removed, flash frozen and stored at -80°C. In the initial adjuvant-induced arthritis study (**chapter 5.2**), vaginal lavage samples were collected and assessed cytologically to determine estrous cycle stage.

2.3 Clinical evaluations

Clinical evaluations were undertaken by Dimitra Vas and colleagues at Attikon University Hospital and Evangelismos District General Hospital in Athens, Greece. Serum and DNA samples were obtained from those institutions with informed consent.

2.4 DNA sequencing and restriction enzyme analysis

2.4.1 DNA isolation, purification and PCR sequencing

Genomic DNA was isolated from human and rat blood lymphocytes using the QIAamp DNA Mini Kit (QIAGEN Sciences, Valencia, CA). PCR amplifications of the human *SERPINA6* promoter and exon sequences were performed using Platinum Pfx polymerase (Invitrogen Canada, Burlington, ON, Canada) and specific oligonucleotide primer pairs (Table 2.1), as described (143). Similar procedures were utilized for PCR amplification of the rat *Serpina6* proximal promoter and exons; rat specific oligonucleotide primers are given in Table 2.2.

The PCR products were purified using MinElute PCR purification or MinElute gel extraction kits (QIAGEN) and were sent to the NAPS unit (University of British Columbia, Vancouver, BC, Canada) for sequencing. Results were analyzed using 4Peaks software (version 1.7.2, mekentosj.com).

2.4.2 Restriction enzyme analysis

To conveniently detect non-synonymous SNP encoding human CBG variants A224S and W371S, PCR amplification was followed by restriction enzyme digestion. The restriction fragments were detected by electrophoresis in 6-8% polyacrylamide gels stained with ethidium bromide, as indicated in Table 2.3.

2.5 Production and purification of recombinant human CBG proteins

2.5.1 Database screening for non-synonymous *SERPINA6* SNP

Human *SERPINA6* SNPs were identified in public databases, i.e. the National Center for Biotechnology Information (NCBI) dbSNP (202), the NIH Heart, Lung and Blood Institute (NHLBI) Exome Sequencing Project – Exome Variant Server (203), and the 1000 Genomes

Project (204). In **chapter 3**, we analyzed CBG variants encoded by 32 uncharacterized non-synonymous SNPs in the 2012 releases of these databases (Table 2.4).

2.5.2 Site-directed mutagenesis and plasmid purification

Wild-type (WT) human CBG cDNA, encoding a Ser at position 224, in the pRc/CMV expression vector was used as a template for site-directed mutagenesis with specific primers (Table 2.4) using the QuikChangeII kit (Agilent Technologies), according to the manufacturer's instructions. The expression vector encoding CBG D367N was constructed previously (16). The mutated cDNAs were sequenced to confirm that only targeted mutations had occurred.

2.5.3 Expression of recombinant human CBG in CHO Cells

To establish WT and variant human CBG producing CHO cell lines, 2 μg of expression vectors were transfected into CHO cells using Lipofectamine 2000®, as described (115). Stably transfected CHO cells were grown to 90% confluence in 150 mm plates, washed with phosphate-buffered saline (PBS) and cultured for five days in 25 ml of SMF4CHO-Utility media (Thermo Scientific) containing 100 nM cortisol and 1% penicillin-streptomycin.

2.5.4 Purification of recombinant human CBG proteins

Culture medium was harvested, centrifuged to remove debris, and filtered using 0.22 μm filters (Millipore). Samples were then either concentrated using Amicon Ultra 3K centrifugal filters (Millipore) and buffer exchanged with 10 volumes of PBS, or dialyzed using SnakeSkin 10 K dialysis tubing (Thermo Scientific), overnight at 4°C in 20 mM Tris, pH 8.0. Dialyzed samples were then semi-purified by fast protein liquid chromatography (FPLC) using a Mini Q or HiTrap Q FF column on an AKTA Explorer chromatography system (GE Healthcare). Concentrated media and FPLC-purified preparations of recombinant human CBG were stored at 4°C in the presence of 100 nM cortisol and 0.02% sodium azide.

2.6 CBG binding assays

2.6.1 CBG steroid-binding capacity assay

The steroid-binding capacity of CBG was measured using an established radioligand-saturation assay (205), in which dextran-coated charcoal (DCC) is used to separate unbound from CBG-bound radioligands - [^{3}H]-cortisol (human CBG) or [^{3}H]-corticosterone (rat CBG). Briefly, samples were diluted in PBS and stripped of endogenous steroids by incubation with DCC for 30 min at room temperature, followed by centrifugation. To monitor non-specific binding, samples were incubated with labeled ligand in the absence or presence of excess unlabeled cortisol or corticosterone. After separation of free [^{3}H]-cortisol or [^{3}H]-corticosterone by adsorption with DCC for 10 min and centrifugation at 0°C, CBG-bound labeled ligand in the supernatants was determined in a scintillation spectrophotometer.

2.6.2 Apparent dissociation rate (off-rate)

Rat serum samples were saturated with [^{3}H]-corticosterone, as described above (**chapter 2.6.1**), and the apparent dissociation rate of CBG-bound steroid was assessed after incubation with DCC for various lengths of time (0-18 min) at 0°C (205). Dissociation rates at 10 min of DCC exposure were used in rat and mouse CBG-corticosterone binding capacity calculations (**chapter 2.6.1**).

2.6.3 Scatchard analysis

For Scatchard and equilibrium dialysis assays (**chapter 2.6.4**) of recombinant human CBG proteins, CBG concentrations were first established by ELISAs using the 12G2 antibody (see **chapter 2.8**) and approximately equal amounts of CBG were used based on their ELISA immunoreactivity. However, for two variants (CBG L93H, CBG H14R), 2-3 fold higher amounts were used for Scatchard analyses to obtain more accurate affinity constants.

The dissociation-rate constant (Kd), was determined by Scatchard analysis at 4°C, and this involved incubating samples with increasing amounts of [^{3}H]-cortisol (human samples) or [^{3}H]-corticosterone (mouse or rat samples), as previously described (56, 104).

2.6.4 Equilibrium dialysis

Equilibrium dialysis was used to further assess the cortisol-binding activity of recombinant human CBG (101) and CBG in human serum samples. In this assay, 150 µl recombinant CBG (20 nM) or human serum (diluted 1:10 in PBS) were dialyzed for 48 h against PBS containing $1.5x10^5$ cpm [^{3}H]-cortisol per mL. After incubation for 30 min at 37ºC, [^{3}H]-cortisol was measured in the dialysis buffer and sample chamber, with the difference between the two representing the amount of cortisol bound to CBG.

2.7 Proteolytic cleavage assays

2.7.1 Characterization of *Pseudomonas aeruginosa* as a protease of CBG

The following bacteria: *Pseudomonas aeruginosa* (PAO1), *Staphylococcus aureus* (RN4220), *Burkholderia cenocepacia* (ATCC 25416), *Micrococcus luteus* (Davies' lab collection), *Enterococcus faecalis* (OG1RF), *Escherichia coli* (K12 MG1655), *Acinetobacter baumannii* (ATCC 19606), and *Mycobacterium smegmatis* (MC2 155); were obtained to test the ability of secreted proteins to disrupt the cortisol-binding capacity of human CBG. All bacterial cultures were obtained from Dr. Julian Davies (UBC), were considered to have reached a stationary phase of growth when harvested, and the supernatants were filter-sterilized after removal of the bacteria by centrifugation. In the initial screening, 90 μl of culture medium from each organism, or an equal volume of Dulbecco's phosphate-buffered saline (DPBS), was incubated with 10 μl of diluted (1:10) human serum for 16 hours at 37°C prior to CBG-cortisol binding capacity assays (see **chapter 2.6.1**).

Time-course experiments using *P. aeruginosa*-conditioned medium were completed to further characterize the ability of this protease to cleave CBG. Reactions containing 1 μl of human serum were incubated in the presence or absence of 1 μl *P. aeruginosa*-conditioned medium in a volume of 100 μl with DPBS (pH 7.4) for 5 min, 15 min, 1 h, 4 h, 8 h, and 16 h at 37°C or 41°C. Reactions were stopped by addition of 5 mM EDTA and stored briefly at 4°C until analysis of CBG-cortisol binding capacity (see **chapter 2.6.1**).

To test temperature and pH effects, human serum and *P. aeruginosa* media were incubated as above with DPBS (pH 6, 7, or 8) for 8 h at 37°C, 39°C, or 41°C. Human serum (1 μl) was also incubated in the presence or absence of *P. aeruginosa*-conditioned medium (1 μl), in the presence or absence of the following protease inhibitors: 5 mM EDTA, 1 mM PMSF, or 50 μM TPEN in a volume of 100 μl with DPBS for 16 h at 37°C.

Protease IV is a protease secreted by *P. aeruginosa* that is irreversibly inhibited by TLCK (206). To determine if Protease IV was able to proteolytically cleave human CBG we pre-incubated 0.05 mM TLCK with 1 μl of *P. aeruginosa*- medium in a volume of 100 μl in DPBS, or with DPBS alone for 1 h at 37°C. The reactions were applied to 0.5 ml 10K Amicon Ultra Centrifugal filter spin columns (Millipore) and washed with 10 volumes of DPBS, in order to remove excess TLCK because it has an inhibitory effect on CBG-cortisol binding. *P. aeruginosa* medium was filtered and washed, as above, as the reference. Retentates were adjusted to 50 μl with DPBS and incubated with 1 μl human serum in 50 μl of DPBS for 16 h at 37°C prior to steroid-binding capacity assays (see **chapter 2.6.1**).

To determine if *P. aeruginosa* elastase (PAE or LasB), another protease secreted by *P. aeruginosa*, was able to disrupt CBG-cortisol binding, we examined the proteolytic activity of PAE in a time-course experiment where human serum (1 µl) was incubated with 1 µg of purified

PAE for 4, 8 and 16 hours at 37°C prior to a cortisol-binding capacity assay (see **chapter 2.6.1**). In addition, PAE-deficient *P. aeruginosa* strains (PW7302 and PW7303, from the PA two-allele library) were prepared (207) and tested for their ability to alter the CBG-cortisol binding capacity.

2.7.2 Cleavage assays with recombinant CBG proteins

Neutrophil elastase (0.2 μg) was added to FPLC-purified recombinant CBG samples in 100 μl PBS. The mixtures were incubated for 5 min at 37°C, and the reactions were stopped by the addition of 3 μl of 100 mM PMSF. Reactions of recombinant CBG samples with bovine (2 μg) or human (0.4 μg) chymotrypsin were prepared in 100 μl Tris-HCl (pH 7.5) buffer containing 0.5 M NaCl. CBG-chymotrypsin reactions were incubated for 20 min at 37°C and stopped by the addition of 10 mM PMSF. *P. aeruginosa* reactions were prepared by adding 20 μl *P. aeruginosa* medium to recombinant CBG samples in 100 μl PBS, followed by incubation for 16 h at 37°C. CBG-*P. aeruginosa* reactions were stopped by the addition of 5 mM EDTA.

Time-course experiments were also performed to determine the kinetics of CBG-cortisol binding capacity loss for CBG variants with higher residual cortisol-binding activity when compared to CBG WT in the initial screening assays.

2.7.3 Cleavage assays with human serum samples from ICU patients

To test the ability of *P. aeruginosa*-conditioned media to cleave plasma CBG of ICU patients, reactions containing1 μl of human serum were incubated in the presence or absence of 1 μl *P. aeruginosa*-conditioned medium in a volume of 100 μl with DPBS (pH 7.4) for 16 h at 37°C. Reactions were stopped by addition of 5 mM EDTA and stored briefly at 4°C until analysis of CBG-cortisol binding capacity (see **chapter 2.6.1**).

To determine which chromatographic fractions (**chapter 2.11**) could inhibit CBG cleavage by *P. aeruginosa* conditioned media, reactions containing 2 μl of human serum were incubated with 48 μl of each fraction in the presence or absence of 50 μl of *P. aeruginosa* conditioned media in a total volume of 100 μl with 1x PBS, overnight at 37°C. Samples were then immediately analyzed for their CBG-cortisol binding capacity (see **chapter 2.6.1**).

Based on size exclusion chromatographic data, it was suspected that alpha-2-macroglobulin (α2M) was responsible for the inhibition of *P. aeruginosa* mediated cleavage of CBG. To test this, 0.7-11.25 μg of purified α2M (Sigma) were incubated with 2 μL of human serum in the presence or absence of 50 μL of *P. aeruginosa* conditioned media in a total volume of 100 μl with 1x PBS. Reactions were incubated overnight at 37°C and immediately analyzed for their CBG-cortisol binding capacity (see **chapter 2.6.1**). An optimal A2M concentration was then utilized to determine the capability of α2M to inhibit the cleavage of plasma CBG by *P.aeruginosa* conditioned media and purified PAE. Reactions containing 2 μL of human serum, 3.32 μL of ZnCl and 50 μL of *P. aeruginosa* conditioned media or 10 μL of purified PAE were incubated in the presence or absence of 2.8 μg of purified α2M in a volume of 100 μl with 1x PBS, 16 hr at 37°C. A control sample containing 2 μL of human serum and 3.32 μL of ZnCl in a volume of 100 μl with 1x PBS was also incubated overnight at 37°C. Reactions were then analyzed for their CBG-cortisol binding capacity (see **chapter 2.6.1**).

2.8 CBG ELISAs

Concentrations of human CBG were measured by ELISAs. These ELISAs are specific for human CBG and utilize a polyclonal immobilization antiserum and monoclonal antibodies that specifically recognize different epitopes within the CBG structure, including an undefined epitope (clone 12G2) and an epitope located in the RCL region (clone 9G12) (143, 208, 209).

2.9 Western blotting of CBG proteins

Western blots were performed to semi-quantify recombinant CBG in media and cell extracts, and to assess the integrity and molecular size of CBG in these samples. In addition, western blots were used to assess the integrity of CBG in human and rat serum or plasma. It has previously been shown that when human neutrophil elastase cleaves the CBG RCL, its apparent molecular size is reduced by approximately 5 kDa, and this size difference can be resolved by SDS-PAGE (115) and non-denaturing (native) PAGE (13). In addition, when unheated samples are subjected to native PAGE, cleaved human CBG yields a thermostable protein that is less prone to aggregation than uncleaved CBG (13).

Prior to western blot analyses, recombinant human CBG expressing-CHO cell pellets were homogenized in ice-cold buffer containing 50 mM Tris, 300 mM NaCl, 10% glycerol, 0.1% NP-40, and 0.1 mM PMSF. When required, protein concentrations were determined by Bradford assay (Bio-Rad).

2.9.1 SDS-PAGE

Prior to SDS-PAGE analyses, samples were denatured and reduced by boiling at 95°C, for a minimum of 10 min, in the presence of β-mercaptoethanol and 1% SDS. SDS-PAGE was used to assess recombinant human CBG in semi-purified media (10 µl) and cell protein extracts (25 µg), as well as CBG in diluted (1:200) rat serum or plasma. For Western blot analysis of protease digests, approximately 50 ng of purified CBG was incubated with 5 μl of *P. aeruginosa medium* or 20 μl of active chromatographic fractions (see **chapter 2.11**) for 16 h at 37°C, or with 300 ng of neutrophil elastase for 10 min at room temperature, in a total volume of 50 μl using 20 mM Tris buffer. Each reaction (10 μl) was then used in SDS-PAGE and proteins were resolved with the use of 5% stacking and 10% resolving gels.

2.9.2 Non-denaturing PAGE

Non-denaturing PAGE (7.5%) was used to assess CBG protein integrity (i.e. cleaved vs. uncleaved) in diluted (1:150) human plasma. Prior to native PAGE analyses, diluted samples were either gradually heated in a thermocycler (13) or remained unheated. To gradually heat samples, the temperature was increased by 0.1°C/s until a final temperature of 70°C was reached, after which the temperature was held at 70°C for 3 min before cooling to 4°C. All native PAGE was performed at 4°C.

2.9.3 Gel transfer and detection

After SDS or native PAGE, proteins were transferred to PVDF membranes which were blocked with PBS buffer containing 5% milk and 0.1% Tween 20. Human samples (recombinant CBG or serum) were incubated with a polyclonal rabbit anti-human CBG antiserum (130) (1:5,000) and a horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Sigma-Aldrich) (1:10,000). Rat plasma or serum samples were incubated overnight at 4°C with polyclonal rabbit anti-mouse CBG antiserum (1:4,000) (22) in 5% milk-PBST, followed by a horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Sigma-Aldrich) for 1 hr at room temperature.

Immunoreactive CBG was detected using the ECL Prime chemiluminescent reagent (GE Healthcare) and an ImageQuant LAS4000 apparatus (GE Healthcare) or CL-XPosure x-ray films (Thermo Scientific).

2.10 *Serpina6* qRT-PCR

2.10.1 RNA isolation and cDNA synthesis

Total RNA from CHO cells expressing WT and variant human CBGs was extracted with Trizol reagent. Reverse transcription was performed after DNAse I treatment using Superscript II and random hexamer primers (Invitrogen).

Trizol reagent was also used to extract total RNA from rat liver samples, after which the RNA was purified using an RNeasy kit (Qiagen), as per the manufacturer's instructions. RNA integrity was assessed using an absorbance ratio (A_{260}/A_{280}) to ensure sample purity. To obtain cDNA, 1 μg of RNA was reversed transcribed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems).

2.10.2 qRT-PCR

For qRT-PCR of cDNA extracted from CHO cells, reactions were prepared with Power SYBR green master mix (Applied Biosystems) and oligonucleotide primers for human CBG (forward: 5´-CAGTCATCGCTGCACTGAGCC; reverse: 5´-GCGTCCTGGGTGATGCGTGAG) and GAPDH (forward: 5´-TCGCC GAGTATGTTGTGGAATCTACTG; reverse : 5´-TGGTGGTGCAGGACGCATTG), as the housekeeping gene.

Real time quantitative PCR was completed on cDNA obtained from rat liver samples using a pre-validated rat *Serpina6* PrimeTime Std qPCR Assay (Integrated DNA Technologies (IDT): Assay ID# Rn.PT.58.02619945) and 5 ng of cDNA per reaction. Hepatic rat *Serpina6* mRNA levels were normalized to those of rat *Gapdh* (IDT: Assay ID# Rn.PT.58.35727291).

Quantitative PCR measurements were performed on an Applied Biosystems 7500 Fast Real-Time PCR System. Samples were run in duplicate and relative CBG mRNA abundance was obtained using the delta-delta Ct method.

2.11 Purification and identification of *P. aeruginosa* proteases that disrupts CBG-cortisol binding and proteolytic cleavage sites within the RCL of CBG.

To purify the protein(s) secreted by *P. aeruginosa* that disrupt the cortisol-binding activity of CBG, dialyzed *P. aeruginosa* media was subjected to FPLC using an ion exchange

column (HiTrap Q FF, GE Healthcare), followed by a size exclusion column (Superdex75, GE Healthcare). Active chromatographic fractions, i.e. fractions that disrupted CBG-cortisol binding activity, were separated by SDS-PAGE and protein bands were excised and analyzed using tandom matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to obtain peptide maps and peptide fragmentation for protein identification (210).

After the incubation of purified human CBG with the chromatographic fraction of *P.aeruginosa* that had the greatest cortisol-disrupting activity (as described in **chapter 2.7.1**), the proteolytic fragments of CBG were analyzed on a Voyager Biospectrometry Workstation (Applied Biosystems), in order to identify the proteolytic cleavage site(s) of CBG (211, 212).

2.12 Purification of ICU patient plasma to identify the protein responsible for inhibiting *P. aeruginosa* mediated CBG cleavage

To determine what plasma component was responsible for inhibiting *P. aeruginosa* induced cleavage of plasma CBG in ICU samples, an ICU plasma sample was subjected to FPLC using anion exchange columns (HiTrap Q FF and Mini Q, GE Healthcare), followed by a size exclusion column (Superdex75, GE Healthcare). First, diluted (1:10) ICU plasma was applied to a HiTrap Q FF anion exchange column and 1 mL fractions were collected. Active chromatographic fractions, i.e. fractions that inhibited the disruption of CBG-cortisol binding activity by *P. aeruginosa* conditioned media, were then combined and loaded onto a Mini Q anion exchange column. Active Mini Q chromatographic fractions were then concentrated to 500 µL using a 3K Amicon Ultra Centrifugal filter spin column (Millipore) and applied to a Superdex 200 gel filtration column.

2.13 HACA-Sepharose separation

To separate and characterize intact CBG and CBG that had undergone proteolysis in rats with severe inflammation, plasma samples were treated with DCC to remove endogenous steroids and applied to an 11β-hydroxy-andros-4-en-3-oxo-17β-carboxylic acid (HACA)-Sepharose affinity column (130). After washing the column with 100 mM Tris-NaCl, steroid-bound CBG was eluted with 200 μM cortisol prepared in the same buffer. The flow-through, wash and eluent fractions were analyzed by corticosterone-binding capacity assays (**chapter 2.6.1**) and western blotting (**chapter 2.9**), as described.

2.14 Measurement of cytokines

In **chapter 5.3**, plasma cytokines (IL-4, IL-5, IL-6, IL-10, IL-13, IFN-ɣ, and TNF-α) were measured in samples collected at termination (stored at -80°C until analyzed) using the Proinflammatory Panel 2 (rat) V-PLEX kit (MSD, catalog #: K15059D-1). The cytokine assays were performed according to the established MSD protocol. The assay plate was read using a MESO QuickPlex SQ 120 and data were analyzed using the MSD Discovery Workbench software v. 4.0. Lower limits of detection for cytokines (pg/mL) were as follows: IL-4 (0.16), IL-5 (6.89), IL-6 (7.18), IL-10 (6.18), IL-13 (0.45), TNF-α (1.04), IFN-ɣ (1.48).

2.15 Measurement of corticosterone

2.15.1 Commercial assays

In human studies (**chapter 3.4**), total cortisol was measured with a commercial radioimmunoassay (MP Biomedicals, UK). Details of assay specificity and sensitivity can be found at: http://www.radmed.com.tr/usr_img/urunler/Cortisol-07221102.pdf. Free cortisol was measured by ELISA following equilibrium dialysis. Briefly, dialysis tubing (12–14 kD, Medicell, London, UK) was heated to 80°C for 30 min in 2% sodium bicarbonate and 1 mM

EDTA before overnight dialysis of plasma into phosphate buffered saline containing 1% gelatin at 37°C. The quantity of dialyzed free cortisol was then measured by a commercial ELISA (Salimetrics Europe Ltd, Newmarket, UK).

In rodent studies (**chapter 5.2**), total corticosterone was measured in rat plasma using the ImmuChem Double Antibody Corticosterone ^{125}I radioimmunoassay kit (MP Biomedicals, LLC, Orangeburg, NY, USA), respectively, as per the manufacturers instructions. The minimum detectable concentration for corticosterone was 7.7 ng/mL, with intra- and inter-assay coefficients of variation of <10 %.

2.15.2 Free corticosterone calculations

In humans (**chapter 3.4**), free cortisol was calculated from measured total plasma cortisol and CBG levels using the Coolen's equation (213).

In rat studies (**chapter 5.2**), levels of CBG-bound corticosterone were calculated using the mass action equation, according to Boksa (214), using a Kd of 45 nM, as determined for rat CBG at 37°C (215). The CBG-bound corticosterone levels were then used to calculate the free corticosterone levels using the following equation: free corticosterone = [total corticosterone] – [CBG-bound corticosterone].

2.16 Statistics

In **chapter 3.3**, two-way analyses of variance (ANOVAs) were performed to evaluate the effect of time and sample in protease incubation time-course experiments completed in.

In **chapter 4.3**, the assumption that the data was normally (Gaussian) distributed was analyzed using the Kolmogorov-Smirnov normality test. Following the confirmation of a normal distribution, data were analyzed using a one-way ANOVA and Tukey's or Dunnett's *post hoc* tests to examine significant effects.

In **chapter 5.2**, data were analyzed using ANOVAs for the factors of colony and inflammation severity, or colony and dose, as appropriate, with repeated measures as required, followed by Fisher *post hoc* tests to examine significant main effects and interactions. The assumption of sphericity was examined using Mauchly's test and when violated, F-values were corrected using Greenhouse-Geisser estimates of sphericity. In line with our hypotheses that Charles River and Harlan rats would differ in severity of inflammation and in hormone and cytokine responses, planned pairwise comparisons were carried out as indicated.

Data from **chapter 5.3** were analyzed using t-tests or ANOVA, with repeated measures as required, followed by Fisher or Tukey's *post hoc* tests to examine significant main effects.

Statistical analyses were performed using using GraphPad Prism 6 software (GraphPad Software, Inc.). For all studies, differences were considered significant at $p \leq 0.05$, and trends ($0.06 > P > 0.05$) were examined, as appropriate. Data are expressed as mean ± SD or SEM, as indicated.

Table 2.1 Oligonucleotide primers used for human *SERPINA6* DNA sequencing

Sequence	PCR and Sequencing Primers	PCR Product
Promoter and Exon 1	Forward: 5'-GAATGGCCTTGGATTTGAATC Reverse: 5'-CTGTGGATGACTCACAGACTC	822 bp
Exon 2	Forward: 5'-CCCATTGACTCAGAGACTGC Reverse: 5'-GTGGATGGGCCTTCAGATGG	721 bp
Exon 3	Forward: 5'-CTGCCCTACAGACCTGTCTC Reverse: 5'-TGAACCTCACAGGGAAAGGG	561 bp
Exon 4	Forward: 5'-CAGCCCTGGAATGTCTAACTC Reverse: 5'-GGTAGAAGGCTCAATATGATCC	562 bp
Exon 5	Forward: 5'-CAGACGTTTTGCTGACTTCAG Reverse: 5'-GTCAGACAGTGCTGAGGCTC	720 bp

Table 2.2 Oligonucleotide primers used for rat *Serpina6* DNA sequencing

Sequence	PCR and Sequencing Primers	PCR Product
Promoter A	Forward: 5'- CCAGCATGCATAAGGTTCAGC Reverse: 5'- GGTTGTGCTTTGCTGCCCAGG	591 bp
Promoter B	Forward: 5'- CCTATCCATTGTCCTCTGAGG Reverse: 5'- GCTGAACCTTATGCATGCTGG	500 bp
Exon 1	Forward: 5'- CCAGCAAACAAGATTTAGTAGG Reverse: 5'- GCTGTGTTCTGGAGTGCAGC	199 bp
Exon 2	Forward: 5'- GCCAATGTGAAGGAAGGATAGG Reverse: 5'- CCAACCTGGTAGAGATTGGC	862 bp
Exon 3	Forward: 5'- GCAGGTGGCTGCATAGCTGG Reverse: 5'- GGCTAGAGAACCTCACAGCC	556 bp
Exon 4	Forward: 5'- CCTATCCCCAAGTTTAACCAGG Reverse: 5'- GGGTTTGTCATTTGGGACC	460 bp
Exon 5	Forward: 5'- GGCTATTTCACCTTCCATGG Reverse: 5'- CCTCTTTCTCAGTGCTCCCTTC	462 bp

Table 2.3. Restriction enzymes used for human CBG variant identification

CBG variant	Exon	Restriction Enzyme	Restriction Fragments (bp)
A224S	3	Hpy188I	Ala/Ala – 561 Ala/Ser – 158, 403, 561 Ser/Ser – 158, 403
W371S	3	XhoI	Trp/Trp – 720 Trp/Ser – 77, 673, 720

Table 2.4. Non-synonymous single nucleotide polymorphisms (SNPs) in human *SERPINA6* and corresponding mutagenic oligonucleotide prime pairs used to produce CBG variants

CBG variant[a]	SNP ID	Nucl. change	Forward primers (5'-3')[b]
C(8)F	rs139544351	G>T	CTCCTCCTGTACACCT**T**TCTTCTCTGGCTGCCCACCAGCG
L(9)F	rs150568135	C>T	CTCCTCCTGTACACCTGT**T**TTCTCTGGCTGCCCACCAGCG
T(14)S	rs142631353	C>G	CTGTCTTCTCTGGCTGCCCA**G**CAGCGGCCTCTGGACCGTC
T(14)I	rs142631353	C>T	CCTGTCTTCTCTGGCTGCCCA**T**CAGCGGCCTCTGGACCG
V(20)I	rs139446936	G>A	CACCAGCGGCCTCTGGACC**A**TCCAGGCCATGGATCCTAAC
P3S	rs146176684	C>T	GACCGTCCAGGCCATGGAT**T**CTAACGCTGCTTATGTGAAC
H14R	rs148218218	A>G	TGTGAACATGAGTAACCATC**G**CCGGGGCCTGGCTTCAGCC
H14Q	rs143058829	C>A	TGTGAACATGAGTAACCATCA**A**CGGGGCCTGGCTTCAGCC
I48N	rs370353870	T>A	CCCCTGTGAGCA**A**CTCCATGGCCTTAGCTATGC
R64L	rs199541890	G>T	CCTGTGGCCACACAC**T**GGCCCAGCTTCTCCAGGGCC
H89Y	rs187253929	C>T	GAGATCCACCAGGGTTTCCAG**T**ACCTGCACCAACTCTTTGC
L93H	rs113418909	T>A	CCAGCACCTGCACCAAC**A**CTTTGCAAAGTCAGACACC
S131L	rs141500229	C>T	CAAGCACTACTATGAGT**T**AGAGGTCTTGGCTATGAATTTC
S131T	rs374185317	T>A	CAAGCACTACTATGAG**A**CAGAGGTCTTGGCTATGAATTTC
V133A	rs151248615	T>C	CACTACTATGAGTCAGAGG**C**CTTGGCTATGAATTTCCAGG
L134F	COSM78469	G>C	GAGTCAGAGGTCTT**C**GCTATGAATTTCCAGGACTGG
G158E	n/a	G>A	GCTATGTCAAGAATAAGACACAGG**A**GAAAATTGTCGAC
D162N	rs182173676	G>A	CAGGGGAAAATTGTC**A**ACTTGTTTTCAGGGCTGGATAGCCC
I179V	rs367840035	A>G	CGTCCTGGTCAACTAT**G**TCTTCTTCAAAGGCACATGG
Q187E	rs142314764	C>G	CTTCAAAGGCACATGGACA**G**AGCCCTTTGACCTGGCAAGC
Q187P	rs148747799	A>C	CTTCAAAGGCACATGGACAC**C**GCCCTTTGACCTGGCAAGC
R195T	rs144992509	G>C	CTTTGACCTGGCAAGCACCA**C**GGAGGAGAACTTCTATGTGG
A224S	rs2228541	G>T	CCATCAGTTACCTTCATGAC**G**CAGAGCTCCCCTGCCAGC
P246Q	n/a	C>A	GGGACTGTCTTCTTCATCCTTC**A**GGACAAGGGGAAGATG
A256T	rs374119759	G>A	GGGGAAGATGAACACAGTCATC**A**CTGCACTGAGCCGGG
R260L	COSM26307	G>T	CGCTGCACTGAGCC**T**GGACACGATTAACAGGTGG
I279F	rs374191911	A>T	GCAGCCAGGTGGACCTGTAC**T**TTCCAAAGGTCACC
D301Y	rs200758120	G>T	GGGCATTGCA**T**ACTTGTTCACCAACCAGGC
N332S	rs147297630	A>G	GCTGTGCTGCAACTCA**G**TGAGGAGGGTGTGGACACAGCTGGC
T338A	rs138483168	A>G	CAATGAGGAGGGTGTGGAC**G**CAGCTGGCTCCACTGGGGTCAC
T349A	rs147101740	A>G	GGGGTCACCCTAAACCTG**G**CGTCCAAGCCTATCATCTTGC
R356L	rs149012523	G>T	GACGTCCAAGCCTATCATCTTGC**T**TTTCAACCAGCCCTTC
R356H	rs149012523	G>A	CCAAGCCTATCATCTTGC**A**TTTCAACCAGCCCTTC
D367N	rs28929488	G>A	CCATCAGTTACCTTCATGAC**G**CAGAGCTCCCCTGCCAGC
W371S	rs267607282	G>C	CGACCACTTCACCT**C**GAGCAGCCTTTTCCTGGCG
S373N	COSM42757	G>A	CCACTTCACCTGGAGCA**A**CCTTTTCCTGGCGAGGG

[a] = When indicated in brackets, amino acid numbering refers to the signal peptide. Otherwise, numbering refers to the sequence of the mature CBG.

[b] = Forward mutagenic primers are presented, with the base change indicated in bold and underlined. Sequences of reverse primers used in mutagenesis reactions correspond to reverse complement sequences of forward primers. Bold underlined characters represent mutagenesis sites.

Chapter 3: Genetic variants: sequence variations in the regulatory and coding regions of *SERPINA6*

3.1 Introduction

Several non-synonymous SNPs have been reported in the *SERPINA6* gene, which alter the production or function of plasma CBG. Clinically, these patients presented with low total plasma cortisol levels in association with a variety of clinical conditions, including chronic pain, fatigue, depression, hypotension, and excess body weight (14-16, 141, 142). However, not all individuals with CBG variants that are produced or function abnormally display these clinical features (101, 141, 142). Although previously identified CBG variants have been considered rare, recently, a CBG A51V variant has been found to occur at a frequency of 1:35 in Han Chinese (143), with similar frequencies present in Dai Chinese, Japanese and Kinh Vietnamese populations (1000 genomes database). The high prevalence of the CBG A51V variant, in addition to the recent identification of a number of CBG SNPs in public databases (NCBI dbSNP, 1000 genomes and Exome), suggests that they may occur more frequently than observed clinically.

In an initial study, the *SERPINA6* gene was examined in a female patient referred for low morning cortisol levels that suggested a CBG deficiency. The origin and segregation of *SERPINA6* SNPs in the proband's immediate family, and their effects on CBG protein structure and function were also studied (**chapter 3.2**).

In a second study, we set out to characterize naturally occurring CBG variants encoded by thirty-two newly reported non-synonymous human *SERPINA6* SNPs that were identified in recent SNP database releases (**chapter 3.3**).

In addition, I contributed to the CORtisol NETwork (CORNET) consortium's genome-wide association study (GWAS) to investigate genetic contributions to variations in plasma cortisol (**chapter 3.4**). This GWAS revealed that *SERPINA6* SNPs represent the major genetic influence on plasma cortisol levels through alterations in plasma CBG-cortisol binding activity. I further characterized a subset of these samples in an attempt to determine what was causing a discrepancy in plasma CBG values measured by CBG-cortisol binding capacity and ELISAs.

3.2 Two different corticosteroid-binding globulin variants that lack cortisol-binding activity in a Greek woman

3.2.1 **Results**

3.2.1.1 Endocrine evaluations

A 56-yr-old woman of Greek origin was referred for possible secondary adrenal insufficiency, suspected on the basis of a low morning cortisol level (52.4 nmol/l and 110.4 nmol/l, ref. range: 179.3-717.3 nmol/L) with a corresponding ACTH of 6.6 pg/ml (ref. range: 5-60 pg/ml). These tests were ordered because of non-specific features suggesting Cushing's syndrome. On clinical examination, the patient had central obesity (BMI: 32 kg/m^2 with a waist-to-hip ratio of 0.98), facial fullness and mild supraclavicular fat pads. However, she had no buffalo hump, thin skin, acne, hirsutism, striae or proximal myopathy. Axillary and pubic hair was normal. Blood pressure was 130/80 mmHg with no postural hypotension. The patient had no symptoms of adrenal insufficiency. She did not suffer from fatigue, drowsiness, or myoskeletal pain, and denied use of steroids. Her past medical history was unremarkable with no change in weight for several years. She reported regular menstrual cycles preceding menopause at age 45, four normal pregnancies and one abortion. Her four children were delivered uneventfully at term:

three, 26, 24 and 22 yr-old, girls and a 16 yr-old boy, whose birth weights were between 3050g and 4050g.

The proband's parents originate from the same village on an island close to the mainland of central Greece but are not consanguineous. Her family members, including her 82 yr-old father, 77 yr-old mother, dizygotic twin sister, 53 yr-old sister and four children, had no relevant medical conditions.

Initial endocrine investigations of the proband confirmed the low morning serum cortisol levels (113.1 nmol/L) with a plasma ACTH of 6.3 pg/ml. She subsequently underwent stimulation testing with 250 μg ACTH intravenous (tetracosactrin (1-24); Synacthen, Defiante Farmaceutica SA, Funchal, Portugal); serum cortisol was 22.1 nmol/L at baseline with a subnormal increase to 320.0 nmol/L at 30 min. An insulin tolerance test also revealed a subnormal peak response of serum cortisol (438.4 nmol/L), suggesting secondary adrenal insufficiency. Nevertheless, serum dehydroepiandrosterone sulfate (DHEA-S) levels (2.8 μmol/L, ref. range: 0.5-5.6 μmol/L) and 24 h urinary free cortisol levels (105 μg/24h, ref. range: 35-135 μg/24h) were normal. Evaluation of other anterior pituitary hormones was normal; she had normal thyroxine stimulating hormone (TSH) and free thyroxine (T_4) levels, normal increase of GH levels during insulin tolerance test (15 ng/ml), normal prolactin and appropriately increased gonadotrophins for a post-menopausal woman. Plasma renin activity and serum aldosterone were normal. A pituitary magnetic resonance imaging scan showed no abnormalities. Routine biochemical evaluation showed elevated fasting glucose (107 mg/dl) with a concomitant insulin value of 14.4 μUI/ml (homeostasis model assessment insulin resistance index: 3.8) and glycosylated hemoglobin (HbA1c) of 5.6%. The blood cell count, renal and liver function,

electrolytes, albumins and lipids were all normal. The patient received a short course of hydrocortisone replacement without notable changes.

Although the endocrine evaluation was suggestive of isolated ACTH deficiency, the lack of consistent clinical signs and symptoms and any subjective improvement while on hydrocortisone, as well as normal 24 h urinary free cortisol levels and normal DHEA-S levels, raised the possibility of a CBG deficiency. This was supported by normal serum free cortisol levels; i.e., 21.7 nmol/L (ref. range: 7.2–35.6 nmol/L) measured by mass spectrometry after separation of the unbound cortisol fraction in serum by temperature-controlled ultrafiltration (101), with a corresponding total cortisol of 135 nmol/L by electrochemiluminescence immunoassay (Elecsys, Roche Diagnostics, Switzerland, Basel). In a second 250 μg Synacthen test, the total cortisol (399 nmol/L) failed to increase above 500nmol/l, but the free serum cortisol increased to 69.1 nmol/L. Serum CBG level by RIA (Mayo Medical Laboratories, Rochester, MN) was borderline subnormal (18 mg/L, ref. range: 19-45 mg/L).

3.2.1.2 Sequence, structure and segregation of the CBG variants

Sequencing of the proband's *SERPINA6* promoter and four coding exons revealed that she was heterozygous for a c.1282G>C transversion in exon 5, which causes an amino acid substitution, p.Trp393Ser. This substitution occurs at residue 371 in the mature CBG polypeptide sequence, and is therefore designated as W371S for reference within the protein structure. The proband was also heterozygous for two other known non-synonymous SNP, found within exons 3 and 5 of *SERPINA6*, which encode the CBG A224S and CBG D367N variants, respectively. She was also heterozygous for two synonymous SNP within exon 2 (rs3748320) and exon 4 (rs1042394) (Figure 3.1).

The W371S, A224S and D367N substitutions are shown in a tertiary structure model of human CBG (Figure 3.2A). This illustrates the close proximity of Trp371 and Asp367 to the steroid-binding site, whereas Ala224 resides on the surface of the protein and is not close to the steroid-binding pocket. The W371S substitution is located on β-sheet B, and the tryptophan in this position directly interacts with steroid ligands. As illustrated in a close-up view of the steroid-binding pocket, the indole side chain of Trp371 lies immediately above the plane of the steroid (Figure 3.2B). This tryptophan residue hydrogen bonds with rings A, B and C of cortisol and is predicted to interact with Arg15 to stabilize the conformation of the CBG steroid-binding site (11). A serine substitution of Trp371 (Figure 3.2B) will therefore not only disrupt these critical interactions with the steroid ligand, but will also destabilize the entire steroid-binding pocket.

The proband's mother is the only member of her immediate family who is heterozygous for the c.1282G>C transversion that encodes CBG W371S (Figure 3.3B). Her father and all four of her children are heterozygous for the CBG D367N variant (Figure 3.3A). Pedigree analysis demonstrated that the CBG W371S and CBG D367N variants segregate independently, and the CBG W371S mutation segregates with Ala224. The proband's father and one child are homozygous for CBG Ser224, and the proband's mother is homozygous for Ala224, whereas the other family members are heterozygous for the CBG A224S polymorphism (Figure 3.3A,B).

3.2.1.3 Analysis of steroid binding properties of the CBG variants in serum

When subjected to a cortisol-binding capacity assay using DCC to separate CBG-bound from free [^{3}H]-cortisol (205), we were unable to detect binding of [^{3}H]-cortisol to CBG in the proband's serum (Figure 3.3C). Equilibrium dialysis was therefore used as an alternative method to measure low affinity binding of [^{3}H]-cortisol to CBG, and this confirmed that CBG in the

proband's serum has no detectable cortisol-binding activity. The proband's family members, excluding the proband's tested sister, had on average 56% (range, 41-70%) lower cortisol binding capacity values when compared to CBG levels by ELISA (Figure 3.3C). By contrast, the proband's sister, who did not carry the CBG D367N or W371S variants, had a serum cortisol-binding capacity (414 nM) that matched the CBG concentration by 12G2 ELISA (413 nM), and these values are within the normal references ranges of these assays (143, 205).

3.2.1.4 Physiochemical characterization of the W371S and D367N human CBG variants

An ELISA was used to quantify the recombinant CBG WT and CBG variant proteins produced by CHO cells. Using the CBG concentrations obtained by 12G2 ELISA, equivalent amounts of WT and variant CBG proteins were subjected to a cortisol-binding capacity assay. This demonstrated that the cortisol-binding capacity of CBG W371S, CBG D367N + A224 and CBG D367N + S224 variants fell below the assay detection limit (5 nM), and were not considered measurable (Figure 3.4A). When equivalent amounts of WT or variant CBGs, based on their 12G2 ELISA values, were subjected to equilibrium dialysis to assess their cortisol-binding activities, CBG D367N had approximately 10% of the cortisol-binding capacity of CBG WT (Figure 3.4B) and CBG W371S exhibited no steroid-binding activity (not shown). There was also no difference between the CBG D367N + A224 and D367N + S224 variants in terms of equilibrium dialysis values (Figure 3.4B**).** The cortisol-binding affinities of wild-type CBG containing either an Ala or a Ser at residue 224 were also determined by Scatchard analysis, and their dissociation constants, 2.94 and 2.97 nM, respectively, did not differ.

3.2.2 **Discussion**

A woman of Greek origin was identified as a heterozygous carrier of two different non-synonymous SNP that severely disrupt the cortisol-binding activity of CBG. The W371S and

D367N substitutions in the mature CBG sequence both reduced the cortisol-binding affinity of CBG to levels that preclude accurate measurements of cortisol-binding kinetics. A marked 4-fold reduction in cortisol-binding affinity has been reported for the CBG D367N variant, and the percentage of free cortisol in plasma of individuals homozygous for CBG D367N are higher (8.7-9.7%) than the 2.9-3.9% observed in individuals homozygous for wild-type CBG (16). An individual homozygous for another CBG variant (G237V) had an even higher percentage (19.8%) of free cortisol, and this is consistent with the fact that CBG G237V has no measurable cortisol-binding activity (101). Given that the new CBG variant we identified with a W371S substitution is also characterized by a profound loss of cortisol binding activity, this explains why the proband, with both CBG D367N and CBG W371S variants in her blood, also had a higher percentage of free plasma cortisol (16.1%) when compared to individuals homozygous for the CBG D367N variant.

The CBG W371S variant was the second CBG variant identified with no measurable cortisol-binding activity, the first one being CBG G237V (101). It is easy to appreciate how the W371S substitution disrupts steroid binding because Trp371 resides within the inter-strand loop connecting β-sheets 4B and 5B, and plays a key role in anchoring steroid-ligands in the binding site (11, 37). The loss of cortisol binding in the CBG W371S variant was also anticipated because substitution of this tryptophan with threonine or lysine has been shown to severely disrupt the steroid-binding activity of human CBG (46). It should also be noted that Trp371 is located only four residues C-terminal from Asp367. The Asp367 residue resides on the rim of β-sheet 4B and points towards helix D, and while it does not make direct contact with ligands in the steroid-binding site, the D367N substitution likely influences steroid-binding mechanisms (37). By contrast, the common A224S substitution, which was also found within this Greek

pedigree, occurs within the loop between β-sheets 1B and 2B, far from the steroid-binding site (37), and we confirmed that it does not alter the steroid-binding affinity of CBG either alone or in combination with CBG D367N. It therefore remains unclear why the CBG A224S variant is linked to clinical symptoms, such as lethargy, previously associated with CBG variants (144).

Pedigree analysis revealed the parental origins and familial segregation of the CBG A224S, D367N and W371S variants. The *SERPINA6* alleles of the proband's mother both encoded an alanine at residue 224, whereas her father was homozygous for the SNP encoding a serine in this position. The novel CBG W371S variant was inherited from the proband's mother, and was not passed on to her children. By contrast, CBG D367N appears to have been inherited from the proband's father, and was transmitted to the proband's children. This allowed us to deduce that CBG D367N and W371S segregate independently. In addition, CBG W371S segregates with Ala224, while CBG D367N is linked to Ser224, as previously reported in the original pedigree in which CBG D367N was identified (16). Interestingly, the family we have studied is now the fifth pedigree of Mediterranean/Middle Eastern descent reported to carry the CBG D367N variant (15, 16, 103, 142, 216), suggesting that the SNP (rs28929488) responsible for CBG D367N originated in this geographic region and may occur more frequently than previously estimated in some populations, as suggested recently (142).

The proband's family members who carry either the CBG D367N or W371S variants alone have approximately half the CBG levels measured by cortisol-binding capacity when compared to those obtained using an immunoassay. Even more striking is the complete loss of serum cortisol-binding capacity in the proband with both the CBG D367N and W371S variants, despite having CBG values within the normal range by 12G2 ELISA. This pronounced divergence in serum CBG values obtained using binding-capacity assays and immunoassays has

been observed previously (16, 101, 103, 140, 143), and illustrates the limitation of using immunoassays that do not reflect the steroid-binding activity of CBG. Thus, while measurements of CBG by immunoassay may have utility in monitoring changes in CBG levels in response to changes in physiological state or disease, it is becoming increasingly evident that CBG measurements by immunoassays cannot be used reliably in mathematical calculations of serum free cortisol levels (213). In this context, it is important to note that although CBG variants with abnormal cortisol-binding affinity, such as the new CBG W371S variant we have identified may be relatively rare in a global context, it is likely that certain variants are much more highly represented in some ethnic groups or isolated populations (142, 143). There is therefore an urgent need to develop robust clinical chemical assays that measure both the levels and steroid-binding activity of CBG in conjunction with serum cortisol measurements.

The current consensus is that CBG is more than just a steroid transport protein, and modulates cortisol action (17, 107) by promoting the targeted delivery of cortisol to inflammatory sites (9). In addition, CBG production by the liver and other tissues may have important roles in fetal and early postnatal development (18, 22). However, the diagnosis and physiological consequences of defective CBG production and/or function remain elusive because CBG variants, for instance, are not always associated with non-specific signs of adrenal insufficiency, such as fatigue, chronic pain or hypotension (17, 107). Indeed, our patient with very low serum total cortisol levels and unmeasurable CBG-cortisol binding activity did not present with a particular clinical phenotype.

Most cases of CBG deficiency have been identified in patients with low serum cortisol levels, and some present with symptoms of adrenal insufficiency (17, 142). Recently, in one CBG-deficient subject, abnormalities in cortisol pulsatility were observed, implicating altered

hypothalamic-pituitary-adrenal dynamics and stress responses (101). This finding, in addition to the increased susceptibility of *Cbg*$^{-/-}$ mice to lipopolysaccharide-induced lethal shock (102), raises concerns that patients with defective CBG variants may be at increased risk for an adverse outcome in the case of critical illness (101). Nevertheless, as reported here and elsewhere (101), an almost complete lack of CBG-cortisol binding activity does not result in an overt phenotype. However, the number of subjects with CBG deficiencies who have been evaluated clinically is far too limited to permit accurate assessments of their overall pathophysiological significance.

In conclusion, identifying patients with CBG deficiencies presents a diagnostic challenge. It is recommended that a CBG deficiency should be suspected in patients with low morning cortisol levels who have normal salivary cortisol, urinary cortisol, or free serum cortisol levels. These results, also clearly demonstrate that immunoassays fail to provide an accurate measurement of the functional activity of CBG, limiting their utility in calculations of serum free cortisol values. Direct measurements of total and free serum cortisol levels are therefore recommended in conjunction with serum CBG measurements by both immunoassay and cortisol-binding capacity assays, in order to identify CBG variants with defects in their ability to bind cortisol.

3.3 Naturally occurring mutations of human corticosteroid-binding globulin

3.3.1 **Results**

3.3.1.1 Production and cortisol-binding properties of CBG variants

The 12G2 ELISA was used to determine the amounts of the CBG variants secreted by CHO cells, and these values were compared with corresponding cortisol-binding capacity values (Figure 3.5). This comparison allowed for the identification of variants with defects in CBG production or cortisol-binding activity. For instance, when comparing the variants to CBG WT,

low values in both assays suggest low production or secretion while a high 12G2 ELISA to steroid-binding capacity ratio suggests a decreased cortisol-binding capacity or affinity. Among the CBG variants tested, CBG I48N and CBG P246Q appeared to be produced or secreted at abnormally low levels, while CBG H14R, CBG H14Q, CBG H89Y, CBG I179V, CBG R260L, and CBG I279F were identified as candidates for abnormally low steroid-binding capacity or affinity.

Interestingly, CBG A256T exhibited a normal cortisol-binding capacity value but it was undetectable by ELISA when using the 12G2 monoclonal antibody (Figure 3.5), whereas it was detected at normal levels when the 9G12 monoclonal antibody was used. By contrast, CBG T349A was detected at a higher level (2-fold) in an ELISA with the 9G12 antibody when compared to ELISA values obtained using the 12G2 antibody.

3.3.1.2 Assessment of CBG variant production and secretion

The *SERPINA6* mRNA abundance in CHO cells was measured for variants exhibiting abnormal ELISA or cortisol-binding capacity values (i.e. CBG H14R, H14Q, I48N, H89Y, I179V, P246Q, R260L, and I279F) (Figure 3.6A). Variation of +/- 2-fold in *SERPINA6* mRNA abundance was observed for cells expressing CBG variants versus CBG WT, and this likely reflects differences in the proportion of stably-transfected cells expressing the cDNA constructs. Moreover, variation in *SERPINA6* mRNA abundance was unrelated to differences in CBG levels in culture media.

Variants suspected of production or secretion defects (Figure 3.5), i.e. CBG I48N and CBG P246Q, were further analyzed by Western blotting to compare the relative amounts of CBG in cell culture medium with those in the corresponding CHO cell extracts (Figure 3.6B). These results excluded the possibility that these variants escaped detection or reacted poorly in the

ELISA, and indicated that CBG I48N is secreted at very low levels when compared to CBG WT, whereas CBG P246Q is not secreted.

3.3.1.3 Steroid-binding properties of CBG variants

Variants with suspected abnormal steroid-binding affinity or capacity (Figure 3.5) were subjected to Scatchard analyses to determine their dissociation constant (Kd) for cortisol (Figure 3.7A). The Kd values of CBG H14Q, CBG H89Y, and CBG I279F are 2 to 5.5-fold higher than that of CBG WT, thus confirming a reduced cortisol-binding affinity for these CBG variants. CBG L93H (Leuven) was also tested as a reference, and its Kd value (14.62 nM) was 4.9-fold higher than CBG WT, which is in agreement with the previously reported 3.5-fold decrease in affinity for this variants (14). The very low binding values of CBG H14R, along with a Kd of 3.92 nM, suggest that this variant has a low steroid-binding capacity, i.e. that a small proportion of the CBG molecules bind cortisol with an affinity similar to CBG WT. This was confirmed by an equilibrium dialysis assay, as was the essentially undetectable cortisol-binding activity of R260L (Figure 3.7B).

3.3.1.4 Sensitivity of CBG variants to proteases

In screening experiments, the cortisol-binding capacity of variants was tested after incubation with proteases that target the CBG RCL. For CBG WT and all CBG variants, apart from CBG I179V and CBG I279F, the residual cortisol-binding capacity (cortisol-binding capacity of protease incubation/negative control without protease; mean ± SD, n=3) after incubations with neutrophil elastase, bovine chymotrypsin, human chymotrypsin or *P. aeruginosa* medium, were 16.2 ± 8.7%, 16.1 ± 14.8%, 16.6 ± 14% and 9.7 ± 16.4%, respectively. However, the residual cortisol-binding capacity for CBG I179V (32.6%, 54.9%,

65.1% and 41.1%, respectively) and CBG I279F (30.4%, 46.9%, 53.9% and 27.9%, respectively) fell outside the ranges observed for CBG WT and the other variants.

In time course experiments, CBG I179V and CBG I279F showed slower rates of cortisol-binding capacity loss than CBG WT after exposures to neutrophil elastase, chymotrypsin or *P. aeruginosa* medium (Figure 3.8A-C). In general, CBG I179V was more resistant than CBG I279F and the effect was most pronounced after incubation with human chymotrypsin. The RCL cleavage of CBG I179V and I279F after incubation with the different proteases was assessed by Western blotting and was similar to that of CBG WT (Figure 3.8D). After incubation with different proteases, these variants were subjected to an ELISA using the RCL-specific monoclonal antibody 9G12, and a loss of immunoreactivity confirmed that cleavage of their RCLs had occurred.

3.3.2 **Discussion**

We identified several previously uncharacterized human CBG variants with abnormalities in cortisol-binding affinity (CBG H14Q, H89Y, R260L, I279F), cortisol-binding capacity (CBG H14R), production/secretion (CBG I48N and P246Q), as well as sensitivity to proteolytic cleavage of the RCL (CBG I179V and I279F) or recognition by monoclonal antibodies used for ELISAs (CBG A256T and T349A) (Table 3.1 and Figure 3.9A-D). Most of these substitutions involve highly conserved residues in CBGs across mammalian species, or in structurally related SERPINs, such as thyroxin-binding globulin (*SERPINA5*) or alpha1-antitrypsin (*SERPINA1*). Several are in close proximity to previously identified naturally occurring CBG mutations that cause defects in CBG production or function (see below), but none are in positions corresponding to mutations identified in rat, mouse or pig *Cbg*s (37).

Crystal structures of rat (11) and human (13) CBG provide insight into how specific amino acid substitutions influence CBG structure or function. They have shown how residues outside the immediate steroid-binding site may affect steroid-binding affinity (11, 12) and our data further illustrate this. For instance, the highly conserved His14 is not located in the steroid-binding site (Figure 3.9A,B) but it resides next to Arg15, which plays a critical role in positioning Trp371 and allowing it to interact with the steroid (11). Thus, His14 probably plays an important role in generating the optimal steroid-binding site topography of CBG along with Arg15. Interestingly, while CBG H14Q exhibits a decreased cortisol-binding affinity, CBG H14R has a reduced cortisol-binding capacity; the reason for which is unclear. Among the variants tested, CBG R260L was the only one with a complete loss of cortisol-binding activity. This was anticipated because Arg260 is highly conserved and is positioned within helix H (Figure 3.9A,B), where it is in direct contact with steroid ligands through hydrophobic interactions (11).

Binding and release of steroids from CBG normally occurs in a state of dynamic equilibrium, through an allosteric mechanism involving the partial insertion of the RCL into the central beta-sheet A in the ligand free state, and the winding of helix D and expulsion of the RCL upon ligand binding (11, 12, 37). CBG H89Y is of interest in this context, as it exhibits an approximately 5.5-fold decreased cortisol-binding affinity that resembles that observed for the previously identified CBG L93H (Leuven) (14, 140). Both His89 and Leu93 are located in helix D (Figure 3.9A,B), and their substitution may thereby affect the allosteric mechanism that determines CBG-steroid binding affinity. Furthermore, it is important to note that helix D is one of the regions of CBG that is most affected by the conformational changes that occur following cleavage of the RCL (13).

After proteolysis by neutrophil elastase, the cleaved RCL fully inserts into beta-sheet A to form a novel beta-strand (s4A), and this occurs along with a displacement and unwinding of helix D, which disrupts the steroid-binding site (11, 12). Although it has not been formally demonstrated, we assume that a similar structural rearrangement occurs after proteolysis of the RCL by chymotrypsin (120) or the PAE enzyme, secreted by *P. aeruginosa* (**chapter 4.2**). A higher residual cortisol-binding activity was observed after protease cleavage of CBG I179V and I279F, when compared to WT CBG. Further, our data also suggest a difference in the sensitivity of these variants to the proteases, with a trend for CBG I179V to be more resistant than CBG I279F.

The isoleucine that is normally present at position 179 is located in s3A, while the isoleucine at position 279 is located between helix H and beta-strand s5A (Figure 3.9A,C), and both positions are conserved across related SERPINs. The substitution in CBG I279F also likely affects the allosteric mechanism that determines steroid-binding affinity, through direct interactions or a domino-like effect, because this variant also showed a decreased steroid-binding affinity (11, 12, 37). We suspect that the residual cortisol-binding activity observed after protease cleavage of these two variants may be due to an abnormal insertion of the RCL and/or an altered conformational change of the steroid-binding site after insertion. However, a difference in the targeting and cleavage of the RCL by proteases cannot be ruled out, but this seems unlikely because a similar effect was observed following RCL cleavage with neutrophil elastase (11, 12), chymotrypsin (120) or *P. aeruginosa* (**chapter 4.2**), all of which cleave the RCL in different locations. Although a comparable residual steroid-binding activity after cleavage of the RCL by neutrophil elastase has been observed in human CBG variants with amino acid substitutions in

the RCL, including CBG T338P (37), one of the naturally occurring variants we studied with a different substitution at the same position, CBG T338A, did not exhibit this property.

The very low level of CBG I48N production/secretion is similar to that observed for CBG A51V (143), and these residues are both located in the hydrophobic core of the protein (Figure 3.9A,D). In the case of the CBG P246Q variant that is not secreted, the substitution occurs immediately after beta-sheet s3B (Figure 3.9A,B), and it likely contributes to the positioning of the loop between s3B and helix H. These amino acids are highly conserved and likely influence the folding and/or stability of SERPIN structures.

Human CBG A256T is the first variants to be identified with a loss of recognition in an immunoassay, while retaining a normal cortisol-binding activity. The position of Ala256 within an α-helix at the surface of the protein (Figure 3.9A), suggests that this residue resides within the epitope recognized by the monoclonal antibody 12G2 used in the CBG ELISA. The observation that CBG T349A, which results in a loss of *N*-glycosylation at Asn347, exhibited unexpectedly high values in an ELISA using the 9G12 monoclonal antibody, was also of interest. This antibody was raised against a synthetic polypeptide containing part of a consensus sequence (N-L-T) for *N*-glycosylation at Asn347, which is utilized in approximately 85% of CBG molecules in human plasma (133), as well as the elastase cleavage site (after Val344) in the CBG RCL sequence (209). Since only residues Ser341 to Leu348 (STGVTLNL) were part of the synthetic RCL polypeptide that the 9G12 antibody was raised against (217), the substitution of alanine for threonine at residue 349 would not be expected to directly alter epitope recognition by the antibody. We therefore conclude that for CBG T349A, a loss of glycosylation in the RCL is likely responsible for increased epitope recognition by the 9G12 antibody, either through

increased accessibility or by altering the conformation of the epitope to more closely resemble the synthetic peptide.

The identification of variants with a loss of recognition by immunoassays together with the growing number of variants identified with abnormal steroid-binding activity (Table 3.1) illustrates the limitations of using steroid-binding capacity assays or immunoassays in clinical evaluation and indicates that both assays should be used when CBG deficiencies are suspected. In this regard, normal free cortisol levels measured either in saliva or urine in subjects with low total plasma cortisol, along with a lower than normal increase in total plasma cortisol after exogenous glucocorticoid administration or ACTH stimulation test, are highly indicative of a CBG deficiency.

The minor allele frequencies of CBG variants reported in current SNP databases (and which can be readily found therein) are based on general population studies of limited numbers of individuals of undefined health status from selected ethnic groups, and are constantly being updated as more extensive population studies are completed. However, some SNPs that are listed in the SNP databases as being very rare are highly enriched in certain geographic regions/ethnic groups. For example, CBG A51V is found at a frequency of approximately 1:35 in Han (143) and Dai Chinese, as well as in Japanese and Kinh Vietnamese (1000 genome database), while CBG Lyon (D367N) is highly enriched in some Mediterranean locations and lineages (17).

A clear definition of the clinical consequences of CBG deficiencies has been hampered by the fact that almost all CBG variants with severely compromised production or function occur rarely in genetically diverse populations (17). However, the concentrations and steroid-binding properties of CBG both influence the plasma distribution of endogenous glucocorticoids, progesterone, as well as some synthetic glucocorticoids, such as prednisolone (2), and will

thereby affect their biological activities, metabolism and clearance. In addition, circadian and ultradian rhythms of cortisol secretion modulate the expression of glucocorticoid-regulated genes in tissues (218), which may be amplified in CBG deficient patients where transient increases in glucocorticoids likely exceed the buffering capacity of CBG. It is also possible that inherited CBG defects contribute to altered physiological states through epigenetic modifications caused by abnormal levels of bioavailable glucocorticoids (219). Further, CBG deficiencies may have consequences in pregnancy when CBG levels increase by approximately 2-fold but progesterone levels increase by almost two orders of magnitude (17), as well as during in utero development when glucocorticoids are required for the maturation of several organ systems, including the lungs (220). Nevertheless, as reported in **chapter 3.2** and elsewhere (101), an almost complete lack of CBG-cortisol binding activity does not result in an overt clinical phenotype. However, the number of subjects with CBG deficiencies who have been evaluated clinically is far too limited to permit accurate assessments of their overall pathophysiological significance.

Fluctuations in glucocorticoid levels may also lead to abnormal responses to acute and chronic stress, and CBG deficiencies may be most relevant clinically in life-threatening situations when a lack of a glucocorticoid reserve or an inappropriate control of glucocorticoid bioavailability play important roles, such as during septic shock. Indeed, lethality in mice treated with TNF-α has been linked to the *Serpina6* gene locus (182), and $Cbg^{-/-}$ mice exhibit much greater mortality than wild-type mice when subjected to a lipopolysaccharide challenge (102). Screening for CBG mutations in patient groups where acute inflammation is life-threatening may therefore reveal how CBG deficiencies are implicated in pathophysiological states with adverse outcomes related to abnormal cortisol levels, for example in trauma, infections, and inflammatory diseases.

While acknowledging the limitations of our *in vitro* studies, it will now be important to examine the expanded list of CBG variants (Table 3.1) in both normal physiological and clinical contexts. Moreover, it can also be anticipated that genome-sequencing projects involving large numbers of subjects in relation to specific disease groups, will reveal many more *SERPINA6* SNPs in the future. In fact, since this study was completed, at least 15 new non-synonymous *SERPINA6* SNPs have appeared in various databases. In this regard, these and other new *SERPINA6* SNPs will need to be characterized to identify those that may be functionally significant, in order to eventually determine their relevance in the context of various clinical conditions.

3.4 Genome-wide association meta-analysis of plasma cortisol

3.4.1 **Results**

3.4.1.1 Genome-wide association meta-analysis

A discovery genome wide meta-analysis of ~2.5 million SNPs investigated morning plasma cortisol levels in 12,597 participants of European origin. The results indicated that <1% of the variance in plasma cortisol is accounted for by genetic variation in a single region of chromosome 14 (14q32) spanning the *SERPINA6* (CBG) and *SERPINA1* (α1-antitrypsin) loci. Only SNP found within the *SERPINA6* locus (Table 3.2) reached genome-wide significance. Furthermore, the association between plasma cortisol and *SERPINA6* SNPs was replicated in an additional 2,795 participants.

3.4.1.2 Functional consequences of genetic variation in the SERPINA6 region

Detailed biochemical investigations were undertaken in 316 participants from the CROATIA-Korcula cohort to further explore the association between plasma cortisol and

SERPINA6 SNPs (rs11621961, rs12589126, rs2749527). Together, these SNP explained 0.54% of the variance in total plasma cortisol in the cohort.

After adjusting for age and sex, all three *SERPINA6* SNPs were found to influence total CBG-cortisol binding activity in plasma; rs11621961, P=0.016; rs12589126, P=2.3x10^{-5}; rs2749527, P=4.3x10^{-4}, respectively (Figure 3.10A-C). Exome chip data for the *SERPINA6/SERPINA1* region was then used to identify non-synonymous variants in CROATIA-Korcula study participants. In this population, the previously identified *SERPINA6* SNP, CBG Leuven (L93H) (14), was found at a minor allele frequency (MAF) of 0.017. Biochemical analyses demonstrated that individuals with the CBG L93H variant had lower (P=1.9x10^{-6}) CBG-cortisol binding activity (Figure 3.10D) and total cortisol levels, but normal CBG immunoreactivity and free cortisol levels (Table 3.3).

3.4.1.3 Investigations of samples with discrepant ELISA values

In addition to CBG-cortisol binding activity, two different ELISAs were utilized to measure plasma CBG levels in the CROATIA-Korcula study samples. One of the monoclonal antibodies, designated 12G2, is able to detect all or "total" CBG, whether the protein is intact or proteolytically cleaved. The other antibody, designated 9G12, was raised against a peptide containing the CBG RCL and therefore, is only able to detect "intact" or uncleaved CBG. In terms of *SERPINA6* SNP, the minor T allele at rs12589136 was associated (P=9.4x10^{-11}) with lower 9G12 ELISA CBG levels, as well as higher CBG-cortisol binding activity and total cortisol levels (Table 3.3).

Plasma CBG levels determined by cortisol-binding activity and both CBG ELISAs were compared, irrespective of genetic status, to identify "discrepant" samples, in which CBG levels measured by the RCL ELISA did not match CBG values obtained from the other assays. In a

majority of samples, all three CBG values correlated relatively well, with a moderate correlation found between CBG-cortisol binding capacity and 12G2 ELISA values (r^2=0.32), in comparison to a lack of correlation when comparing CBG-cortisol binding capacity to 9G12 ELISA values (r^2=0.15) (Figure 3.11A). Yet, a small subset were identified with much lower (50-60%) 9G12 ELISA CBG levels when compared to CBG levels measured by cortisol-binding activity or the 12G2 ELISA (Figure 3.11B).

Scatchard plots were utilized to compare the cortisol-binding affinity (Kd) of plasma CBG in a "normal" sample, where all three CBG values correlated, with a "discrepant" sample, where the CBG value measured by 9G12 ELISA was much lower than either the cortisol-binding capacity or 12G2 ELISA values (Figure 3.11C). No differences were observed, as the normal and discrepant samples had binding affinities (Kd) of 0.21 nM and 0.23 nM, respectively. To determine if RCL cleaved CBG could be observed in a discrepant sample, non-denaturing PAGE was used to compare the physicochemical properties of CBG in a normal versus discrepant sample (Figure 3.11D). In this assay, thermostable RCL-cleaved human CBG is less prone to aggregation following heat treatment than uncleaved human CBG (13). As positive controls, both samples were also incubated with neutrophil elastase, to induce CBG cleavage. As shown in Figure 3.11, the results demonstrate that there is no evidence of CBG RCL cleavage in the discrepant sample.

3.4.2 **Discussion**

In the genome-wide meta-analysis study investigating genetic influences on plasma cortisol, we identified the *SERPINA6*/*SERPINA1* locus, on chromosome 14, as a major contributor to individual differences in plasma cortisol levels. Notably, only SNPs (rs11621961, rs12589126, rs2749527) found in the *SERPINA6* gene locus reached genome-wide significance.

Further biochemical studies revealed that the major genetic influence on plasma cortisol is likely to be mediated by variations in the cortisol-binding capacity of CBG. Although these variants only accounted for <1% of the variance in plasma cortisol, the inherent difficulty in obtaining unstressed cortisol samples at a fixed time of day may have obscured more significant results, and it would not be surprising if these variants contribute considerably more to the 30-60% heritability estimate of cortisol (221-223).

Clinically, the significant association between these common *SERPINA6* SNP and plasma cortisol levels is important because variations in plasma cortisol levels are associated, in population-based studies, with cardio-metabolic (224-228), inflammatory (224, 229) and neuro-cognitive (230-232) traits and diseases. Overall, these findings reveal a novel common genetic source of variation in the binding of cortisol by CBG, and reinforce the key role of CBG in determining plasma cortisol levels.

Indeed, the CBG Leuven variant, which has been shown to lead to 3 to 4-fold reductions in CBG-cortisol binding affinity (14), was associated with significantly reduced CBG-cortisol binding activity and total cortisol levels in this study. All of the individuals identified with the CBG L93H variant were heterozygous carriers. Despite decreased CBG-cortisol binding capacity and total cortisol levels in CBG L93H carriers, free cortisol levels did not differ from individuals without this CBG variant. This finding is similar to that found in individuals carrying other CBG variants, including CBG G237V (101), CBG D367N (16) and W371S (**chapter 3.2**), all of which have either reduced or negligible CBG-cortisol binding activities. Although the percentage free cortisol was not calculated in this or previous studies involving the CBG L93H variant, it would be expected that similar to CBG D367N, which has a 4-fold reduction in cortisol-binding affinity (16), CBG L93H would result in elevations in the percentage of plasma free cortisol. If so, a

percent free cortisol of ~9% would be expect for individuals heterozygous for L93H, which is increased from the 3 - 5% observed in individuals with CBG WT (16).

The minor allele frequency (MAF) of CBG L93H provided by the 1000 genomes and dbSNP databases are 0.001 and 0.0046, respectively, with values from both databases being determined from a mixed representation of individuals from various continents. Here, within a Croatian population, the MAF for CBG L93H was 0.017, which is much higher than the aforementioned values. A similar observation is made for CBG A51V, where the MAF provided by SNP databases (1000 genomes (204) and dbSNP (202)) is 0.004, but a frequency of 1:35 was reported in Han Chinese (143). Notably, in the Exome database, where individuals are classified by whether they are of European or African American decent, MAFs of 0.2558 and 0.0227, respectively, are given for CBG L93H, with an overall MAF of 0.1768. Further, the initial identification of the SNP responsible for CBG L93H came from a patient with sepsis of central European descent (14), with CBG L93H subsequently identified in a Polish kindred (140). Together, this suggests that the SNP responsible for L93H originated in Europe and similar to other CBG variants, it likely occurs more frequently in specific geographic/ethnic populations than that estimated by some SNP databases.

Through the parallel use of the highly specific 12G2 and 9G12 monoclonal antibodies, which discriminate between CBG with an intact versus cleaved RCL, it has been reported that cleaved CBG is normally present in the human blood circulation, making up approximately 25-30% of total CBG levels (209). Yet, direct evidence, such as a size reduction in CBG consistent with RCL cleavage, has never been observed in human blood samples, consistent with the notion that human CBG is rapidly cleared from the blood following RCL cleavage (233). The fact that plasma concentrations of functional CBG, such as those assessed by cortisol-binding capacity

assay, were not measured in conjunction with the ELISAs, sheds further uncertainty on the interpretation that a lower 9G12 versus 12G2 ELISA CBG value is the result of CBG RCL cleavage.

Given that it has been previously shown that RCL cleaved CBG lacks high-affinity steroid binding (9), if 25-30% of circulating CBG has a cleaved RCL (209), CBG levels measured by cortisol-binding capacity assays would be expected to correlate closely with CBG levels obtained by the 9G12 ELISA, and 12G2 ELISA CBG levels would be 25-30% higher. Yet, in this study, where CBG levels were measured by cortisol-binding capacity, as well as 12G2 and 9G12 ELISAs, the strongest correlation (r^2=0.32) was observed between CBG-cortisol binding capacity and 12G2 ELISA values. In addition, for the common rs1289136 variant, lower 9G12 ELISA CBG levels corresponded with higher CBG-cortisol binding activity. If interpreting lower 9G12 ELISA CBG levels to represent CBG RCL cleavage, elevated CBG-cortisol binding activity is contradictory, as cleaved CBG has a lower affinity for cortisol. Evidence that RCL cleaved CBG is not present in the circulation was also provided by non-denaturing PAGE, where upon heat treatment, plasma samples fully polymerized, which is characteristic of CBG with an intact RCL (13).

When comparing discrepant and non-discrepant samples using non-denaturing PAGE, no discernable differences were observed and there was no evidence of CBG RCL cleavage. This finding, combined with normal, highly correlated CBG-cortisol binding capacity and 12G2 ELISA values, suggests that CBG RCL cleavage has not occurred in discrepant samples. The ELISAs utilized in this study have been developed using specific monoclonal antibodies that recognize surface epitopes. CBG detection by the 12G2 ELISA antibody is unaltered following conformational changes associated with CBG RCL cleavage and insertion and as mentioned, this

antibody correlates well with CBG-cortisol binding capacity measurements. On the other hand, discrepancies are observed when utilizing the 9G12 ELISA antibody, which was raised against a synthetic RCL polypeptide. Notably, human CBG contains an *N*-glycosylation site within the RCL (Asn347) that has been shown to be occupied 85% of the time, contains a relatively high degree of fucosylation (35%) and is composed of bi-, tri- and tetra-antennary glycans (133). This is important, as the synthetic RCL peptide utilized to develop the 9G12 ELISA antibody was unglycosylated. In support of *N*-glycosylation playing an important role in 9G12 antibody epitope recognition, as mentioned above, a loss of the *N*-glycosyation consensus sequence within the RCL that occurs in the CBG variant T349A (**chapter 3.3**) lead to an increased 9G12 ELISA value. Therefore, the presence of glycosylation, as well as differences in the composition of glycans attached at Asn347, may alter 9G12 antibody recognition of plasma CBG either through steric hindrance or by inducing subtle changes in the conformation of the surface epitope, and may account for the discrepancy seen in some samples. Further studies need to be undertaken to confirm this hypothesis.

In conclusion, in this genome wide association study, CBG was confirmed to be the primary determinant of circulating plasma cortisol levels in humans. Importantly, these results were consistent across multiple cohorts, in addition to gene-based hypothesis-free analysis and in candidate gene analysis. In this Croatian cohort, a MAF of 0.017 for the CBG Leuven variant (L93H), which is much greater than that provided by databases containing largely mixed population representations, combined with the previous identification of L93H in individuals of central European descent (14, 140), suggests that similar to W371S (**chapter 3.2**), this CBG variant may occur more frequently in specific geographic/ethnic populations. Furthermore, our findings suggest that differential *N*-glycosylation of Asn347 within the CBG RCL, rather than

CBG RCL proteolysis, likely alters 9G12 antibody recognition, and leads to discrepancies in CBG levels when comparing the CBG value obtained by 9G12 ELISA to those obtained by CBG-cortisol binding capacity and/or 12G2 ELISA.

Table 3.1. List of all known functionally relevant *SERPINA6* mutations

CBG variant	SNP ID	Nucl. change	Functional defect	Reference
Leu5CysfsX26	n/a	deletion	Premature stop codon, fatigue	(25)
Trp11Stop	n/a	G>A	Premature stop codon, fatigue	(24)
H14R	rs148218218	A>G	Decreased capacity	*
H14Q	rs143058829	C>A	Decreased affinity	*
I48N	rs370353870	T>A	Low production/secretion	*
A51V	rs146744332	C>T	Low production/secretion	(23)
H89Y	rs187253929	C>T	Decreased affinity	*
L93H	rs113418909	T>A	Decreased affinity	(18)
E102G	n/a	A>G	Decreased capacity	(23)
I179V	rs367840035	A>G	Remaining activity after RCL cleavage	*
A224S	rs2228541	G>T	Association with fatigue	(26)
A256T	rs374119759	G>A	Antibody recognition difference	*
G237V	n/a	G>T	No binding activity	(21)
P246Q	n/a	C>A	No secretion	*
R260L	COSM26307	G>T	No binding activity	*
I279F	rs374191911	A>T	Remaining activity after RCL cleavage, decreased affinity	*
T349A	rs147101740	A>G	Antibody recognition difference	*
D367N	rs28929488	G>A	Decreased affinity, fatigue	(20)
W371S	rs267607282	G>C	No binding activity	(22)

* = newly identified

Table 3.2. Association of SNPs in the *SERPINA6* region with morning plasma cortisol.

SNP ID	Position on chromosome 14‡	Position relative to *SERPINA6*	Alleles	P[a]
rs11621961	94769476	1 kb downstream	C>T	$4.0x10^{-8}$
rs12589136	94827068	4 kb upstream	G>T	$3.3x10^{-12}$
rs2749527	94793686	30 kb upstream	C>T	$5.2x10^{-11}$

‡ Based on GRCh37.p13 Assembly

[a] adjusted for age and sex

Data were obtained by the CORtisol NETwork (CORNET) Consortium

Table 3.3 Functional consequences of SNPs in the *SERPINA6* region, in the Croatia-Korcula cohort.

rs11621961				
	TT	**CT**	**CC**	**P[a]**
Total cortisol[b]	655 (129)	664 (411)	675 (357)	0.305
Free cortisol	14 (27)	14 (70)	13 (67)	0.933
Cortisol-binding activity	503 (56)	492 (125)	531 (136)	0.016
9G12/12G2 CBG ELISA	0.76 (56)	0.77 (125)	0.76 (136)	0.940
rs12589136				
	TT	**GT**	**GG**	**P[a]**
Total cortisol[b]	706 (53)	681 (302)	655 (540)	0.017
Free cortisol	13 (39)	N/A	13 (125)	0.777
Cortisol-binding activity	557 (52)	552 (47)	491 (216)	$2.3x10^{-5}$
9G12/12G2 CBG ELISA	0.68 (52)	0.69 (47)	0.80 (216)	$9.4x10^{-11}$
rs2749527				
	CC	**CT**	**TT**	**P[a]**
Total cortisol[b]	659 (251)	668 (431)	674 (212)	0.495
Free cortisol	16 (60)	11 (51)	13 (53)	0.246
Cortisol-binding activity	486 (102)	507 (122)	545 (92)	$4.3x10^{-4}$
9G12/12G2 CBG ELISA	0.76 (102)	0.76 (122)	0.76 (92)	0.824
rs113418909 (Leuven, L93H)				
	AA		**AT**	**P[a]**
Total cortisol[b]	673 (792)		562 (28)	0.001
Free cortisol	14 (147)		13 (5)	0.924
Cortisol-binding activity	512 (279)		347 (9)	$1.9x10^{-6}$
9G12/12G2 CBG ELISA	0.76 (279)		0.82 (9)	0.146

[a]adjusted for age and sex
[b]Data for total cortisol is from the whole Croatia-Korcula sample cohort, n=898
Data presented are the mean (n). Total cortisol, free cortisol and cortisol-binding activity are given in nmol/L.
Total and free cortisol measures were completed by various collaborators.

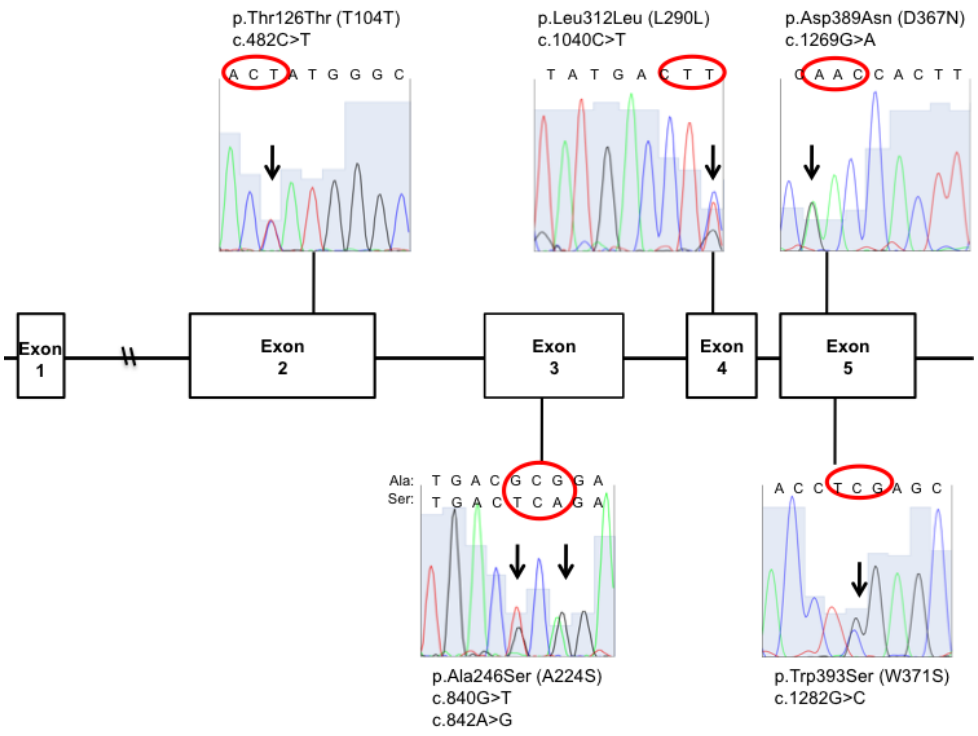


Figure 3.1. Sequence analysis of *SERPINA6* in genomic DNA (NCBI reference number NG_011796.1) from the proband. Two synonymous SNP were identified: one in exon 2 in the codon for Thr104 and another in exon 4 in the codon for Lys290. Two separate SNP were identified in exon 3 in the codon for Ala224 and only the G>T transversion resulted in a serine substitution. Exon 5 sequences revealed two non-synonymous SNP; a previously identified SNP (rs28929488) in the codon for Asp367 that results in an asparagine substitution, and a novel SNP (rs267607282) in the codon for Trp371 that introduces a serine residue at this position. Transitions and transversions in the DNA sequence are identified with arrows, and codons containing SNP are circled.

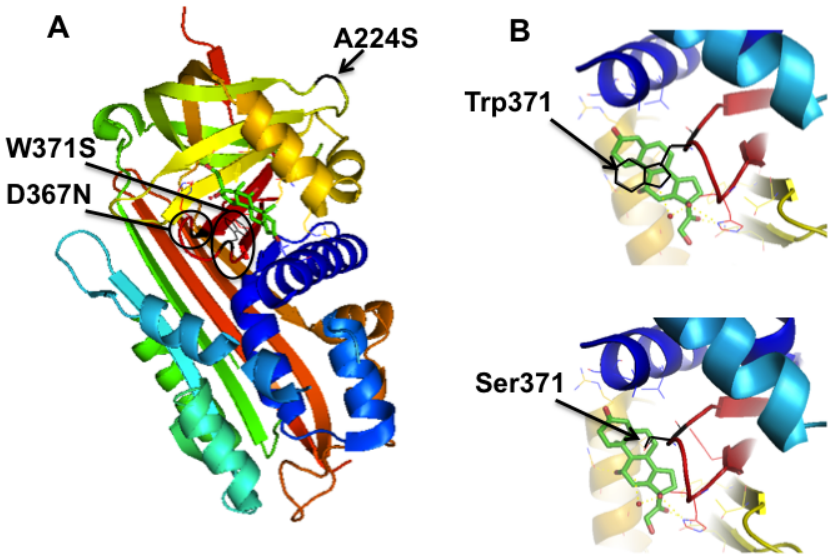


Figure 3.2. Tertiary structure of human CBG showing the location of amino acid substitutions encoded by *SERPINA6* SNP identified in the proband. (A) The position of the novel CBG W371S variant is shown, along with the previously identified naturally occurring CBG D367N and CBG A224S variants. β-sheet B strands 4 and 5, bearing residues Asp367 and Trp371, respectively, are colored *red*. The loop connecting β-sheet B strands 1 and 2, containing A224, is shown in *yellow*. *Black* coloring designates the amino acid specific to each variant within the tertiary structure of CBG. (B) The indole side chain of Trp371 is positioned immediately above the cortisol ligand (*top*), and is known to hydrogen bond with rings A, B and C of the steroid backbone (11). An interaction between this tryptophan and a conserved arginine residue in the amino-terminal region of CBG is also essential for maintaining the conformation of the steroid-binding site (11). Substitution of Trp371 with Ser (*below*) will fail to provide the same degree of interaction with cortisol or the ability to interact with Arg15. Cortisol in the steroid-binding pocket is shown in *green*. The human CBG (PDB ID 2VDY) model was developed using the SWISS-MODEL homology module (234) and illustrations were created using the program PyMol (http://pymol.sourceforge.net)).

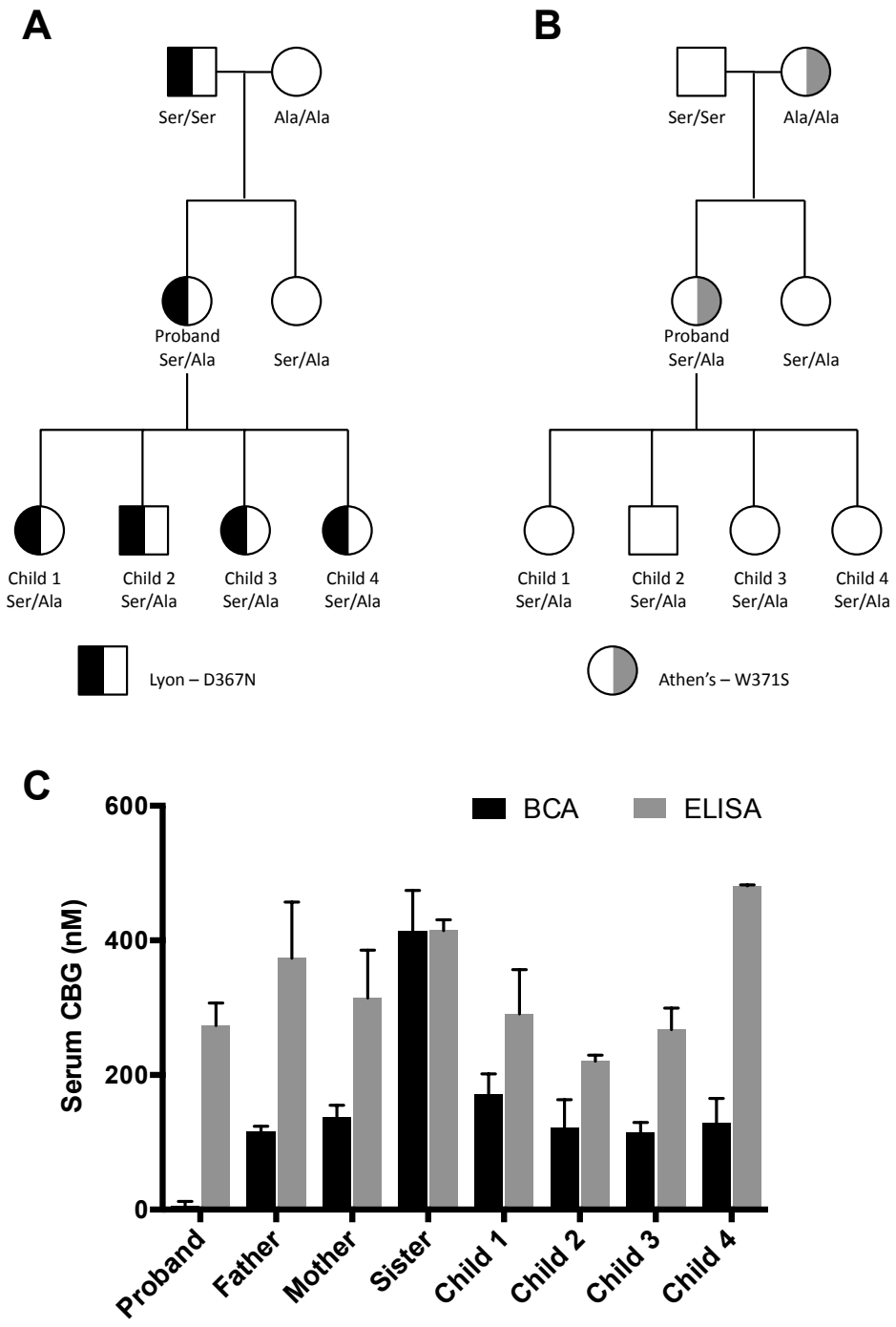

A
Ser/Ser
Ala/Ala
Proband
Ser/Ala
Ser/Ala
Child 1
Ser/Ala
Child 2
Ser/Ala
Child 3
Ser/Ala
Child 4
Ser/Ala
Lyon – D367N
B
Ser/Ser
Ala/Ala
Proband
Ser/Ala
Ser/Ala
Child 1
Ser/Ala
Child 2
Ser/Ala
Child 3
Ser/Ala
Child 4
Ser/Ala
Athen's – W371S
C
BCA
ELISA
Serum CBG (nM)
600
400
200
0
Proband
Father
Mother
Sister
Child 1
Child 2
Child 3
Child 4

Figure 3.3. Pedigree charts (A and B) and serum CBG measurements by cortisol-binding capacity and 12G2 ELISA in the proband's family (C). (A) Six family members were heterozygous for CBG D367N. (B) Two family members were heterozygous for the novel CBG W371S variant. Genotypes for the CBG A224S variant are displayed under the symbol for each individual. *Circles* represent females, *squares* represent males, and *half-filled symbols* represent heterozygous carriers of CBG D367N (black) and CBG W371S (grey). (C) Comparison of serum CBG levels measured by cortisol-binding capacity assay (BCA) and 12G2 ELISA. The compound heterozygous proband showed no cortisol-binding capacity, where as her mother, who is heterozygous for only the CBG W371S variant, had a ~50% reduction in cortisol-binding capacity. The proband's sister was not a carrier of either cortisol-binding deficient CBG variants, and had cortisol-binding capacity within the normal reference ranges (143, 205). The remaining family members were heterozygous for the CBG D367N mutation and demonstrated a ~50% decrease in CBG-cortisol binding.

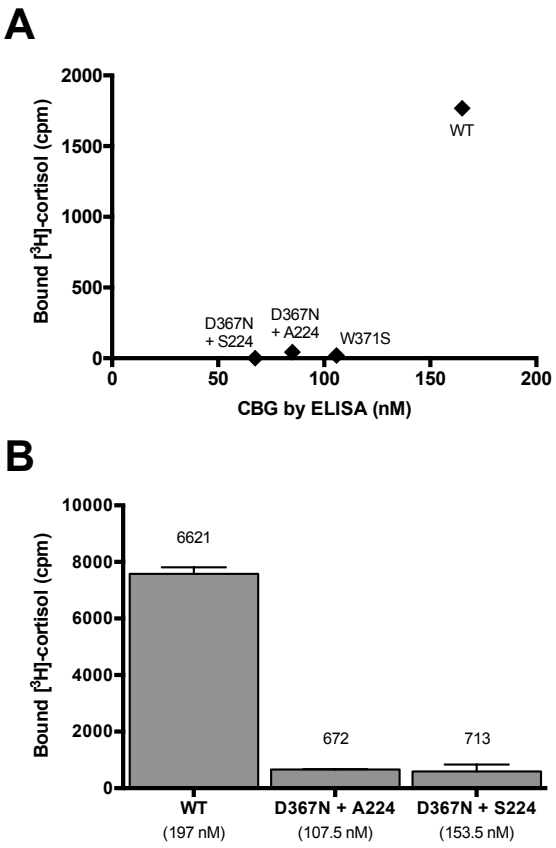


Figure 3.4. Characterization of recombinant wild-type (WT) and variant CBGs by cortisol-binding assay, 12G2 ELISA, and equilibrium dialysis analysis. (A) Recombinant CBG levels were assessed by both cortisol-binding capacity and 12G2 ELISA. The CBG variants, D367N + A224, D367N + S224 and W371S, had negligible cortisol-binding capacities despite normal concentrations by 12G2 ELISA. (B) When subjected to an equilibrium dialysis assay, a small amount of low affinity cortisol-binding was detected in the D367N + A224 and D367N + S224 CBG variants. CBG concentrations of recombinant proteins, as determined by 12G2 ELISA, are displayed in parenthesis.

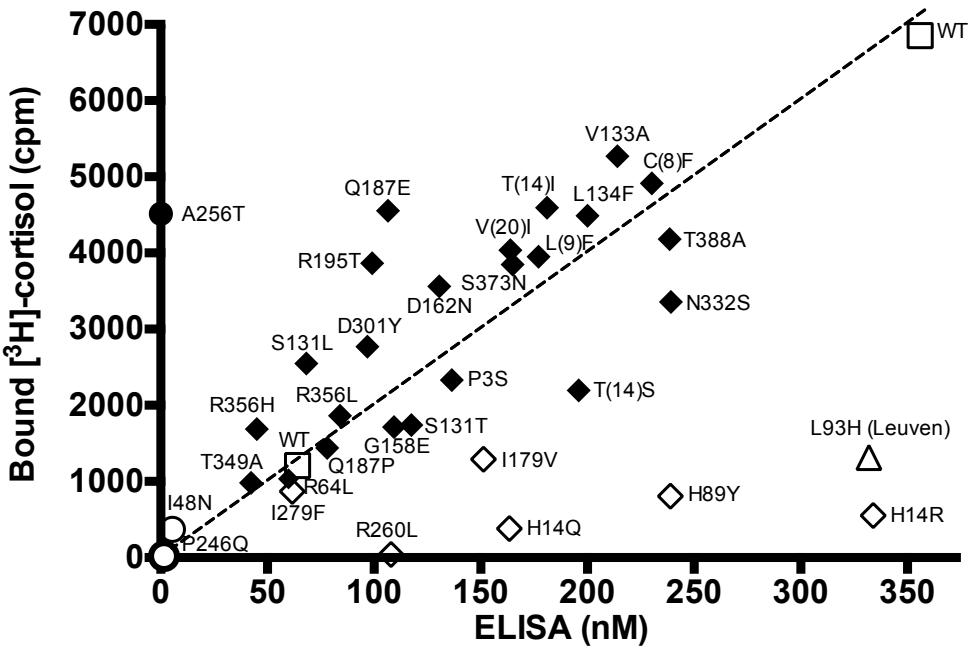


Figure 3.5. Identification of CBG variants that exhibit functional defects. Thirty-two previously uncharacterized CBG variants were produced as recombinant proteins and subjected to 12G2 ELISA and a cortisol-binding capacity assay to determine their CBG concentration and cortisol-binding activity, respectively. Variantss with potentially abnormal steroid-binding activity, i.e., with inappropriately low cortisol-binding capacity values *versus* 12G2 ELISA values, are identified by *white diamonds*, while candidates for decreased production/secretion are indicated by *white circles*. Two CBG wild-type preparations (at low and high concentrations) are indicated as *white squares* and connected by the dashed line. The CBG A256T was not detected by the 12G2 ELISA and is indicated as a *black circle*. The CBG L93H (Leuven), that is known to exhibit a decreased cortisol-binding affinity (14, 140), was included as a reference and is indicated as a *white triangle*.

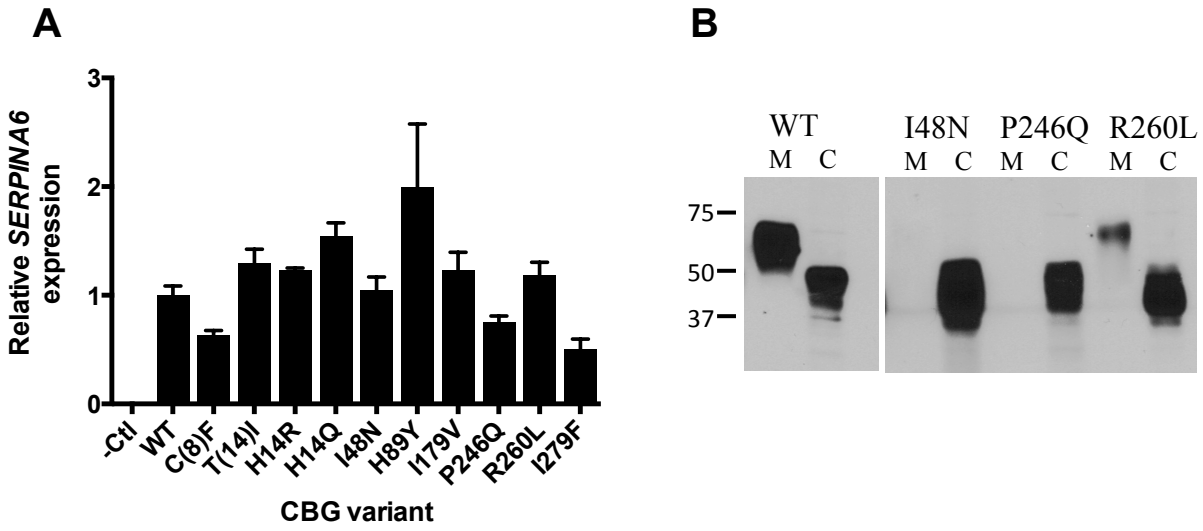


Figure 3.6 Identification of CBG variants that exhibit secretion defects. (A) qRT-PCR was performed on CBG variants that exhibited a functional defect to determine if their *CBG* mRNA levels were different from CBG WT, especially for those with low CBG production/secretion. Transfection efficiency of CHO cells is not responsible for the very low levels of some CBG variants in CHO medium. CBG variants with amino acid substitutions in the signal polypeptide sequence, i.e., C(8)F and CBG T(14)I, were included for comparison purposes. Negative control (-Ctl) represents cDNA prepared from untransfected CHO cells. (B) For CBG variants that exhibited low or negative 12G2 ELISA values, western blot analysis was performed to compare CBG levels in cell media to corresponding cell extracts. Immunoreactive CBG was detected in all cell extracts, indicating that all variants were produced in CHO cells, although no secretion was observed for P246Q, and very low secretion was observed for I48N (a faint band was visible with longer film exposure). The CBG R260L, that showed an absence of cortisol-binding activity, was included to assess protein integrity. The difference in molecular weight for CBG between protein solutions and cell extracts is most likely related to the degree of glycosylation. M: media; C: cell extract. Molecular size markers (kDa) are indicated. Data were obtained by Dr. Marc Simard.

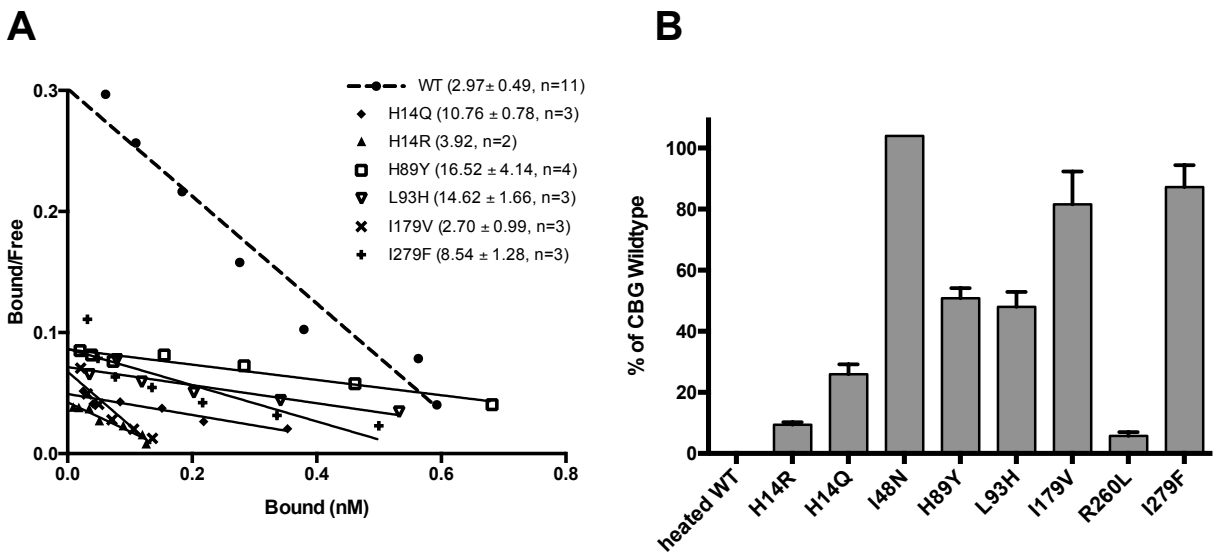


Figure 3.7. Characterization of recombinant CBG proteins by Scatchard analysis and equilibrium dialysis. (A) Scatchard plots obtained using [^{3}H]-cortisol for variants with a suspected binding defect versus CBG WT, with associated dissociation constant (Kd +/- SD) shown in parenthesis. A greater than 2.5-fold decrease in binding affinity was seen in CBG H14Q, H89Y, L115H (CBG Leuven) and I279F, when compared to CBG wild-type (WT). Note that CBG H14R and CBG L93H were assayed at 2-3 times higher amounts than other variants. (B) CBG variants (20 nM CBG, based on 12G2 ELISA values) were also subjected to equilibrium dialysis assays, to determine the relative amount of low affinity binding, in comparison to the CBG WT value that was set at 100%. No difference in binding was seen for CBG I48N, and a small difference was seen for CBG I179V and I279F (81.6 ± 10.7 % and 87.2 ± 7.2 %, respectively). An approximate 50% decrease in binding was observed for CBG H89Y and L93H. Much lower levels of binding were seen for CBG H14R (9.4 ± 0.8 %), H14Q (25.9 ± 3.3 %) and CBG R260L (5.76 ± 1.2 %). Variants and heat deactivated CBG WT (negative control) were assayed in triplicate, except for I48N (n = 2) and H89Y (n=4).

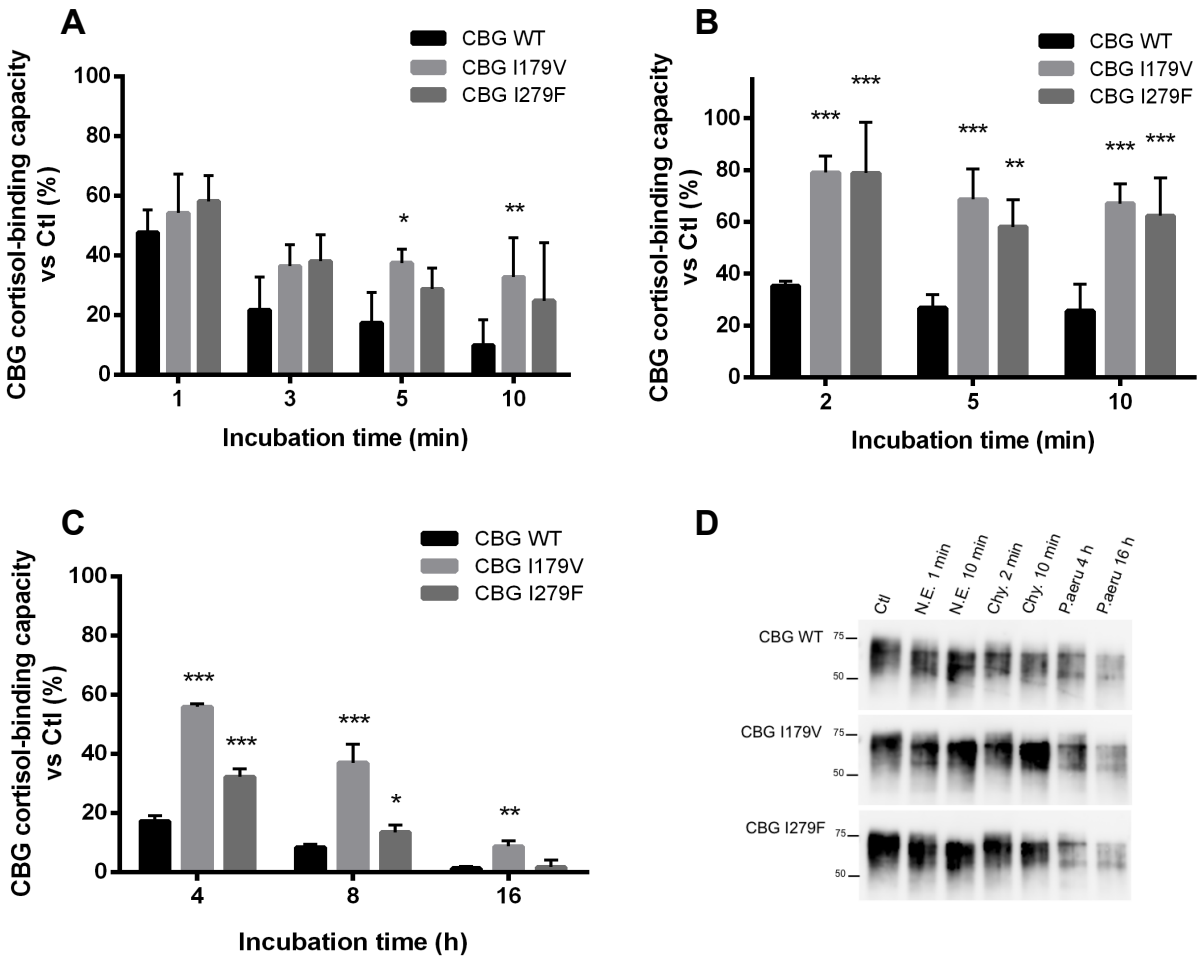

A
CBG WT
CBG I179V
CBG I279F
CBG cortisol-binding capacity vs Ctl (%)
100
80
60
40
20
0
1
3
5
10
Incubation time (min)
*
**
B
CBG WT
CBG I179V
CBG I279F
CBG cortisol-binding capacity vs Ctl (%)
***
***
***
**
***
***
2
5
10
Incubation time (min)
C
CBG WT
CBG I179V
CBG I279F
CBG cortisol-binding capacity vs Ctl (%)
***
***
***
*
**
4
8
16
Incubation time (h)
D
Ctl
N.E. 1 min
N.E. 10 min
Chy. 2 min
Chy. 10 min
P.aeru 4 h
P.aeru 16 h
CBG WT
CBG I179V
CBG I279F
75
50

Figure 3.8 Analysis of CBG variants that exhibit reduced sensitivity to proteolytic disruption of cortisol-binding activity. All CBG variants were tested in screening experiments for their sensitivity to protease cleavage, in which CBG I179V and CBG I279F showed a higher residual cortisol-binding activity than CBG WT and other CBG variants. To further examine this, CBG WT, CBG I179V and CBG I279F were subjected to time-course experiments to determine their rates of cortisol-binding activity loss following incubation with (A) neutrophil elastase, (B) human chymotrypsin, and (C) *P. aeruginosa* media; means ± SD are presented, n=3-5. In two-way ANOVA analysis, significant effects ($P < 0.0005$) of both incubation time and recombinant CBG sample type (CBG WT, CBG I179V and CBG I279F) were observed in time-course experiments after treatment with all three proteases. Furthermore, CBG I179V and CBG I279F exhibited significantly higher levels of residual steroid-binding activity (Tukey's *post hoc*: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$), when compared to CBG WT after treatment with the different proteases. (D) Western blots of CBG WT, CBG I179V and CBG I279F before and after incubations with different proteases at different times as in the time-course experiments. When compared to their untreated control (Ctl) samples, CBG WT, CBG I179V and CBG I279F all produced the typical ~5-10 kDa reduction in apparent molecular size that is consistent with cleavage of the RCL (115). N.E. : neutrophil elastase; Chy. : chymotrypsin; P.aeru : *P. aeruginosa* media. Molecular size markers (kDa) are indicated. Data were obtained by Dr. Marc Simard.

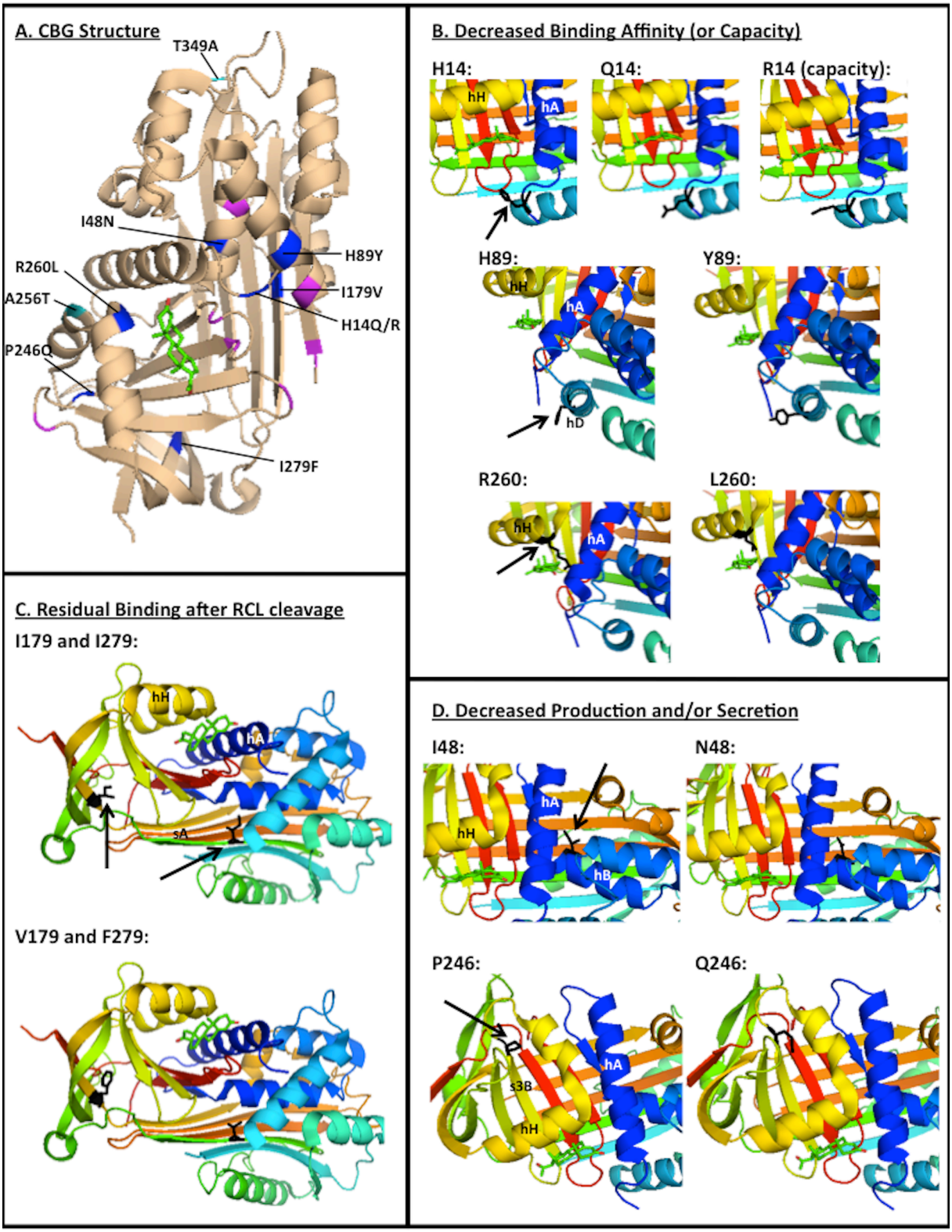

A. CBG Structure
T349A
I48N
H89Y
R260L
I179V
A256T
H14Q/R
P246Q
I279F
B. Decreased Binding Affinity (or Capacity)
H14:
Q14:
R14 (capacity):
hH
hA
H89:
Y89:
hD
R260:
L260:
C. Residual Binding after RCL cleavage
I179 and I279:
sA
V179 and F279:
D. Decreased Production and/or Secretion
I48:
N48:
hB
P246:
Q246:
s3B

Figure 3.9. Amino acid substitutions of CBG variants localized within a human CBG model. (A) The human CBG structure, depicting previously identified (*pink*) and newly identified (*blue*) variations that alter CBG function, immunochemical properties or production. Variants with discrepant ELISA values are shown in *cyan*. Variants that exhibit: (B) a decrease or absence of cortisol-binding activity, (C) abnormal residual cortisol-binding activity after protease incubation, or (D) low or absence of production/secretion, are shown. For each variant (in panels B, C and D), the normal and substituted amino acids are shown, with the specific amino acid indicated in *black*. Helices A, D, and H are colored *dark blue*, *light blue*, and *yellow*, respectively. Beta-sheets s3A and s5A are colored *green* and *light orange*, respectively. Beta-sheets s2B and s3B are colored *yellow*, and beta-sheets s4B and s5B are colored *red*. Progesterone in the steroid-binding pocket is shown in *green*. For representations, the human CBG (PDB ID 4BB2) model was used, as described in **chapter 3.2**.

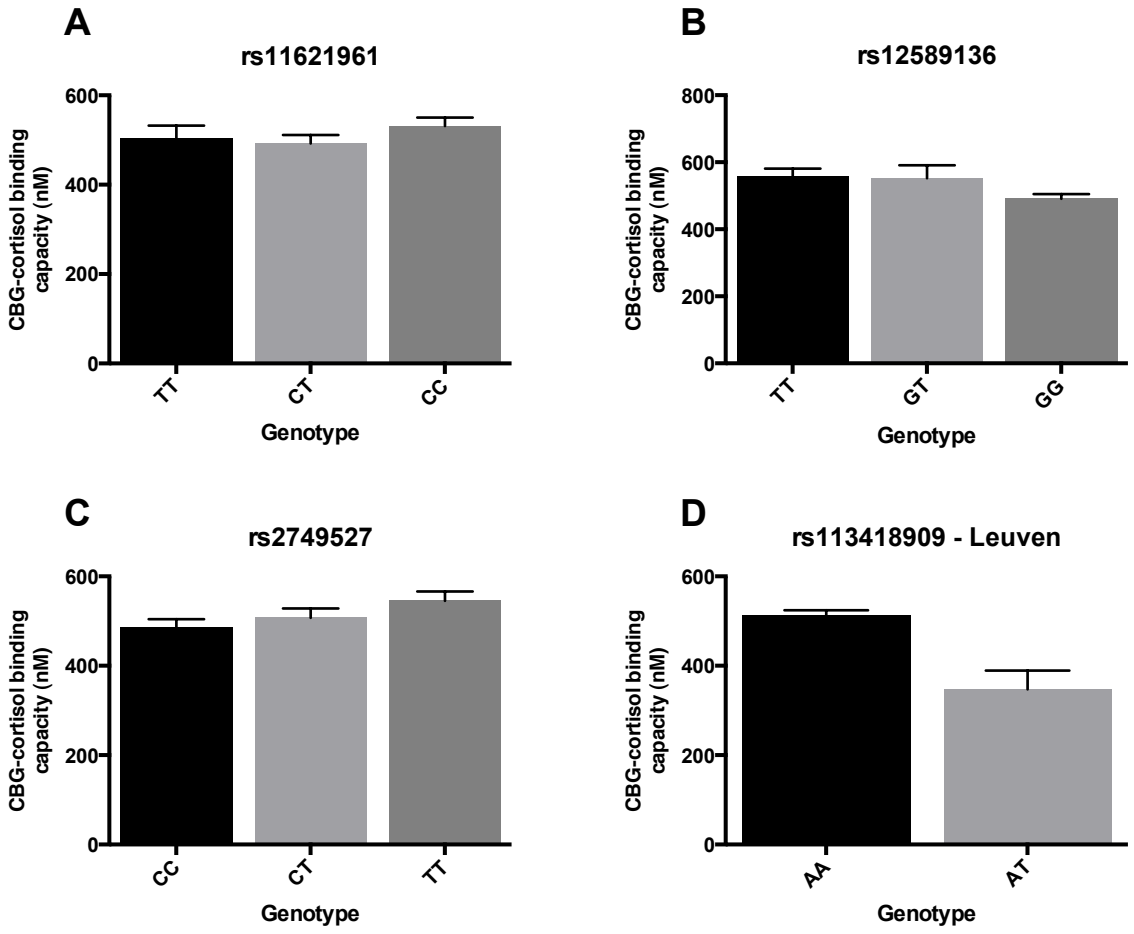


Figure 3.10 Differences in CBG-cortisol binding capacity in relation to variants in the *SERPINA6* locus. The CBG-cortisol binding capacity was measured in CROTIA-Korcula samples (n=317). (A-C) All three *SERPINA6* locus variants that were associated with plasma total cortisol levels were also associated with differences in plasma CBG-cortisol binding activity; P=0.016, P=2.3x10^{-5} and P=4.3x10^{-4}, respectively. (D) The previously identified CBG Leuven (L93H) variant (ref) was also identified and heterozygous carriers were found to have significantly reduced CBG-cortisol binding capacity values, P=1.9x10^{-6}. For each genotype, the data is expressed as the mean ± 95% confidence interval.

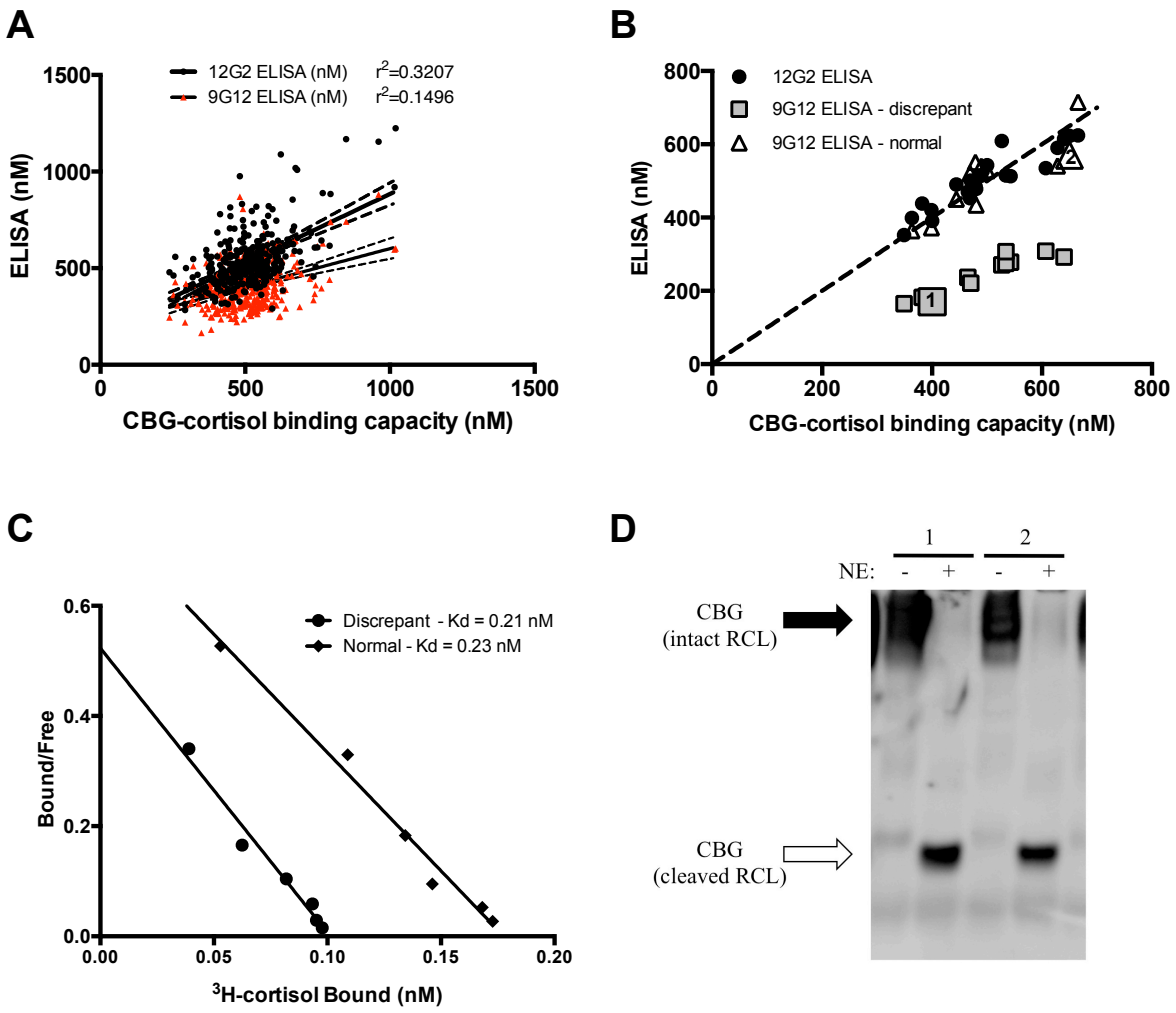

A
12G2 ELISA (nM) r2=0.3207
9G12 ELISA (nM) r2=0.1496
ELISA (nM)
CBG-cortisol binding capacity (nM)
B
12G2 ELISA
9G12 ELISA - discrepant
9G12 ELISA - normal
ELISA (nM)
CBG-cortisol binding capacity (nM)
C
Discrepant - Kd = 0.21 nM
Normal - Kd = 0.23 nM
Bound/Free
3H-cortisol Bound (nM)
D
1
2
NE: - + - +
CBG
(intact RCL)
CBG
(cleaved RCL)

Figure 3.11 The discrepancy in 9G12 ELISA measurements of CBG in human plasma samples is not due to the presence of RCL cleaved CBG. (A,B) Plasma CBG levels were determined by cortisol-binding activity, 12G2 and 9G12 ELISAs. (A) A moderate correlation was found between CBG-cortisol binding capacity and 12G2 ELISA values (*black*, r^2=0.32), whereas there was no correlation between CBG-cortisol binding capacity and 9G12 ELISA values (*red,* r^2=0.15). The best-fit linear regression (*solid*) line and 95% confidence interval (*dotted*) are shown and the slopes of the linear regression lines significantly differed (P<0.0001). (B) In a majority of samples (only representative samples from panel A are shown), the CBG values obtained for all three assays were similar. In a small subset of individuals, the 9G12 ELISA values (*grey square*) were much lower (50-60%) than those obtained by binding assay or 12G2 ELISA. The *dashed* line is provided for reference and represents the unity between CBG-cortisol binding capacity and ELISA values. (C) No differences in the cortisol-binding affinity (Kd) of plasma CBG were observed between a normal (ELISA values match cortisol binding capacity assay values) and a discrepant (large discrepancy between ELISA values) sample; Kd= 0.21 and 0.23 nM, respectively. (D) There is no evidence of CBG RCL cleavage in plasma that exhibits a discrepancy in ELISA values. Plasma samples 1 (normal) and 2 (discrepant) were further analyzed by non-denaturing PAGE after being subjected to a heat ramp (up to 70ºC at a rate of 0.1ºC/s). Under these conditions, CBGs with an intact RCL polymerize (solid arrow) while CBG with an RCL that is cleaved by neutrophil elastase (NE) does not (open arrow) (13).

Chapter 4: Roles of human CBG during infections and acute inflammation

4.1 Introduction

During acute inflammation, CBG responds as an acute phase "negative" protein, with significant reductions in plasma CBG levels noted in individuals with various pathological conditions, including severe burn injury (23), open-heart surgery (24), multi-trauma (169), acute pancreatitis (170), sepsis (25) and septic shock (25, 171, 172). Such reductions in plasma CBG levels during inflammation have been attributed to both proteolytic cleavage (9) and down-regulation of its production by the liver (23, 56, 77).

Similar to other SERPINs, CBG has a surface-exposed RCL that serves as a "protease bait domain" (37). When proteolytically cleaved, the CBG RCL inserts into the protein core and this results in a conformational change that greatly reduces the affinity of CBG for cortisol (115). The loss of CBG steroid-binding activity causes a significant redistribution of plasma cortisol between the albumin-bound and unbound or "free" fractions, enhancing glucocorticoid bioavailability at sites of infection or inflammation (9). When CBG primary structures are compared between species, the RCL represents a poorly conserved region, and it has been proposed that the CBG RCL has rapidly evolved to respond to proteases secreted by species-specific pathogens (235). Prior to these studies, the only protease known to cleave human CBG was neutrophil elastase, and this was shown to occur at a specific site (8, 9, 115).

Studies in **chapter 4.2** came about as an unexpected finding in a crystal structure, where human CBG displayed the typical "relaxed" conformation of a SERPIN that occurs after proteolytic cleavage of the RCL (13). This was surprising, as the protein had not been treated with a protease prior to crystallization. Importantly, RCL cleavage occurred at a different position from the known neutrophil elastase cleavage site, suggesting the presence of an

unknown protease. Given that the RCL of α1-antitrypsin (*SERPINA1*) is cleaved by bacterial proteases (236), we set out to determine whether CBG is specifically targeted by proteases secreted by a variety of bacteria, a number of which are produced by common human pathogens.

In **chapter 4.3**, we sought to characterize plasma CBG levels in an ICU patient cohort using a cortisol-binding capacity assay and two CBG ELISAs that have been used to discriminate between intact and proteolytically cleaved CBG (217). In addition, investigations were undertaken to determine how plasma CBG levels in ICU patients are linked to the basis of their illness and why the cleavage of CBG in many of these samples is resistant to *Pseudomonas aeruginosa*-mediated cleavage of the CBG RCL.

4.2 *Pseudomonas aeruginosa* elastase disrupts the cortisol-binding activity of corticosteroid-binding globulin

4.2.1 Results

4.2.1.1 *P. aeruginosa culture media specifically disrupts human CBG-cortisol binding*

Culture media from various bacterial isolates were examined to determine if they were capable of disrupting the cortisol-binding activity of human CBG (Figure 4.1). After 16 h incubations at 37°C, only medium from *P. aeruginosa* significantly reduced the CBG-cortisol binding capacity (Figure 4.1). When *P. aeruginosa* medium was tested at a 90 times lower concentration, we again observed a significant reduction in the CBG-cortisol binding capacity, demonstrating that the activity in the *P. aeruginosa* medium was substantially greater than that of any other pathogen tested.

4.2.1.2 Loss of CBG-cortisol binding after incubation with P. aeruginosa medium is temperature, pH, and zinc dependent

In time-course incubations of human serum and *P. aeruginosa* culture medium at different physiological temperatures, the CBG-cortisol binding capacity decreased progressively between 1 h and 16 h (Figure 4.2A). Over the time points studied, the CBG-cortisol binding capacity was significantly lower ($P < 0.001$) after incubations at 41°C than at 37°C, with a loss of ~45% and ~70% occurring by 1 h and 4 h, respectively, in the samples incubated at 41°C. In addition, after 8 h incubations, the remaining activity was significantly lower ($P = 0.0065$) at pH 7 than at pH 6 and 8 (Figure 4.2B). At all pH conditions, a greater decrease in cortisol-binding capacity was observed at 41°C than at 37 and 39°C, and this is consistent with results presented in Figure 4.2A.

To characterize the type of protease involved in disrupting CBG-cortisol binding, different protease inhibitors were tested: PMSF as a serine and cysteine protease inhibitor, EDTA as a non-specific chelator of divalent cations, and TPEN as a potent and specific zinc chelator (Figure 4.2C). After incubation with *P. aeruginosa* culture medium at 37°C for 16 h, PMSF failed to protect the loss of CBG-cortisol binding capacity in human serum, while EDTA was partially protective and TPEN was fully protective. These results suggested that a zinc-dependent metalloprotease is responsible for CBG RCL cleavage.

4.2.1.3 Pseudomonas aeruginosa elastase is the protease responsible for CBG cleavage at a specific location within the RCL

To identify the protease(s) responsible for CBG cleavage, several FPLC purification steps were performed on *P. aeruginosa* culture media. First, media was injected onto a HiTrap Q FF strong anion exchange column. Only the flow-through material influenced CBG-cortisol binding,

suggesting that the protease(s) responsible for CBG cleavage are not negatively charged at physiological pH. Concentrated flow-through was injected on a Superdex75 size exclusion column and proteolytic activity was observed in two fractions containing proteins eluting within a 14 - 43 kDa size range. Gel electrophoresis of an active fraction revealed the presence of three bands of approximately 33, 27, and 14 kDa (Figure 4.3A). Tryptic in-gel digestion and peptide extraction were performed on the three bands to allow for mass spectrometry analysis. The ~33 kDa band was identified as *P. aeruginosa* elastase (PAE or LasB), the ~27 kDa was identified as lysyl endopeptidase, (or protease IV), and the ~14 kDa band was identified as a C-terminal portion of PAE.

To determine the protease cleavage site(s), purified CBG (9) was incubated with an active fraction isolated from *P. aeruginosa* media (as described above), and the product was analyzed by mass spectrometry. Four peptides generated from adjacent cleavage sites in the RCL of CBG were identified, with the major cleavage site identified between Asn347 and Leu348 (Figure 4.4).

4.2.1.4 Loss of CBG-cortisol binding after incubation with purified PAE and absence of effect of the protease IV inhibitor TLCK

The ability of 1 µg of purified PAE to cleave human serum CBG was examined in a time-course experiment, and ~50% of CBG-cortisol binding capacity was lost after 16 h of incubation at 37°C (Figure 4.3B), thus confirming the involvement of this metalloprotease in the proteolytic activity of *P. aeruginosa* media.

Protease IV, the other protein detected in the active chromatographic fraction of *P. aeruginosa* medium (Figure 4.3A), is a serine protease that also acts as a virulence factor (237). To determine if protease IV targets CBG, experiments using TLCK, a known irreversible

inhibitor of protease IV, were performed (Figure 4.3C). The results excluded any direct action of protease IV on CBG, as TLCK did not influence the ability of *P. aeruginosa* media to disrupt CBG-cortisol binding capacity.

4.2.1.5 PAE-deficient strains of P. aeruginosa do not disrupt CBG-cortisol binding capacity

To further establish a role of PAE in CBG RCL cleavage, human serum was incubated with media from cultures of PAE-deficient strains of *P. aeruginosa* and the effect on CBG-cortisol binding capacity was compared to that of the parent strain (Figure 4.3D). These results confirm that PAE is the protease secreted by *P. aeruginosa* that is mainly responsible for disrupting the CBG-cortisol binding capacity.

4.2.2 **Discussion**

Bacterial pathogens secrete a number of proteases as virulence factors that act to accentuate their ability to cause disease (206, 238). Previous studies have shown that the RCL of α1-antitrypsin is targeted by several bacterial proteases, including PAE secreted by *P. aeruginosa* (236). Therefore, it is notable that among the bacteria tested, only *P. aeruginosa* potently disrupted CBG-cortisol binding activity through cleavage of its RCL. Our identification of PAE as the *P. aeruginosa* protease responsible for cleavage of the CBG RCL is supported by several observations. First, incubation of human serum with P. *aeruginosa* media was inhibited by the removal of zinc ions that are essential for the activity of this metalloprotease. Second, PAE was identified by mass spectrometry as one of only two proteins in a proteolytically active chromatographic fraction of *P. aeruginosa* medium. Third, commercially available purified PAE disrupted the CBG-cortisol binding capacity, whereas media from PAE-deficient *P. aeruginosa* strains were unable to disrupt the cortisol-binding activity of CBG. Finally, while protease IV

was also detected in the active chromatographic fraction of *P. aeruginosa* medium, our results indicate that this protease has no effect on CBG-cortisol binding.

PAE is a zinc-metalloprotease that has been extensively studied as a virulence factor secreted by *P. aeruginosa* (239, 240). It shows optimum proteolysis at pH 7-8, and its activity and stability are zinc- and calcium-dependent, respectively. PAE is the most abundant extracellular endopeptidase secreted by *P. aeruginosa* and is commonly found in sputum samples of chronically infected cystic fibrosis patients (241). Its proteolytic action causes tissue destruction as well as modulation of the host immune response; for example, PAE is known to degrade matrix components, complement factors, cytokines, and to inactivate host protease inhibitors such as α1-antitrypsin and α-2-macroglobulin (240, 242). Clinically, different inhibitors of PAE have been developed as a potential second-generation class of antibiotics that may minimize the emergence of resistant strains (240, 243). Our results suggest that these inhibitors would likely also modulate CBG cleavage and therefore, the release of bound cortisol.

Mass spectrometry results revealed that CBG incubated with semi-purified *P. aeruginosa* medium was preferentially cleaved within its RCL domain between Asn347 and Leu348, with additional adjacent secondary cleavage sites also identified. It has been reported that PAE favours hydrophobic or aromatic amino acid residues at the P1' position (239, 244), and the main cleavage site identified is consistent with those findings. All cleavage sites identified differ from the one between Val344 and Thr345, observed after cleavage by neutrophil elastase (8, 9) and the one after Thr349 or Ser350, observed in the recent crystal structure of human CBG bound with progesterone (13). Cleavage of the CBG RCL in this crystal structure was unexpected, and a protease produced by another microorganism may have been responsible for this. However, it is also possible that presentation of the RCL of *E. coli*-produced recombinant CBG bound to

progesterone may differ from the RCL of serum CBG bound to cortisol, thereby altering how the RCL is targeted by proteases. In this regard, steroid occupancy of the CBG-steroid binding site may alter the conformation and surface presentation of the RCL (115). In addition, notably, human plasma CBG is extensively glycosylated (133), whereas *E. coli* expressed CBG lacks glycosylation. This is particularly important in the context of CBG RCL cleavage, due to the presence of a consensus sequence for *N*-glycosylation within the RCL. The glycosylation site within the CBG RCL is utilized in about 80% of CBG molecules, with the composition of *N*-glycans attached varying considerably (133). Heterogeneity in glycosylation at this site may explain why we observed several secondary cleavage sites adjacent to the main site. Therefore, proteolytic cleavage of the CBG RCL may be modulated by the presence and/or type of *N*-glycan within the RCL.

After incubation with neutrophil elastase, CBG looses its cortisol-binding activity rapidly (9). In contrast, incubation of human serum with *P. aeruginosa* medium for 5 min only results in a 20% reduction in the cortisol-binding capacity of CBG, and this is followed by a progressive loss over the hours that follow. The explanation for this may be related to differences in the presentation or recognition of the CBG RCL or to the presence of inhibitors in serum, such as α2-macroglobulin, which limit the activities of bacterial proteases (245). The *P. aeruginosa* induced loss of CBG-cortisol binding capacity was consistently more efficient at 41°C when compared to 37°C, and this is of interest because increases in temperature over this range also decrease the steroid-binding capacity of CBG (168). In cases of infections accompanied by fever, any increase in proteolytic activity would act in concert with a decrease in CBG-steroid binding to enhance free cortisol levels. In addition, PAE-induced cortisol release from CBG may vary

according to the pH of an infected area, which will likely be different between, for example infected burn wounds (246) and airways (247).

As one of the most common opportunistic pathogens, *P. aeruginosa* is involved in a variety of pathologic complications involving wounds (248), burn infections (249), septicemia (250), and pneumonia (251), and is a major cause of chronic lung infections in cystic fibrosis patients (252). *P. aeruginosa* infections lead to a pro-inflammatory environment with reductions in bacterial clearance, leukotaxis, and excess immune, inflammatory, oxidative, and proteolytic activities, which trigger further inflammation and tissue damage (253). Recruited, activated neutrophils secrete neutrophil elastase that can rapidly cleave the RCL of CBG, increasing the local concentration of free cortisol that will be available to target cells (8, 9). In addition, we have shown here that the secreted protease, PAE, is also able to cleave CBG. The combined effects of neutrophil elastase and PAE on CBG at sites of infection/inflammation will lead to an increased cortisol release and an accelerated depletion of the cortisol reservoir. Further, PAE may also modulate the cleavage of CBG by neutrophil elastase through its ability to cleave AAT without forming inhibitory complexes, thereby allowing neutrophil elastase to escape capture by AAT (254). However, the production of free radicals by neutrophils has been shown to cause the reversible oxidation of Met358 in the RCL of AAT, greatly reducing its ability to inhibit neutrophil elastase (255). Similarly, the ability of PAE to cleave oxidized α1-antitrypsin has been shown to be reduced (236). Therefore, a complex interplay between various protease and SERPIN interactions most likely occurs, ultimately modulating free cortisol levels, and this will be discussed more fully below (**chapter 4.3**).

In summary, we discovered that the *P. aeruginosa* virulence factor, PAE, specifically cleaves the RCL of human CBG at novel sites, leading to a loss of CBG-cortisol binding activity.

In the control of infectious and inflammatory diseases of tissues like the lung and skin, where *P. aeruginosa* infections are common, the cleavage of CBG by PAE may augment the actions of neutrophil elastase, promoting the localized release of cortisol. Increases in free cortisol levels at these sites will have a broad range of actions, including modifying the production of cytokines, free radicals, prostaglandins and chemotactic factors, by various cell types involved in the inflammatory response, as well as controlling the activity of infiltrating immune cells (256). Understanding the relative contribution of PAE and neutrophil elastase to these processes may be relevant in optimizing the dosage and route of glucocorticoid administration to treat inflammation associated with severe *P. aeruginosa* infections.

4.3 Investigations of plasma CBG in an ICU patient cohort

4.3.1 **Results**

4.3.1.1 CBG-cortisol binding capacity and protein levels

Plasma CBG levels were measured by cortisol-binding capacity and two CBG ELISAs in ICU patients (n=134) admitted for various etiologies. In consultation with Dr. Vassiliadi, patients were grouped based on their diagnoses. Significant differences in plasma CBG-cortisol binding capacity were found when comparing different patient populations (P=0.0238; Figure 4.5). Patients with cardiac and brain related diagnoses had significantly higher ($P < 0.05$) CBG-cortisol binding capacity values when compared to those with inflammatory related diagnoses.

When comparing the mean plasma CBG-cortisol binding capacities of the various patient groups to the mean of a healthy control population from the CROATIA-Korcula cohort (**chapter 3.4**), significant differences were detected (P=0.0007). Significantly lower CBG-cortisol binding capacity values were detected in patients with renal failure and burns ($P < 0.05$), trauma,

pneumonia, respiratory failure and gastrointestinal diagnoses ($P < 0.01$), as well as individuals with inflammation ($P < 0.001$), when compared to the control mean (Figure 4.5).

In addition, plasma CBG levels were assessed using the 12G2 and 9G12 CBG ELISAs, and compared to corresponding CBG-cortisol binding capacity values (Figure 4.6A-C). The strongest correlation was observed between plasma CBG-cortisol binding capacity and 12G2 ELISA values (r^2=0.6351; Figure 4.6A). Correlations between CBG levels determined by 9G12 ELISA, and CBG-cortisol binding capacity (Figure 4.6B) or 12G2 ELISA (Figure 4.6C) were very weak, with $r^2 = 0.1830$ and 0.1427, respectively.

4.3.1.2 Incubation of ICU samples with P. aeruginosa conditioned media

After establishing that *P. aeruginosa* media is able to proteolytically cleave the CBG RCL (**chapter 4.2**), it was of interest to determine if plasma CBG from ICU samples could also be cleaved by neutrophil elastase and *P. aeruginosa*. Incubations with neutrophil elastase resulted in significant reductions (~13-18% of baseline control) in the CBG-cortisol binding capacity of plasma from both healthy controls and ICU patients (Figure 4.7A). By contrast, when a subset of randomly selected ICU plasma samples were incubated with complete *P. aeruginosa* media, all of the ICU samples had high (~85-90%) residual CBG-cortisol binding levels when compared to a healthy control sample, which had approximately 30% residual binding (Figure 4.7B). This suggested that some of the ICU samples contained a factor that specifically inhibited CBG RCL cleavage by *P. aeruginosa* media.

To determine what was responsible for *P. aeruginosa* inhibition, proteins from an ICU plasma sample that exhibited this property were separated and purified by FPLC using anion exchange and gel filtration columns. At each stage of chromatography, fractions were tested for their ability to inhibit cleavage of the CBG RCL by *P. aeruginosa* media. In brief, diluted (1:10)

ICU plasma was applied to a HiTrap Q FF anion exchange column and 1 mL fractions were collected. Eight fractions were able to inhibit CBG RCL cleavage by *P. aeruginosa* media (Figure 4.8A). The active fractions were then pooled and loaded onto a Mini Q anion exchange column, with six fractions demonstrating inhibitory activity (Figure 4.8B). After concentration, the active Mini Q fractions were applied to a Superdex 200 gel filtration column and four fractions with *P. aeruginosa* inhibitory activity were identified (Figure 4.8C).

Based on known elution volumes of standard proteins from the Superdex 200 column, the plasma protein responsible for inhibiting *P. aeruginosa*-mediated CBG RCL cleavage coincided with a molecular size between 440 and 660 kDa, and alpha-2-macroglobulin (α2M) is a broad range protease inhibitor that has a molecular size consistent with this. To determine if α2M was the protein responsible for *P. aeruginosa* inhibition, purified α2M was incubated with *P. aeruginosa* media (Figure 4.8D,E) or purified PAE (Figure 4.8E), and, as predicted, CBG RCL cleavage was inhibited, as indicated by significantly greater CBG-cortisol binding capacity in reactions containing α2M ($P<0.01$).

4.3.2 **Discussion**

During inflammation, human CBG acts as an acute phase negative protein (23, 25, 170), with similar findings made in various animal models of acute inflammation, where decreased CBG levels have been reported after thermal injuries to mice and rats (176, 177) and in pigs treated with lipopolysaccharide (178). In line with these findings, we observed decreased plasma CBG levels in an ICU patient population with a variety of severe illnesses. Differences were seen between the various patient populations, with the lowest plasma CBG-cortisol binding capacity levels observed in individuals presenting with a clinical profile that included inflammation, such as peritonitis and pancreatitis. When compared to healthy controls, significant differences in the

CBG-cortisol binding capacity were not found in patients with cardiac or brain-related illness, including cardiac arrest, cardiogenic shock, subdural hematoma, altered mental status, stroke and cerebral hemorrhaging. In line with previous studies (23, 169), a reduction in plasma CBG occurred following trauma and burn injuries. In the cohort we studied, reductions in plasma CBG levels were also noted in patients with various gastro-intestinal and inflammatory insults, as well as in individuals with renal failure, respiratory failure and pneumonia. Given these findings, there appears to be differences with respect to changes in CBG levels following certain pathological insults, with severe inflammatory conditions leading to reduced plasma CBG levels.

The decreases in plasma CBG-cortisol binding capacity observed here may reflect CBG RCL cleavage and/or a decrease in hepatic CBG production in response to increases in the plasma levels of corticosterone (45) and proinflammatory cytokines (46). The human CBG RCL is a known target for neutrophil elastase (42), resulting in significant decreases in its binding affinity for cortisol (9). Recent studies have shown that chymotrypsin also has a similar but somewhat less specific ability to cleave the human CBG RCL (120), and our studies have extended this to PAE produced by *Pseudomonas aeruginosa (***chapter 4.2**). It has been postulated that human plasma CBG is rapidly removed from the blood circulation following CBG RCL cleavage (233), thereby enhancing its plasma clearance rate. However, an inverse relationship has been observed between plasma IL-6 and CBG levels in humans (23, 79), and studies utilizing HepG2 cells have reported an IL-6 induced reduction in CBG production (77) that has been associated with decreased *SERPINA6* mRNA stability (78).

The dynamic changes in plasma CBG levels observed during inflammation are expected to modulate the availability of cortisol to its target cells, thereby affecting the inflammatory reaction as well as the healing process. Importantly, CBG RCL cleavage by proteases present at

sites of inflammation is believed to allow for the targeted release of anti-inflammatory glucocorticoids (9, 10). In addition, during recovery, the normalization of CBG levels is likely to play an important role in restoring the normal homeostatic balance of glucocorticoids.

Recently, ELISAs that use highly specific monoclonal antibodies have been utilized to discriminate between CBG with an intact versus cleaved CBG RCL (217). Using the 12G2 ELISA, total CBG levels are measured and compared to values obtained using the 9G12 ELISA that only detects CBG with an intact or un-cleaved RCL. Through the parallel use of these ELISAs, it has been reported that RCL cleaved CBG makes up approximately 30% of total CBG levels in healthy human blood samples (209). Using the same immunoassays, altered levels of cleaved CBG have been reported in various patient groups, with paradoxically reduced levels of cleaved CBG found in patients with abdominal obesity (257), alpha-1-antitrypsin deficiency (258) and active rheumatoid arthritis (259). Furthermore, in another similar study investigating plasma CBG levels in patients with sepsis and septic shock, a significant linear trend was observed between plasma levels of cleaved CBG and illness severity (260). However, in all of these studies from the same group of researchers, evidence that cleaved CBG exists within the human blood circulation is based solely on differences observed in immunoassays that rely on the use of specific monoclonal antibodies, and direct biochemical evidence of CBG RCL cleavage in the blood samples analyzed is lacking.

As previously noted (**chapter 3**), there are significant limitations of solely relying on immunoassays to determine plasma CBG concentrations, as they do not reflect the steroid-binding activity of CBG. This is particularly important in the context of assessing CBG levels using the 12G2 and 9G12 ELISAs, where lower 9G12 ELISA values are interpreted as representing the presence of RCL cleaved CBG despite a lack of direct evidence. In this study,

we also assessed the plasma concentration of functional CBG using a cortisol-binding capacity assay, allowing us to compare functional and immunoreactive CBG levels. Similar to **chapter 3.4**, the strongest correlation was seen between CBG-cortisol binding capacity and 12G2 ELISA values (r^2=0.6351), with weak correlations observed between the 9G12 ELISA and CBG-cortisol binding capacity (r^2=0.1427) and 12G2 ELISA (r^2=0.1830) values, respectively. Given that proteolytically cleaved CBG lacks high-affinity steroid binding (9), if alterations in 9G12 ELISA values represent the presence of RCL cleaved CBG within the circulation, CBG-cortisol binding capacity levels would be expected to correlate closely with those obtained by the 9G12 ELISA, which is not the case.

It should be noted that the epitope within the CBG RCL, corresponding to the synthetic unglycosylated peptide that the 9G12 antibody was raised against, contains an *N*-glycosylation site (Asn347) that is almost always utilized (133). Therefore, it is possible that differences in the presence or composition of glycans at Asn347 likely alter the recognition of plasma CBG by the 9G12 antibody and account for the observed difference in CBG levels measured by the cortisol-binding capacity assay and 12G2 ELISA. It is well established that during inflammatory diseases, changes in the glycosylation of serum proteins occur (261) as a result of altered expression and localization of biosynthetic enzymes, such as glycosyltransferases (262). These quantitative and qualitative alterations in glycosylation vary depending on a number of factors, including the type of inflammation, disease activity and the presence of concurrent infection (262). Given this, significant differences in CBG glycosylation are expected between different ICU patient populations and even between individuals with the same diagnosis, depending on disease severity, and this may therefore influence the recognition of plasma CBG by the 9G12 antibody in ways that could either reduce or enhance epitope recognition within the RCL.

Alpha-2-macroglobulin is an abundant general protease inhibitor within plasma, comprising as much as 8-10% of total serum proteins (263). Within the circulation, α2M accomplishes protease inhibition through an irreversible trapping mechanism, whereby cleavage of a bait region on α2M by target proteases results in the formation of a covalent bond between the two molecules (245). Following trapping, the α2M-protease complex is cleared from the blood circulation by receptor-mediated endocytosis (264). As a general inhibitor, α2M shows a broad range of reactivity, inhibiting endogenous and exogenous proteases, including serine, cysteine, aspartate and metallo-proteases (265). Clinically, the interaction of α2M with various proteases plays an important role in septic and systemic inflammatory response syndrome patient outcomes (266). Previously, guinea pig α2M has been shown to inhibit the enzymatic activity of *P. aeruginosa* elastase *in vitro*, and the depletion of plasma α2M *in vivo* increased the susceptibility of animals to lethal shock (267). Building on these findings in guinea pigs, we have now demonstrated that *in vitro*, α2M inhibits *P. aeruginosa* elastase activity in humans.

Interestingly, when compared to healthy controls, plasma from ICU samples had a significantly enhanced ability to inhibit *P. aeruginosa* activity, and through FPLC purification, this inhibition was attributed to α2M. Previous research has shown that patients with sepsis and inflammation have either no change or a decrease in the concentration of plasma α2M. Therefore, an increase in the concentration of α2M is likely not responsible for the increased inhibition of *P. aeruginosa*-mediated CBG RCL cleavage seen in ICU patient plasma. An alternate explanation is provided by research that showed that the capacity of α2M to inhibit exogenous proteases is enhanced in critically ill and septic patients (266). It is then expected that a dynamic interplay occurs at sites of inflammation where *P. aeruginosa* is present, whereby α1-antitrypsin and α2M act to inhibit neutrophil elastase and *P. aeruginosa* elastase, respectively,

modulating CBG RCL cleavage and thereby, the local delivery of cortisol. As mentioned (**chapter 4.2**), AAT activity is modulated by *P. aeruginosa* elastase (254) and free radical production (255), which through different mechanisms, reduce the ability of α1-antitrypsin to inhibit neutrophil elastase and thereby, make neutrophil elastase more accessible to cleave CBG. Thus, a complex network of interactions takes place between various proteases, inhibitors and SERPINs at sites of inflammation, which likely influences the extent of CBG RCL cleavage and the localized release of cortisol.

In conclusion, decreased plasma CBG levels were observed in various ICU patient populations, and differences exist based on etiology, with severe inflammatory conditions leading to the largest decreases in CBG levels. These data suggest a differential role of altered plasma CBG levels depending on the underlying pathophysiology but nonetheless, further supports the idea that CBG plays an important role during inflammatory reactions. In this study the strongest correlation in plasma CBG levels were observed between the cortisol-binding capacity assay and 12G2 ELISA, suggesting that despite lower 9G12 ELISA values, RCL cleaved CBG is not present in the blood circulation. Although further studies are needed to confirm the cause of discrepant 9G12 ELISA values, a likely candidate is differential glycosylation of the *N*-glycan site within the CBG RCL. In addition, building on the work completed in **chapter 4.2**, within ICU plasma, α2M was identified as an endogenous inhibitor of *P. aeruginosa*. Therefore, a complex relationship is envisioned between various proteases and inhibitors, the balance of which will modify the extent of CBG RCL cleavage and the localized release of cortisol at sites of inflammation.

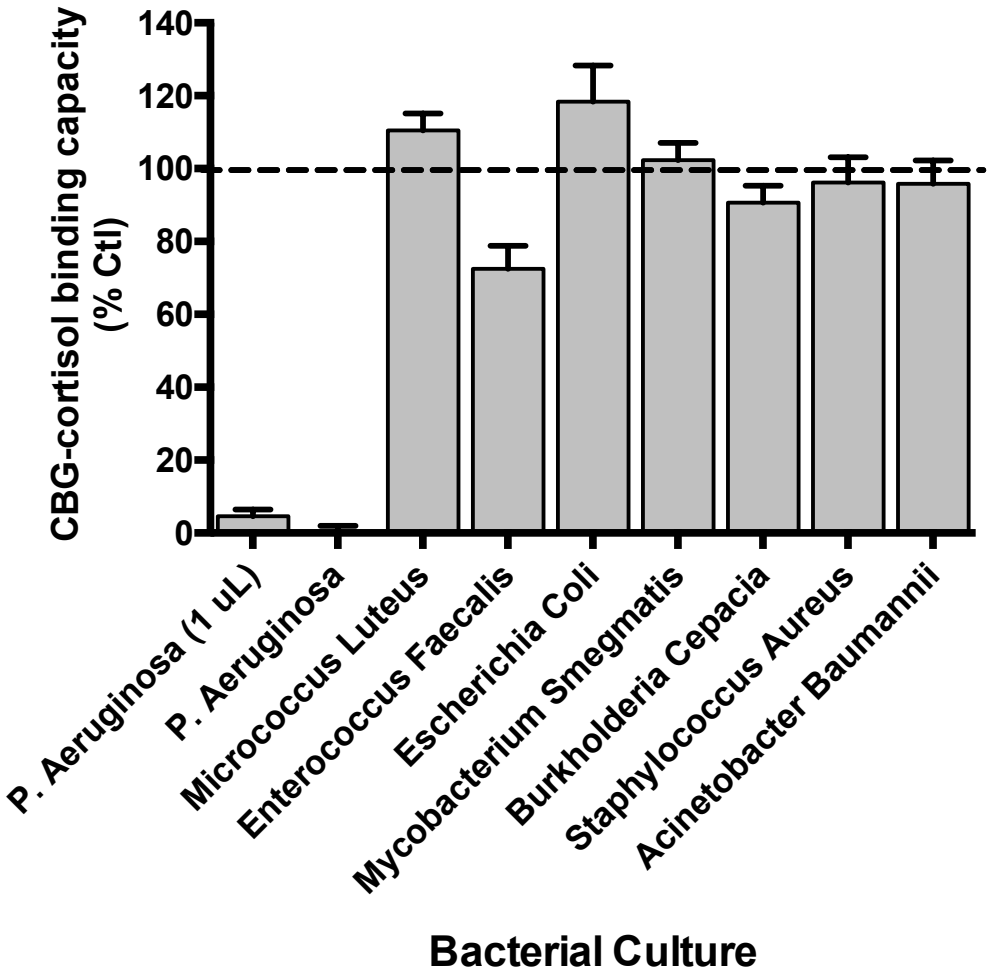
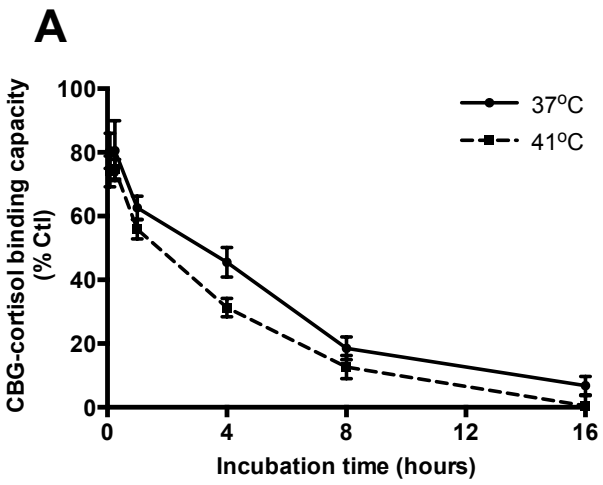
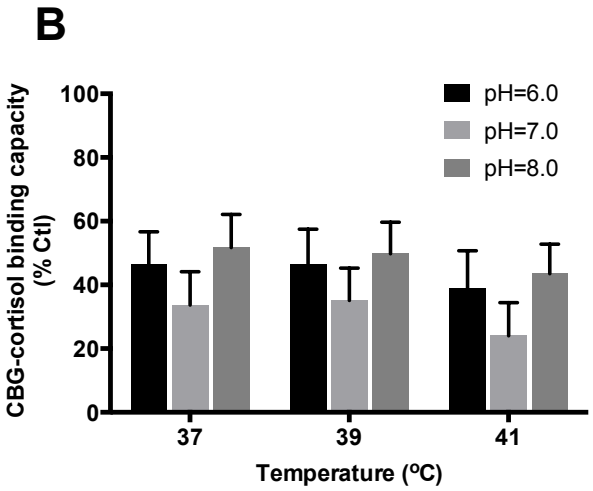


Figure 4.1 Changes in CBG-cortisol binding capacity after incubation of human serum with culture media from various bacteria. The cortisol-binding capacity of CBG was measured after human serum (1 μl) was incubated for 16 h at 37°C with media (90 μl) from bacterial cultures. A marked decrease in cortisol-binding capacity was also observed after incubation with only 1 μl of *P. aeruginosa* medium. Data are presented as mean percentage ± SD of cortisol-binding capacity relative to serum incubated with 90 μl of Dulbecco's PBS instead of bacterial culture media. Incubations were performed in triplicate.


A
CBG-cortisol binding capacity (% Ctl)
100
80
60
40
20
0
37°C
41°C
0
4
8
12
16
Incubation time (hours)


B
CBG-cortisol binding capacity (% Ctl)
100
80
60
40
20
0
pH=6.0
pH=7.0
pH=8.0
37
39
41
Temperature (°C)

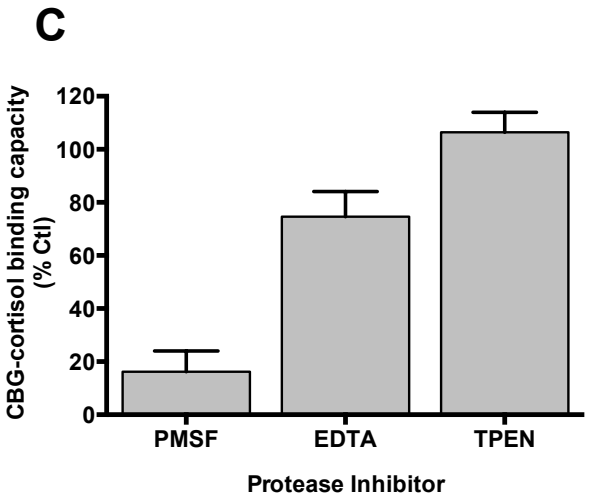

C
CBG-cortisol binding capacity (% Ctl)
120
100
80
60
40
20
0
PMSF
EDTA
TPEN
Protease Inhibitor

Figure 4.2 Reduction in the cortisol-binding capacity of CBG in human serum after incubation with *P. aeruginosa* media is time, temperature and pH dependent, and requires the presence of zinc. (A) The cortisol-binding capacity of CBG was measured after incubation of human serum with *P. aeruginosa* media for 5 min, 15 min, 1 h, 4 h, 8 h, and 16 h at 37°C or 41°C. Incubation at 41°C led to a greater decrease ($P < 0.001$) in activity than incubation at 37°C. (B) Human serum was incubated with *P. aeruginosa* media for 8 h at pH 6, 7, or 8, and at 37, 39, or 41°C. The cortisol-binding capacity of CBG was lower after incubations at pH 7 than at pH 6 and 8 for all temperatures tested ($P = 0.0065$). (C) Human serum was incubated with *P. aeruginosa* media in the presence of 1 mM PMSF (serine/cysteine protease inhibitor), 5 mM EDTA (divalent cation inhibitor), or 50 μM TPEN (specific zinc chelator) for 16 h at 37°C. All incubations were performed in triplicates and data are presented as mean percentage ± SD of steroid-binding activity relative to a control. Data for (B) was obtained by Dr. Marc Simard.

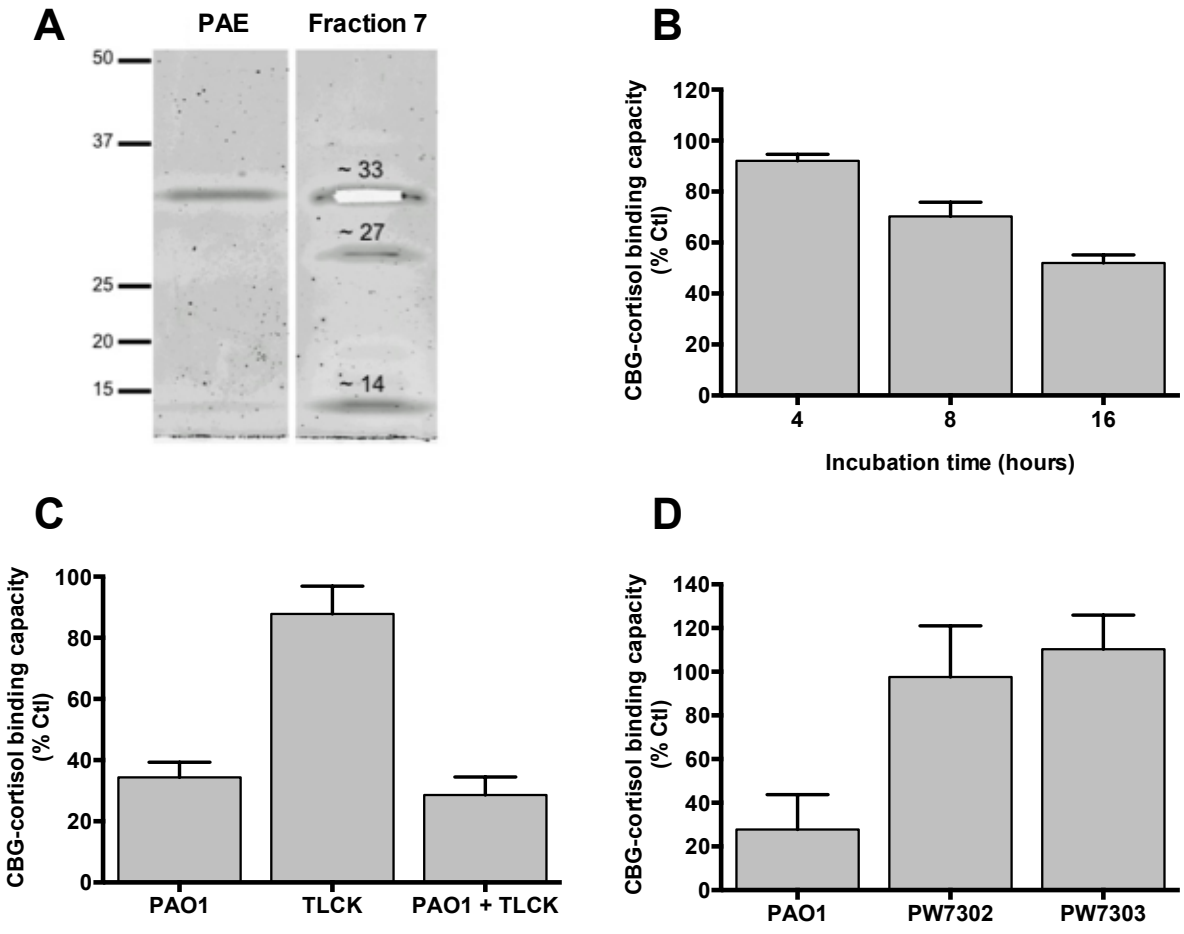

A
PAE
Fraction 7
50
37
25
20
15
~ 33
~ 27
~ 14
B
CBG-cortisol binding capacity (% Ctl)
120
100
80
60
40
20
0
4
8
16
Incubation time (hours)
C
CBG-cortisol binding capacity (% Ctl)
100
80
60
40
20
0
PAO1
TLCK
PAO1 + TLCK
D
CBG-cortisol binding capacity (% Ctl)
140
120
100
80
60
40
20
0
PAO1
PW7302
PW7303

Figure 4.3 Identification of candidate proteases within *P. aeruginosa* media that target and cleave the CBG RCL: PAE and not protease IV is responsible for the disruption of CBG-cortisol binding capacity. (A) An SDS-PAGE gel, stained with Sypro Ruby, of an active chromatographic fraction of *P. aeruginosa* revealed the presence of three bands that were excised for mass spectrometry analysis. The ~33 kDa band was identified as *P. aeruginosa* elastase (PAE), the ~27 kDA band as protease IV and the ~14 kDA band as a C-terminal fragment of PAE. Commercially available PAE is also shown as a reference. (B) The cortisol-binding capacity of CBG in human serum was determined after incubation with purified PAE (1 μg) for 4, 8, or 16 h at 37°C. A progressive decrease in the CBG-cortisol binding capacity was observed, with ~50% remaining after 16 h of incubation. (C) *P. aeruginosa* medium (PAO1) was pre-incubated with 0.05 mM TLCK as an inhibitor of protease IV for 1 h at 37°C, filtered and then incubated with human serum for 16 h at 37°C. Significant reductions in the CBG-cortisol binding capacity were observed after TLCK treatment, suggesting that protease IV is not involved in CBG cleavage. (D) Human serum (1 μl) was incubated with conditioned media from two PAE-deficient clones (i.e., PW7302 and PW7303) of *P. aeruginosa* and from the parental PAO1 strain. No loss of cortisol-binding activity was observed after incubations with the two PAE-deficient clones, confirming the main role of PAE in the disruption of CBG-cortisol binding capacity. Incubations were run in triplicate and the data are presented as mean percentage ± SD of steroid-binding activity relative to controls. Data for (A) and (D) were obtained by Dr. Bernd Keller and Dr. Marc Simard, respectively.

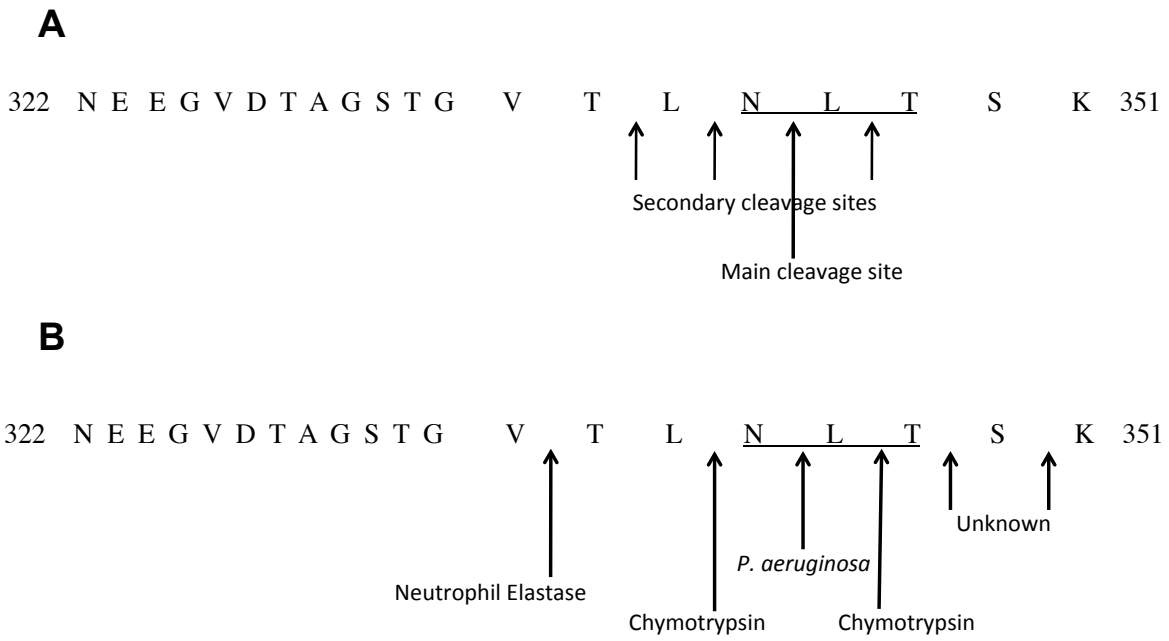


Figure 4.4 Localization of protease cleavage sites within the CBG RCL. The CBG RCL sequence, corresponding to amino acids 322 to 351 within the mature CBG protein, is shown with respective protease cleavage sites notated. (A) After incubation of human CBG with an active *P.aeruginosa* fraction, mass spectrometry identified four cleavage sites within the CBG RCL. The main cleavage site is located after Asn347, which is part of a consensus sequence for *N*-glycosylation (*underlined*). Three additional secondary cleavage sites were also identified after Thr345, Leu346 and Leu348, respectively. (B) CBG RCL cleavage sites identified to date. The endogenous proteases neutrophil elastase and chymotrypsin cleave the CBG RCL after Val344 (8, 9) and Leu346/Leu348 (120), respectively. In a human CBG crystal structure, the CBG RCL was cleaved by an unknown protease after either Thr349 or Ser350 (13).

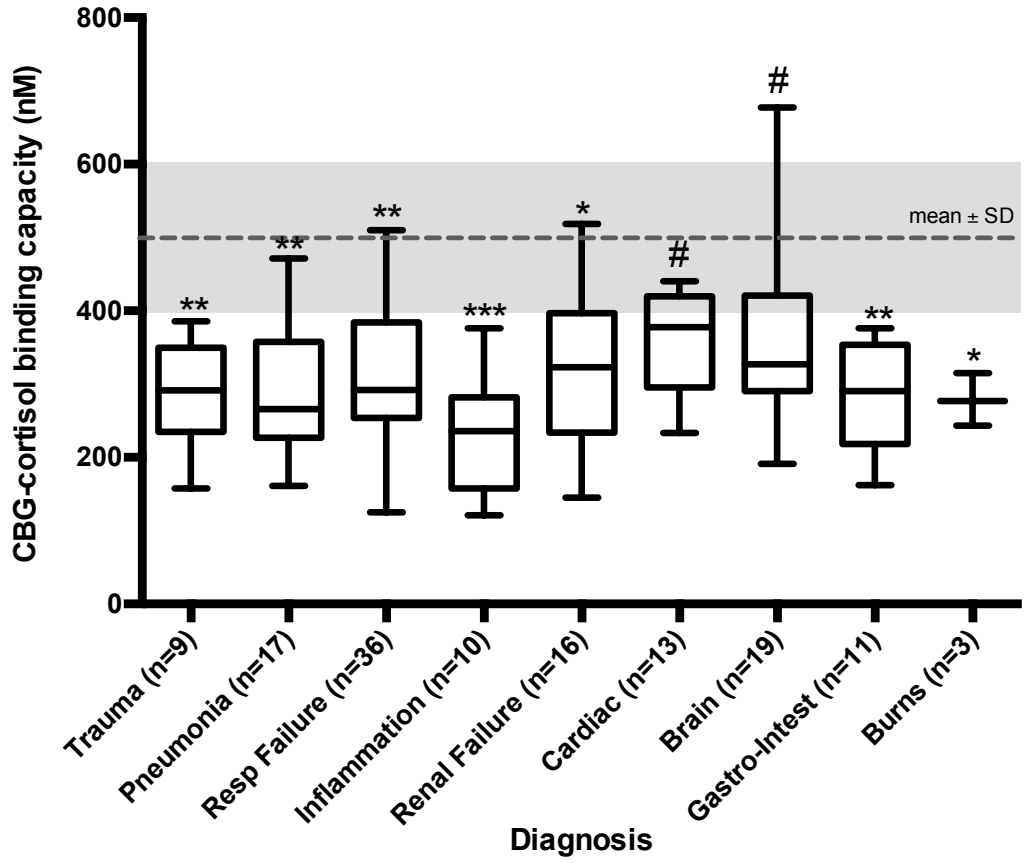


Figure 4.5 CBG-cortisol binding capacity in various patient populations. Box plots for each patient group display the maximum, third quartile, median, first quartile and minimum. When comparing different patient populations, significant differences in plasma CBG-cortisol binding capacity were found (one-way ANOVA, $P = 0.0238$), with higher CBG binding identified in patients with cardiac and brain related diagnoses, in comparison to patients with inflammation (#$P < 0.05$). Patient CBG-cortisol binding capacity values were also compared to the mean of a healthy control population (*mean ± SD shaded grey*) and significant differences were detected (main effect of diagnosis, $P = 0.0007$). Significantly lower CBG-cortisol binding capacity values were found in patients with renal failure and burns (*$P < 0.05$), trauma, pneumonia, respiratory failure (resp failure) and gastro-intestinal (gastro-intest) diagnoses (**$P < 0.01$), as well as patients with inflammation (***$P < 0.001$).

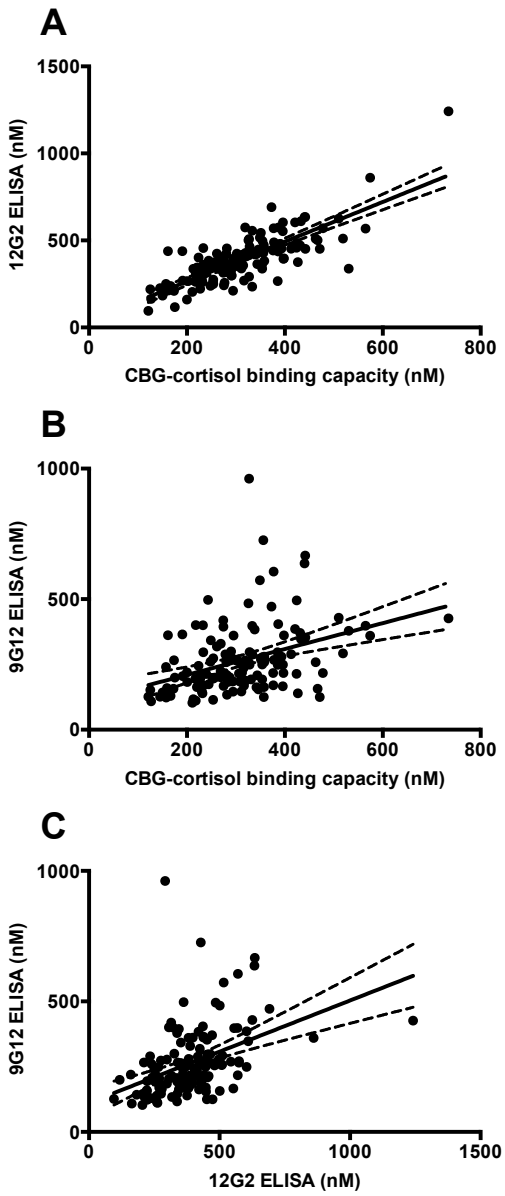


Figure 4.6 Plasma CBG levels measured by cortisol-binding capacity and the 12G2 ELISA are strongly correlated; correlations with the 9G12 ELISA are weak. Plasma CBG levels were measured by cortisol-binding capacity, 12G2 and 9G12 ELISAs. (A) A strong correlation was found between CBG-cortisol binding capacity and 12G2 ELISA values (r^2=0.6351). (B,C) A weak correlation was found between 9G12 ELISA and (B) CBG-cortisol binding capacity (r^2=0.1427) and (C) 12G2 ELISA values (r^2=0.1830), respectively.

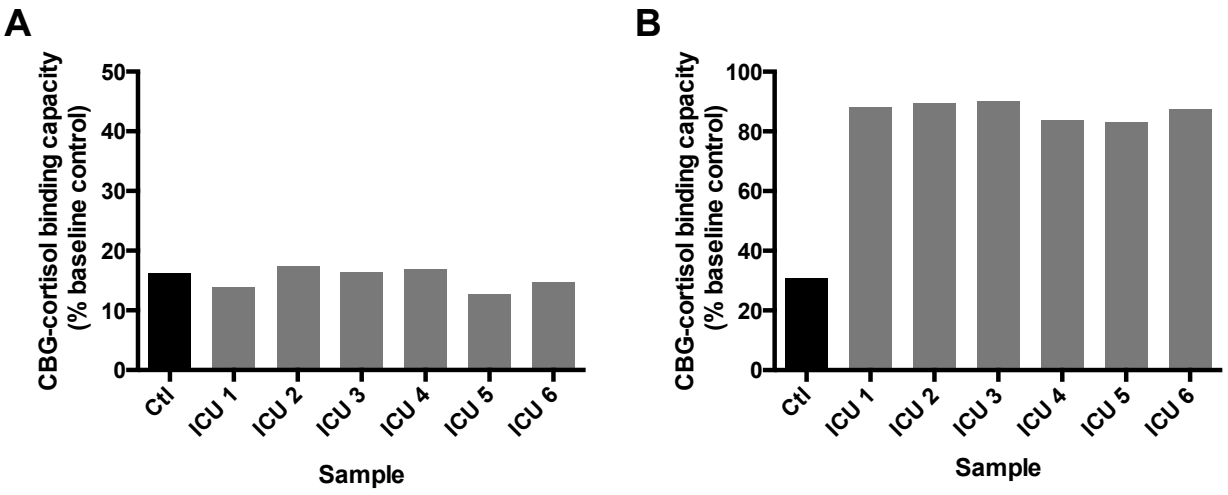


Figure 4.7 ICU plasma inhibits CBG RCL cleavage by *P. aeruginosa* but not neutrophil elastase. (A) Similar reductions in the CBG-cortisol binding capacity were observed after incubations of control (Ctl) and ICU patient plasma with neutrophil elastase. (B) The plasma CBG-cortisol binding capacity of ICU samples was determined after incubation with *P.aeruginosa*. The residual cortisol-binding capacity of CBG in ICU plasma was much greater (83-90%) than a healthy control (Ctl, 30%). Data are presented as a percentage of steroid-binding activity relative to controls that did not contain neutrophil elastase or *P. aeruginosa* media.

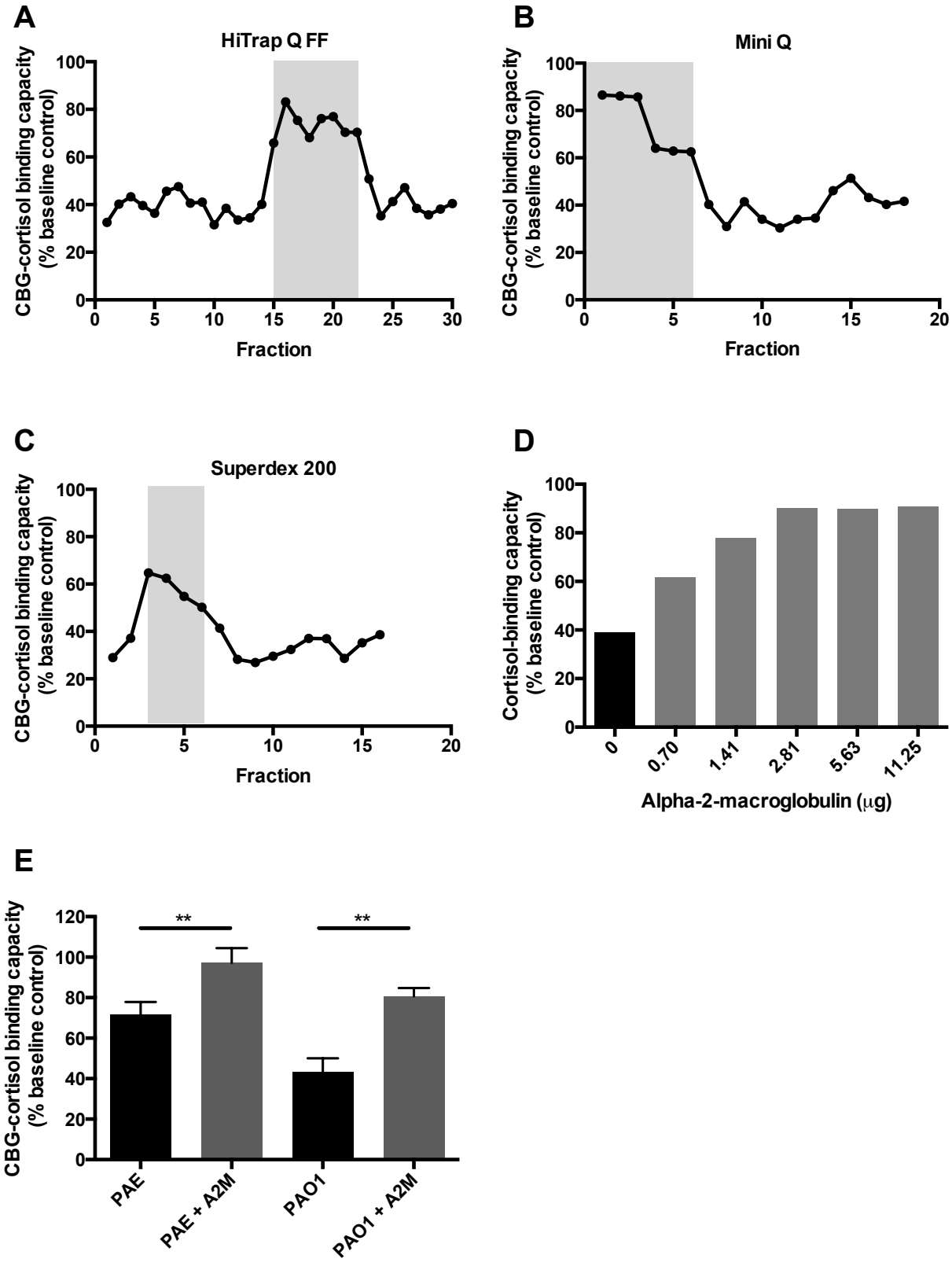

A
HiTrap Q FF
CBG-cortisol binding capacity (% baseline control)
Fraction
B
Mini Q
CBG-cortisol binding capacity (% baseline control)
Fraction
C
Superdex 200
CBG-cortisol binding capacity (% baseline control)
Fraction
D
Cortisol-binding capacity (% baseline control)
0
0.70
1.41
2.81
5.63
11.25
Alpha-2-macroglobulin (μg)
E
CBG-cortisol binding capacity (% baseline control)
**
**
PAE
PAE + A2M
PAO1
PAO1 + A2M

Figure 4.8 Resistance of ICU plasma to *P.aeruginosa*-mediated CBG RCL cleavage is due to alpha-2-macroglobulin (A2M). (A-C) Elution profiles from the chromatographic separation of ICU plasma to identify proteins that media the inhibition of *P.aeruginosa*-mediated CBG RCL cleavage. Fractions with inhibitory activity are highlighted by *grey shading*. (A) Diluted (1:10) ICU plasma was applied to a HiTrap Q FF column and 1 mL fractions were collected and tested for their ability to inhibit CBG RCL cleavage by *P.aeruginosa*. (B) Active HiTrap Q FF fractions were pooled and applied to a Mini Q column. (C) Further purification of active Mini Q fractions was completed using a Superdex 200 gel filtration column. Four fractions, coinciding to a molecular size between 440 and 660 kDa possessed the ability to inhibit *P.aeruginosa*-mediated CBG RCL cleavage. (D) Purified A2M (0.7-11.25 μg) dose-dependently inhibited CBG RCL cleavage by *P.aeruginosa* media. (E) The addition of A2M (2.8 μg) to reactions containing human serum and purified *P.aeruginosa* elastase (PAE, 10 μL) or *P.aeruginosa* media resulted in significantly greater CBG-cortisol binding capacity values (***P*<0.01), indicating A2M is able to inhibit CBG RCL cleavage by *P.aeruginosa* proteases. (D,E) Data (means) are presented as a percentage of steroid-binding activity relative to controls that did not contain *P. aeruginosa* media.

Chapter 5: CBG as a biomarker of inflammation in rats

5.1 Introduction

Although decreases in human plasma CBG have been reported in a number of inflammatory states (23, 25, 170-172), the exact timing and mechanisms of plasma CBG changes during inflammation remains to be determined. Several studies in CBG deficient animals support the idea that CBG plays a vital role in determining susceptibility to inflammation. For instance, a genetic study revealed that C57BL/6 mice are more sensitive to an acute challenge with TNF-α than DBA/2J mice, and this trait was mapped to the *Cbg* (*Serpina6*) locus (182). A key role for CBG in inflammatory responses is further supported by studies in $Cbg^{-/-}$ mice in which survival after an acute inflammatory challenge is compromised (102). Together, these data suggest that lower plasma CBG levels may compromise an adequate response to inflammation.

The rat *Serpina6* sequence is known to be polymorphic with non-synonymous SNPs resulting in reduced plasma levels or corticosteroid-binding activity of CBG noted in some strains (104, 145). Although rats of the same strain are generally considered comparable, genetic drift within colonies may account for differences in hypoxic responses (268), neuropathic pain behaviors (269), as well as differences in metabolic, endocrine, and immune function among SD rats from different vendors (181). In addition, Charles River (Hollister, CA) and Harlan (Indianapolis, IN) Sprague Dawley (SD) rats exhibit different endocrine and immune responses to inflammatory challenge with LPS, turpentine, and IL-1β (181).

To explore these questions, the following studies utilized a model of inflammation in which female rats were given an intradermal injection of complete Freund's adjuvant. In this model, inflammation develops after injection of CFA, as a result of an immune response against components of the cartilage matrix. This is initiated by a T cell response to exogenous antigen,

likely a component of a mycobacterial 65 kD heat-shock protein (270), thought to contain a cartilage mimicking epitope (271).

In an initial study, I examined whether differences in CBG levels may account for differential endocrine and clinical responses to inflammation in Harlan versus Charles River SD rats (**chapter 5.2**). In a follow-up study, using the same model, changes in plasma CBG levels were examined during the onset of acute inflammation in Charles River SD rats and the biochemical basis of changes in plasma CBG levels were examined, and mapped to the severity of the inflammatory response (**chapter 5.3**).

5.2 Colony specific differences in endocrine responses to an inflammatory challenge in female Sprague Dawley rats

5.2.1 **Results**

5.2.1.1 Clinical scores reveal a more severe course of inflammation in Harlan compared to Charles River SD rats

The mean day of inflammation onset (first day of clinical score > 0) did not differ between Harlan and Charles River SD rats, ranging from a mean of day 11.1 (± 1.20) to 13.0 (± 0.63) post-injection for all adjuvant doses examined.

Initially, low (0.3 mg) and high (1.2 mg) doses of adjuvant were tested in rats from both colonies, with an additional lower (0.2 mg) dose tested in Harlan SD rats and an additional intermediate (0.6 mg) dose tested in Charles River SD rats. Stacked bar graphs (Figure 5.1) represent the proportion of rats from all adjuvant doses at each inflammation severity level.

A comparison of severity profiles across all doses demonstrated that at the lowest doses tested in SD rats from each colony (Charles River: 0.3 mg; Harlan: 0.2 mg), there were no cases of severe inflammation. At the other two doses, fewer Harlan (0.3 and 1.2 mg: 13%) than

Charles River (0.6 mg: 33%; 1.2 mg: 38%) SD rats failed to develop clinical signs of inflammation. A direct comparison of Charles River and Harlan SD rats at the 0.3 and 1.2 mg doses (Figure 5.1) demonstrated increased inflammation severity in Harlan SD rats. At the 0.3 mg dose, Charles River SD rats had less of an inflammatory response, as 43% of Charles River SD rats did not develop inflammation, compared to only 13% of Harlan SD rats. In addition, none of the Charles River SD rats developed severe inflammation compared to 38% of Harlan rats. At the 1.2 mg dose, while similar numbers (25%) of Harlan and Charles River SD rats developed severe inflammation, colony differences were again observed when comparing the number of rats that failed to develop inflammation, as 38% of Charles River did not develop inflammation, compared to only 13% of Harlan SD rats.

5.2.1.2 Increased plasma corticosterone and decreased CBG in SD rats with increasing severity of inflammation

Plasma corticosterone levels increased with adjuvant injection (main effect of inflammation severity, $p < 0.001$; Figure 5.2A), with the highest corticosterone levels found in rats with severe inflammation. Free plasma corticosterone also increased with adjuvant injection (main effect of inflammation severity, $p < 0.001$; Figure 5.2B) in both Charles River and Harlan SD rats, with patterns of free corticosterone closely paralleling those of total corticosterone.

Plasma CBG levels decreased with inflammation severity (main effect of inflammation severity, $p < 0.001$), with the lowest levels detected in rats with severe inflammation (Figure 5.2C). Significant differences in plasma CBG levels were also observed between colonies, with lower levels overall in Harlan rats when compared to Charles River SD rats (main effect of colony, $p = 0.003$). Interestingly, in the absence of inflammation, CBG levels were significantly lower in Harlan SD rats when compared to Charles River SD rats ($p < 0.05$; Figure 5.2C).

However, with severe inflammation, CBG levels did not differ between Charles River and Harlan SD rats.

5.2.1.3 Differences in Serpina6 sequences in Harlan and Charles River SD rats

When the *Serpina6* coding sequences for CBG were compared in the two SD rat colonies, we found a synonymous single nucleotide transition (C>T) in exon 2 within the codon for Phe152, and two non-synonymous single nucleotide transitions (A>G) in exon 4 that cause amino acid substitutions (Ile298Met and Met307Val) in Charles River SD rats. However, there were no sequence differences within the 439bp proximal promoter region (36).

5.2.1.4 Plasma CBG-corticosterone binding affinities are similar in Charles River and Harlan SD rats.

To determine if the difference in CBG coding sequences between Charles River and Harlan SD rats alters CBG-corticosterone binding affinity, plasma samples from control (saline-injected) rats from each colony were subjected to Scatchard analysis, using ^{3}H-corticosterone as the radiolabeled ligand. The results indicated that CBG from Charles River and Harlan SD rats have similar dissociation rate constants (Kd) for corticosterone, 1.15 nM and 1.13 nM, respectively (Figure 5.2D), and therefore, similar affinities (affinity constant, Ka = 1/Kd).

5.2.2 **Discussion**

In this study, differences were observed in the incidence and severity of inflammation, as well as in endocrine responses, following adjuvant injection in SD rats obtained from Charles River versus Harlan Laboratories. Overall, Harlan rats were more susceptible to inflammation, developing more severe inflammatory responses compared to Charles River SD rats. In addition, while rats from both colonies showed increasing corticosterone levels with the development of inflammation, Harlan SD rats had lower plasma CBG levels than Charles River SD rats in the

absence of inflammation (control and no inflammation groups). As a result, the decrease in plasma CBG that occurred with the development of inflammation was less pronounced in Harlan SD rats.

In Charles River and Harlan SD rats, increasing plasma basal total and free corticosterone levels were generally associated with inflammation severity, likely due to increased levels of pro-inflammatory cytokines driving increased HPA axis activity, as well as a loss of the corticosterone diurnal rhythm (272). Increased variability in corticosterone levels observed with the onset of severe inflammation is a finding common to the adjuvant-induced inflammation model (273-275), and can be attributed to individual differences in the severity of inflammation.

Importantly, differences in CBG levels were detected, with lower plasma CBG levels in Harlan compared to Charles River SD rats under control conditions. This difference may in part explain previous findings of differences in ACTH levels seen after administration of LPS and turpentine in rats from the same SD colonies studied here (181). Plasma CBG levels were not measured in the latter study, but large increases in plasma corticosterone were observed in all rats treated with LPS or turpentine, irrespective of the SD colony. If CBG levels were different between rats from these different colonies, as we have now observed, it would be expected that the lower levels of CBG in Harlan SD rats would lead to higher free corticosterone levels, which would be available to feedback onto ACTH, thus explaining the lower ACTH levels in Harlan compared to Charles River SD rats, in the latter study (181). In addition, higher CBG levels in Charles River SD rats could explain why they have increased levels of basal corticosterone (181), as more of the steroid would be bound to CBG. In the present study, while ACTH levels were not statistically different between colonies, mean ACTH levels were lower in Harlan

compared to Charles River SD rats in rats with severe inflammation, which may also be suggestive of colony differences in overall HPA activity.

Lower plasma CBG levels in Harlan SD rats suggest a lower corticosterone reservoir, which could play a key role in the observed increased susceptibility of Harlan rats to the immune challenge. It is known that human CBG is targeted by proteases such as neutrophil elastase (9), significantly decreasing its binding affinity for glucocorticoids and allowing for their targeted release at sites of inflammation. Immunoreactive CBG degradation products have also been observed in wound fluids of rats subjected to thermal injury (176). Thus, the larger corticosteroid reservoir in Charles River SD rats may function to more effectively deliver corticosterone to sites of inflammation and result in reduced inflammation in these rats. Of note, with the development of inflammation, CBG levels decreased in SD rats from both colonies, with significant differences in CBG levels detected between controls and rats with severe inflammation. In the severe inflammation state, when CBG levels are significantly lower and total corticosterone levels are significantly higher, there is a substantial increase in free corticosterone, which would also be available at target tissues. The decrease in plasma CBG levels observed with increasing inflammation severity in both Charles River and Harlan SD rats may reflect either an increase in the plasma clearance of CBG (233), and/or a decrease in hepatic CBG production in response to increases in the plasma levels of corticosterone (56) and proinflammatory cytokines (23).

Differences in baseline CBG levels between Harlan and Charles River SD rats were not due to differences in CBG steroid-binding affinity, despite the presence of two amino acid differences. There were also no differences between the two colonies of SD rats in their *Serpina6* proximal promoter sequences, which contain a C/EBPβ regulatory site that is known to mediate the acute phase response (62, 276). Therefore, while we cannot exclude the possibility that the

differences in the CBG coding sequences alter the secretion rates of CBG from hepatocytes *in vivo*, differences in transcription factor levels or differences in regulatory sequences outside of the proximal promoter sequence might account for this.

While we have shown genetic differences between Charles River and Harlan SD rats, supporting the hypothesis that genetic drift has occurred between these two SD rat populations, the role of environmental factors, such as diet and early-life rearing conditions, cannot be discounted. These environmental factors are known to impact physiological parameters and naturally differ between colonies. While early-life environmental conditions may be affecting epigenetic regulation of endocrine and immune genes (277, 278), our studies of *Serpina6* clearly demonstrate that genetic differences exist between Charles River and Harlan SD rats, and the consequences need to be considered when comparing results from different laboratories.

5.3 Corticosteroid-Binding Globulin is a biomarker of inflammation onset and severity in female rats

5.3.1 **Results**

5.3.1.1 Body weights and clinical scores

The body weights of all animals increased over the course of the experiment (i.e., 242.0 ± 8.7 g to 285.0 ± 10.4 g), but there were no differences in body weight between groups. Five of the 20 adjuvant-treated animals (25%) developed inflammation, as indicated by their clinical scores (Figure 5.3 in parenthesis), which generally increased over time. The mean time of inflammation onset (i.e. the first day of a clinical score > 0) was 12.8 ± 0.8 days.

5.3.1.2 Time-course of changes in plasma CBG levels

Adjuvant-treated rats had a 30-50% reduction in plasma CBG levels 24 hrs post injection, irrespective of whether or not they developed inflammation, and this decrease resolved by day 3

post-injection (Figure 5.3). This was not seen in the saline-treated control rats. When compared to baseline values, 40-80% decreases in CBG-corticosterone-binding capacity were subsequently observed over the experimental period in rats that developed inflammation (Figure 5.3), but mean plasma CBG levels were unchanged in saline-treated rats (not shown) or in adjuvant-treated rats that did not develop inflammation (Figure 5.3).

Overall, the magnitude of decline in plasma CBG levels matched the severity of inflammation, with animals that developed severe inflammation showing the largest decreases in CBG levels (Figure 5.3). In two severely inflamed rats, 50% reductions in plasma CBG were seen as early as day 8 post-injection, which was 4 days before any clinical signs of inflammation were evident. In these animals, CBG levels reached as low as 20% of baseline by 12-13 days post injection. The two rats with mild-moderate inflammation also had significant reductions (35% and 55% of baseline values at 13-14 days post-injection) in CBG levels. Total protein levels in plasma samples did not differ in rats over the course of the study in relation to the severity of inflammation (data not shown).

5.3.1.3 Plasma CBG and hepatic Serpina6 mRNA levels in relation to clinical score

Plasma CBG levels at termination declined in relation to clinical score (main effect of inflammation severity, $P = 0.0001$; Figure 5.4A). Rats with severe inflammation had significantly lower CBG levels when compared to saline-treated controls or adjuvant-treated rats that did not develop inflammation ($P < 0.001$). Significant differences were also seen in *Serpina6* mRNA levels in livers collected on the day of termination (main effect of inflammation severity, $P < 0.02$; Figure 5.4B). When compared to saline-treated rats or adjuvant-treated rats that did not develop inflammation, *post hoc* tests revealed that animals with severe inflammation had lower *Serpina6* mRNA levels ($P < 0.05$).

Western blotting was used to assess the integrity of CBG in plasma samples taken at termination. An ~5 kDa reduction in the apparent molecular size of CBG was evident in rats with mild-moderate and severe inflammation (Figure 5.4C, lanes 4 and 5), consistent with cleavage of its RCL (13). This was also observed in some adjuvant-treated rats without clinical signs of inflammation (Figure 5.4C, lane 3), suggesting the presence of an underlying inflammatory state in those animals. As expected, this evidence of CBG proteolysis coincided with decreased plasma CBG levels as determined in the corticosterone-binding capacity assay (Figure 5.4C). Therefore, on the day of termination, decreases in the corticosterone-binding capacity of plasma CBG in animals with severe inflammation are associated with decreased *Serpina6* mRNA levels, as well as evidence of proteolytic cleavage of CBG.

5.3.1.4 Plasma CBG and hepatic Serpina6 mRNA levels in relation to CBG proteolysis

Plasma samples taken at termination were classified into two groups depending on the integrity of CBG as assessed by western blotting: i.e., samples in which CBG proteolysis was either evident (cleaved) or not (intact), as illustrated in Figure 5.4C. Reductions in CBG levels were found in rats with evidence of cleaved CBG (Figure 5.5A), when compared to rats with intact CBG ($P < 0.0001$). When classifying samples in this way, no differences were found in corresponding liver *Serpina6* mRNA levels (Figure 5.5B), suggesting that CBG proteolysis precedes changes in liver *Serpina6* mRNA levels.

Next, we investigated whether cleaved CBG in plasma samples retained high-affinity corticosterone-binding activity. Plasma samples from rats with severe inflammation (Figure 5.5C, lanes 2 and 3) were pooled and CBG was captured by HACA-Sepharose-affinity column chromatography. Cleaved CBG, as indicated by an ~5 kDa size reduction, failed to bind the affinity column and eluted in the flow through fractions (Figure 5.5C, lanes 4 and 5). Moreover,

the flow through fractions containing the cleaved CBG had no detectable CBG-corticosterone binding activity. There was no immunoreactive CBG or corticosterone-binding activity in the wash fraction (Figure 5.5C, lane 7). Importantly, the CBG eluting from the affinity column using excess (200 μM) corticosterone (Figure 5.5C, lane 8) appeared to be intact and retained full corticosterone-binding activity.

5.3.1.5 Plasma cytokine biomarkers of inflammation and spleen weight

Pro- and anti-inflammatory cytokine levels were measured in plasma samples collected at termination (Figure 5.6A-G), as a means to assess the inflammatory response and are not necessarily glucocorticoid regulated. Measurements in samples from saline-treated control animals and adjuvant-treated animals were classified according to whether CBG was intact or cleaved (Figure 5.4C). No significant differences were found in plasma levels of IL-5 (Figure 5.6A). Trends for a main effect of CBG proteolysis were found for the anti-inflammatory cytokines IL-4 (Figure 5.6B, $P = 0.051$) and IL-10 (Figure 5.6C, $P = 0.056$), as well as the pro-inflammatory cytokine IFN-ɣ (Figure 5.6D, $P = 0.057$). Planned pairwise comparisons revealed significantly elevated IL-4, IL-10 and IFN-ɣ levels only in rats with cleaved CBG, when compared to saline-treated controls ($P < 0.05$).

A significant main effect of CBG proteolysis was found for TNF-α ($P < 0.004$), IL-13 ($P = 0.007$) and IL-6 ($P < 0.001$), respectively. A treatment effect was found for the pro-inflammatory cytokine TNF-α, with *post hoc* tests revealing that adjuvant treated animals (both intact and cleaved CBG) had higher cytokine levels compared to control animals (Figure 5.6E, $P < 0.01$). A different pattern was observed for the anti-inflammatory cytokine IL-13 for which *post-hoc* tests indicated that plasma levels were significantly increased in rats with cleaved CBG, when compared to saline-treated controls ($P < 0.001$) or rats with intact CBG ($P < 0.05$),

respectively (Figure 5.6F). In terms of changes in plasma cytokine levels, the largest increases were seen for IL-6 (Figure 5.6G), with *post-hoc* tests indicating significant increases in rats with cleaved CBG, as compared to the saline-treated controls or rats with intact CBG ($P < 0.001$). Similar to IL-6, spleen weight (Figure 5.6H) was very significantly associated with CBG proteolysis (main effect of CBG proteolysis, $P < 0.001$). *Post-hoc* tests also indicated that rats with cleaved CBG had significantly elevated spleen weights, when compared to the saline-treated controls or adjuvant-treated rats with intact CBG ($P < 0.001$). Furthermore, there were strong negative correlations between plasma CBG and IL-6 levels (Figure 5.7A, $r^2=0.71$) and between plasma CBG levels and spleen weights (Figure 5.7B, $r^2=0.73$) but not between plasma CBG and IL-13 levels ($r^2= 0.22$).

5.3.2 **Discussion**

Changes in plasma CBG levels occur during inflammation in humans, with very low levels reported during sepsis (25), septic shock (25, 171, 172), burn injuries (23) and after open heart surgery (24). In addition, decreased CBG levels have been reported after thermal injuries to mice and rats (176, 177) and in pigs treated with lipopolysaccharide (178). These latter studies model acute, all-or-none, inflammation, while the adjuvant-induced inflammation model we have used allows those rats that developed inflammation to be compared with those that did not. In doing so, it was possible to monitor temporal changes in the plasma levels of CBG and inflammatory markers, as inflammation developed at different rates and degrees of severity.

In adjuvant-treated rats, the corticosterone-binding capacity of plasma CBG decreased 30-50% at 24 hrs post-injection, irrespective of whether or not rats eventually developed inflammation. Based on previous reports (270), we suspect that an initial inflammatory response to the complete Freund's adjuvant is responsible for decreases in plasma CBG levels because this

did not occur in saline-treated controls. However, this initial response was short-lived and CBG levels in adjuvant-treated animals returned to baseline by day 3 post-injection. Over the subsequent experimental period we found consistent decreases in plasma CBG levels of 40-80% in those animals that developed inflammation, and the magnitude of this decline aligned with the severity of inflammation, as indexed by clinical scores. Notably, marked (50%) decreases in plasma CBG levels occurred 4 days before any clinical symptoms of inflammation were evident. Such dynamic changes in CBG levels prior to or during inflammation are expected to modulate the availability of corticosterone to its target cells, thereby affecting the inflammatory reaction as well as the healing process. When considered together, these results suggest that CBG may be a useful biomarker of inflammation onset and severity.

As noted in our previous study (**chapter 5.2**), significant decreases in plasma CBG levels occurred in adjuvant-treated rats that developed severe inflammation, and we have now defined the mechanisms responsible for this. At day 14 or 16 post-adjuvant injection (termination), rats with severe inflammation had reduced plasma CBG levels and this was associated with an ~5 kDa reduction in CBG molecular size by western blotting. Changes in the carbohydrate composition of plasma proteins have been reported to occur during acute inflammation, including decreases in core fucosylation (261). However, small changes in the composition of the six *N*-linked oligosaccharides associated with rat CBG will not result in detectable differences in molecular size by western blotting. It is also known that the complete loss of *N*-glycosylation of human CBG at Asn238 causes a loss of steroid-binding activity (44), however there is no evidence that compositional changes in the *N*-linked glycosylation of CBG, in any species, adversely effects its steroid-binding activity. Differences in *N*-linked oligosaccharide composition will not account for the substantial reduction in the molecular size of CBG, or the

complete loss of steroid-binding activity associated with immune-reactive CBG that does not interact with the steroid-affinity chromatography matrix, and we therefore conclude that these observations reflect proteolytic cleavage.

It has been reported that cleaved CBG can be detected in human blood samples using ELISAs with highly specific monoclonal antibodies that discriminated between CBG with an intact versus cleaved RCL (209), but direct evidence that RCL cleavage of CBG actually occurred in these samples is lacking. A size reduction of CBG consistent with RCL cleavage has never been observed in human blood samples, and it has been postulated that human CBG is rapidly removed from the blood circulation after RCL cleavage (233). In rats, this does not seem to be the case and it appears that cleaved CBG is cleared more slowly from the circulation. However, the site of RCL cleavage of rat CBG and the protease responsible for this remain to be identified.

The steroid-binding activity of CBG is undetectable in rat plasma samples in which CBG appears to be have undergone proteolysis, and this is in line with a marked loss in cortisol-binding affinity observed when the RCL of human CBG is cleaved by neutrophil elastase (9), chymotrypsin (279) or the bacterial proteinase, PAE (**chapter 4.2**). However, our observations of *in vivo* rat CBG proteolysis under pathophysiological conditions contrasts with a previous report that *E-coli* produced rat CBG, mutated to allow for cleavage by human neutrophil elastase, only undergoes a 2-fold reduction in binding affinity (13). We attribute this discrepancy to the fact that *E.coli* expressed CBG is not glycosylated and has about a 10-fold lower affinity for corticosterone when compared to native CBG in rat blood samples (13). It is known that the *N*-glycosylation of human CBG is critically important for the formation of a high-affinity binding site (7, 44) and the RCLs of human and rat CBGs both contain *N*-glycosylation sites, but in

different locations (10). It is possible that *N*-glycosylation within the RCL of rat CBG influences how, and in what location, it is cleaved by a protease that allows for RCL insertion and the subsequent protein conformational rearrangement that disrupts the high affinity steroid-binding properties of CBG (37). Unexpectedly, plasma CBG proteolysis was also evident in rats that developed mild-moderate inflammation, as well as in a subset of rats that did not display clinical signs of inflammation. However, significant reductions in plasma CBG were evident in rats where CBG proteolysis appears to have occurred, despite the fact that this did not coincide with a reduction in liver *Serpina6* mRNA levels, which suggests that plasma CBG proteolysis occurs prior to changes in liver *Serpina6* mRNA levels.

Plasma CBG production by the liver is down regulated by glucocorticoids (56) and cytokines (77). Although plasma corticosterone levels were not measured in this study, they were increased in our previous study (**chapter 5.2**) using the same experimental protocol, with the highest levels found in animals with severe inflammation. In this model, increased levels of IL-6, as seen here, and corticosterone, as seen previously (**chapter 5.2**), are likely contributors to the significant decreases in liver *Serpina6* mRNA levels that we have now observed in rats with severe inflammation. This is not entirely surprising given the presence of a *cis*-regulatory element for IL-6 in the rat *Serpina6* promoter (48) and the established role of IL-6 in the acute phase response during inflammation (280). This is also in line with inverse relationships between IL-6 and plasma CBG levels in humans (23, 79) and studies in HepG2 cells where an IL-6 induced reduction in CBG production (77) was associated with decreased *Serpina6* mRNA stability (78). Moreover, the strong negative correlation we observed between plasma CBG and IL-6 levels, further supports the proposition that IL-6 inhibits CBG production during inflammation. Together, our results suggest that the mechanisms responsible for decreases in

plasma CBG levels during inflammation are multi-factorial and occur in a sequential manner. First, the RCL of CBG undergoes proteolysis rendering it essentially non-functional as a steroid-binding protein, thereby amplifying free plasma corticosterone levels. Further decreases to plasma CBG levels are then caused by decreases in liver *Serpina6* mRNA levels, mediated by increased IL-6 and corticosterone levels.

Activated immune cells produce a wide variety of cytokines and cytokine levels increase in the circulation during inflammation (281, 282). We have now found that animals with evidence of CBG proteolysis also had increased cytokine (IFN-γ, TFNα, IL-4, IL-6, IL-10 and IL-13) levels, in association with large increases in spleen weight, which is a recognized marker of inflammation. These changes in plasma cytokine levels are expected to act together with increases in corticosterone levels and bioavailability to repress the production of cytokines in an attempt to alleviate cytokine-mediated tissue damage in rats with cleaved CBG. Increases in the plasma levels of positive acute phase plasma proteins, including other SERPINA family members, such as AAT (*SERPINA1*) that inhibits the activities of neutrophil elastase in humans, may have occurred in animals that developed inflammation. Although this may afford some protection to CBG proteolysis, there is little information about the roles of AAT and other related SERPINAs, or their target proteases, in rats in relation to inflammation.

The fact that both plasma IL-6 and IL-13 levels are markedly elevated in animals with cleaved CBG, while only IL-6 levels were inversely correlated with plasma CBG levels, suggests that these cytokines function in different ways. In contrast to IL-6, the lack of any relationship between plasma CBG and IL-13 levels suggests that IL-13 does not influence CBG production or proteolysis. Moreover, the coincidence of increased cytokine levels and spleen weights in rats with cleaved CBG indicates an underlying inflammatory response, even in the absence of clinical

symptoms. Although some rats with cleaved CBG did not display overt signs of inflammation over the experimental period, it is possible that these may have developed if the study had been extended. Nevertheless, these data indicate that evidence of CBG proteolysis in plasma samples is a potential pre-symptomatic biomarker of inflammation.

In conclusion, our time course study of CBG changes during inflammation demonstrates that significant decreases in the corticosterone-binding capacity of plasma CBG occur in rats that developed inflammation, with the magnitude of the decline matching the clinical severity. Notably, large decreases in plasma CBG levels occur prior to clinical signs of inflammation. At termination, significant decreases in the corticosterone-binding capacity of CBG appear to coincide with CBG proteolysis that causes a loss of its steroid-binding activity. Significant increases in pro- and anti-inflammatory plasma cytokine levels, as well as increased spleen weights, were all associated with evidence of CBG proteolysis. These novel findings suggest that CBG proteolysis is a marker of active inflammation and, perhaps even more importantly, is a prognostic indicator of inflammation onset. In addition, the fact that rats with evidence of plasma CBG proteolysis had similar liver *Serpina6* mRNA levels to rats with intact CBG, suggests that cleavage of plasma CBG occurs before any reductions in hepatic CBG production occur. Overall, our data suggest that changes in CBG, particularly CBG proteolysis, are an early, pre-symptomatic, marker of inflammation and a useful biomarker of inflammation onset and severity.

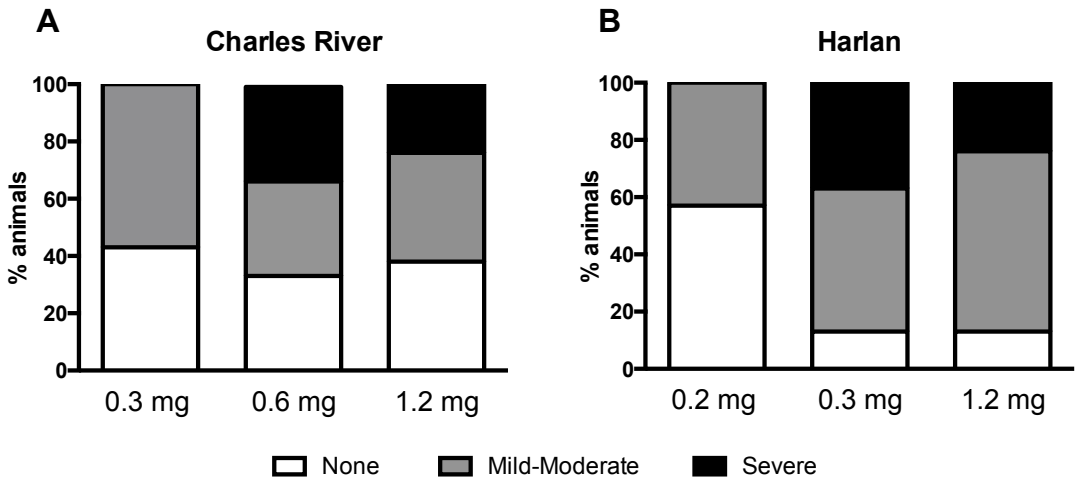


Figure 5.1 Severity of inflammation following complete Freund's adjuvant injection in Charles River and Harlan SD rats. (A, B) Arthritis severity profiles (% of rats in each inflammation category at day 16 post-injection: none, mild-moderate or severe) across the three CFA doses for Charles River (A: 0.3, 0.6, 1.2 mg) and Harlan (B: 0.2, 0.3, 1.2 mg) SD rats. N=7-8 rats per vendor per dose. Data was obtained by Dr. Tamara Bodnar.

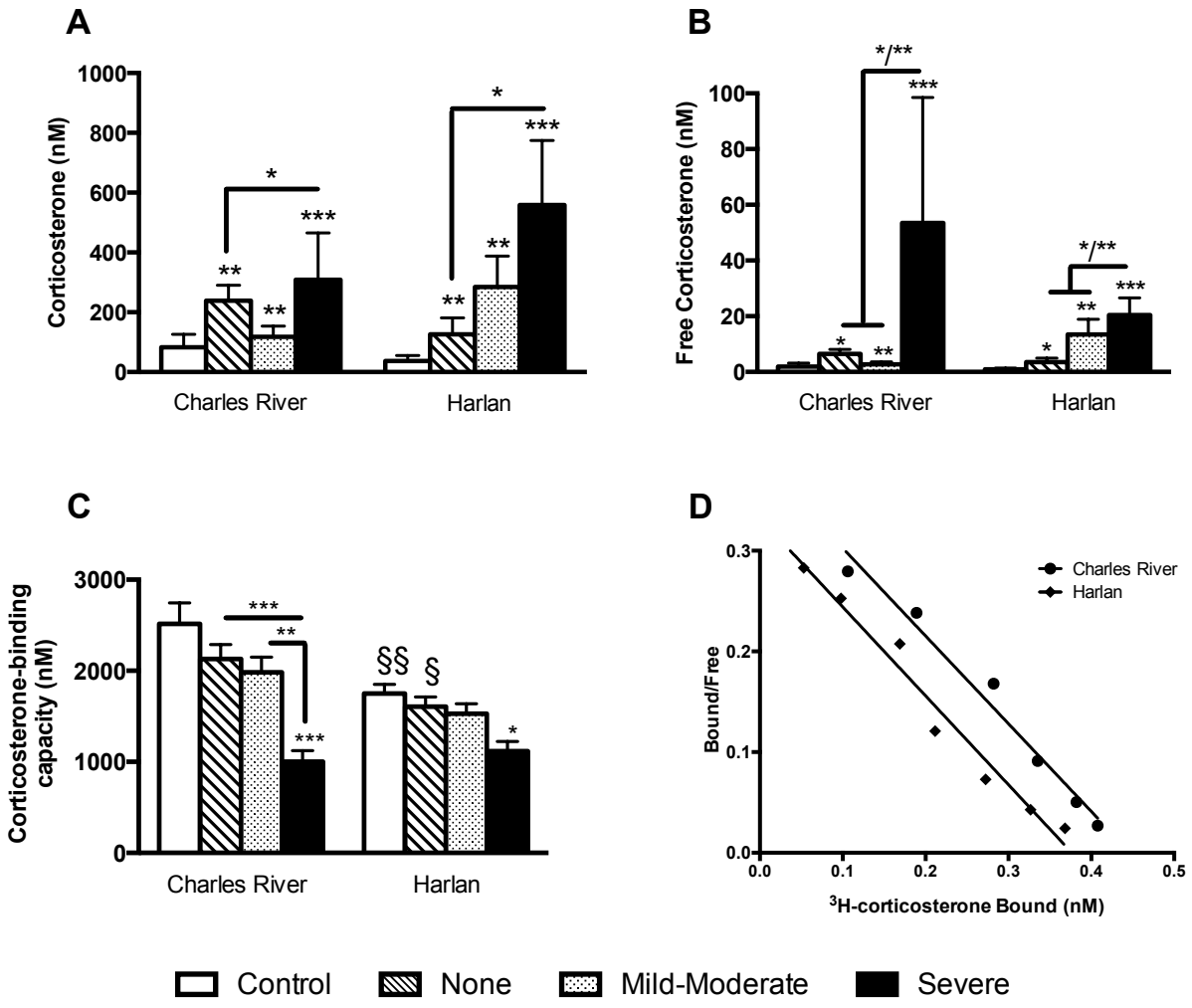

A
Corticosterone (nM)
1000
800
600
400
200
0
Charles River
Harlan
B
Free Corticosterone (nM)
100
80
60
40
20
0
Charles River
Harlan
C
Corticosterone-binding capacity (nM)
3000
2000
1000
0
Charles River
Harlan
D
Bound/Free
0.3
0.2
0.1
0.0
0.0
0.1
0.2
0.3
0.4
0.5
3H-corticosterone Bound (nM)
Charles River
Harlan
Control
None
Mild-Moderate
Severe

Figure 5.2. Plasma corticosterone (total and free) and CBG levels, and characterization of plasma CBG by Scatchard plot. (A) Plasma corticosterone levels increased in adjuvant-treated rats from both colonies [main effect of inflammation severity, $F(3, 49) = 7.00$, $P < 0.001$]. (B) Free corticosterone levels also increased in adjuvant-treated rats from both colonies [main effect of inflammation severity, $F(3, 48) = 8.40$, $P < 0.001$]. Note: Corticosterone (total (A) and free (B)) levels were log-transformed for statistical analysis; untransformed levels are presented here. (C) Plasma CBG levels decreased with increasing inflammation severity [main effect of inflammation severity, $F(3, 49) = 10.99$, $P < 0.001$], with differential effects by colony [main effect of colony, $F(1, 49) = 9.72$, $P < 0.01$]. Harlan rats had lower plasma CBG levels in both saline-treated control rats and adjuvant-treated rats without clinical signs of inflammation (None), when compared to Charles River rats (§§/§). Data (A – C) are presented as mean ± SEM of measurements collected from animals at all doses (Harlan: 0.2, 0.3 and 1.2 mg; Charles River: 0.3, 0.6 and 1.2 mg) of adjuvant used. Control, Charles River: n = 7, Harlan: n = 6; None, Charles River: n = 14, Harlan: n = 6; Mild-Moderate, Charles River: n = 3, Harlan: n =12; Severe, Charles River: n = 4, Harlan: n = 5. Post hoc: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ with comparisons made to controls, unless indicated otherwise. Pair-wise comparisons between colonies: §, $P < 0.05$ and §§, $P < 0.01$. (D) Scatchard plots indicate no difference in the rate dissociation constant (Kd) between Charles River (Kd = 1.15 nM) and Harlan (Kd = 1.13 nM) control rats, indicating no difference in CBG-corticosterone binding affinities between SD colonies. Data for (A) and (B) were obtained by Dr. Tamara Bodnar.

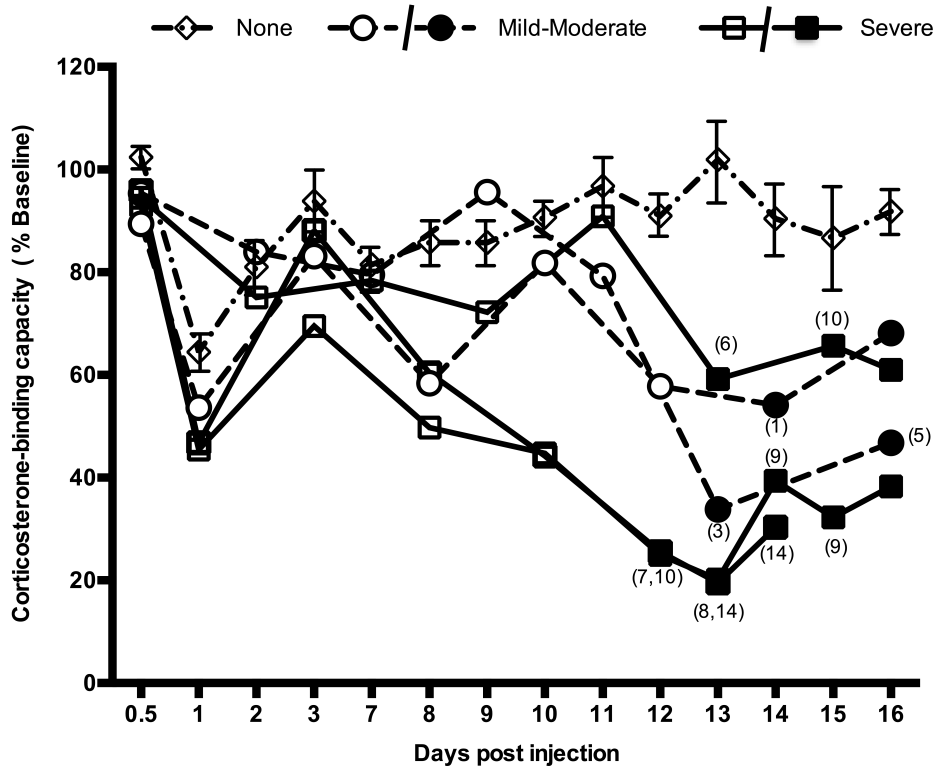
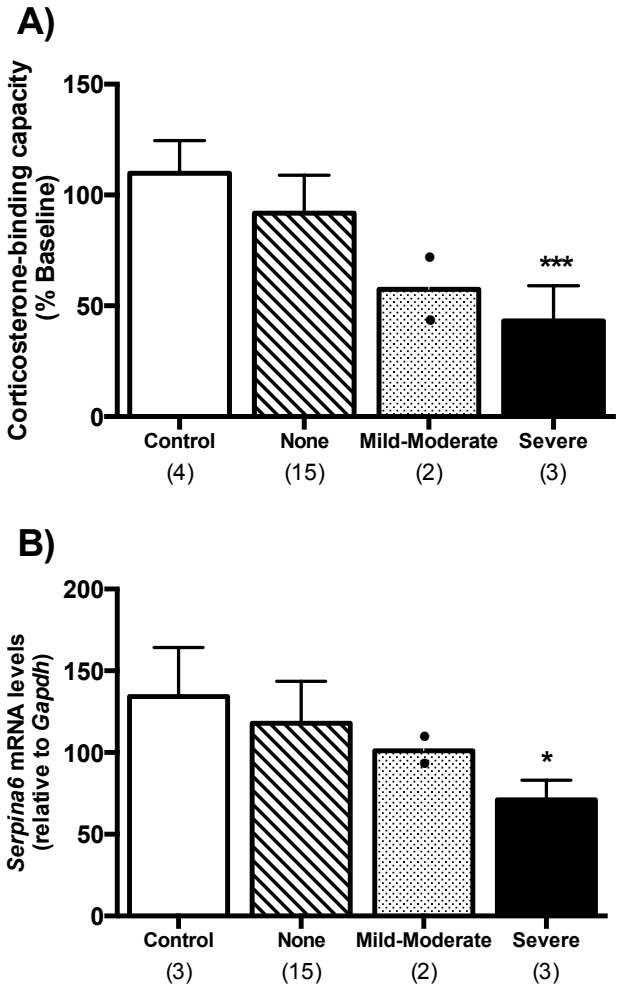


Figure 5.3. Time course of plasma CBG levels during inflammatory responses. Blood samples were collected from each animal prior to treatment (baseline values) and after CFA injection (0.6 mg), as indicated. A reduction in the corticosterone-binding capacity of serum CBG was observed 24 hrs post injection in all adjuvant-treated animals. Following this, rats that developed inflammation had large decreases in CBG levels, whereas the mean CBG levels in animals without clinical signs of inflammation (none, *diamonds*) were unchanged. Rats with mild-moderate inflammation (*circles*) displayed reduced CBG levels 12-13 days post-injection. Two of three rats with severe inflammation (*squares*) had reduced CBG levels as early as day 8, several days before clinical signs of inflammation were evident. Solid symbols denote the presence of clinical symptoms with the corresponding clinical score given in parentheses, while open symbols denote the absence of clinical signs of inflammation. Data for rats (n=15) without inflammation are presented as mean ± SEM for comparison.


A)
Corticosterone-binding capacity (% Baseline)
150
100
50
0
***
Control
(4)
None
(15)
Mild-Moderate
(2)
Severe
(3)
B)
Serpina6 mRNA levels (relative to Gapdh)
200
150
100
50
0
*
Control
(3)
None
(15)
Mild-Moderate
(2)
Severe
(3)

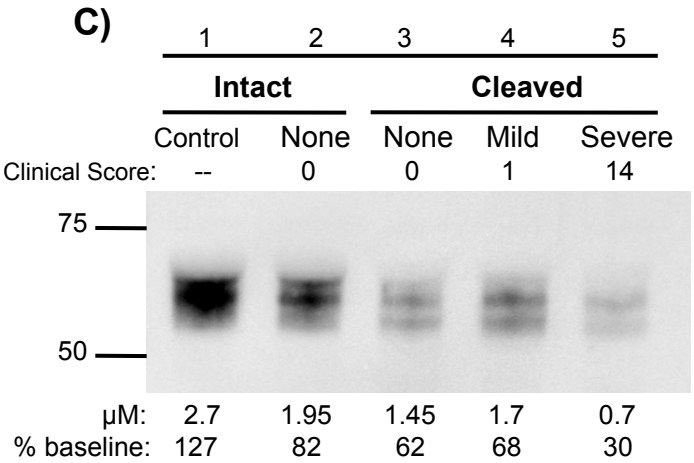

C)
1
2
3
4
5
Intact
Cleaved
Control
None
None
Mild
Severe
Clinical Score:
--
0
0
1
14
75
50
µM:
2.7
1.95
1.45
1.7
0.7
% baseline:
127
82
62
68
30

Figure 5.4. Plasma CBG (A) and liver *Serpina6* mRNA (B) levels, and evidence of CBG proteolysis (C) in relation to clinical score (None, Mild-Moderate, or Severe). In A and B, the numbers of animals in each group are shown in parentheses, and rats (n=2) with mild-moderate inflammation were not included in statistical analyses. Rats with severe inflammation were compared to both saline-treated control animals and adjuvant-treated animals without clinincal signs of inflammation (None). (A) Plasma CBG levels decreased in relation to clinical score with a significant decrease in rats with severe inflammation [main effect of inflammation severity, $F(2,19) = 14.85$, $P = 0.0001$]. (B) Liver *Serpina6* mRNA levels were significantly lower in animals with severe inflammation [main effect of inflammation severity, $F(2,17) = 5.59$, $P < 0.02$]. Data in A and B are presented as mean ± SD. Tukey's Post hoc: *$P < 0.05$; ***$P < 0.001$. (C) Representative western blot illustrating the proteolysis of plasma CBG, as indicated by an ~5 kDa size reduction (lanes 3-5), in concert with reductions in CBG corticosterone-binding capacity (μM) when compared as a percentage of pretreatment values (shown below). Note that CBG proteolysis was also observed in some animals prior to clinical signs of inflammation (None, lane 3).

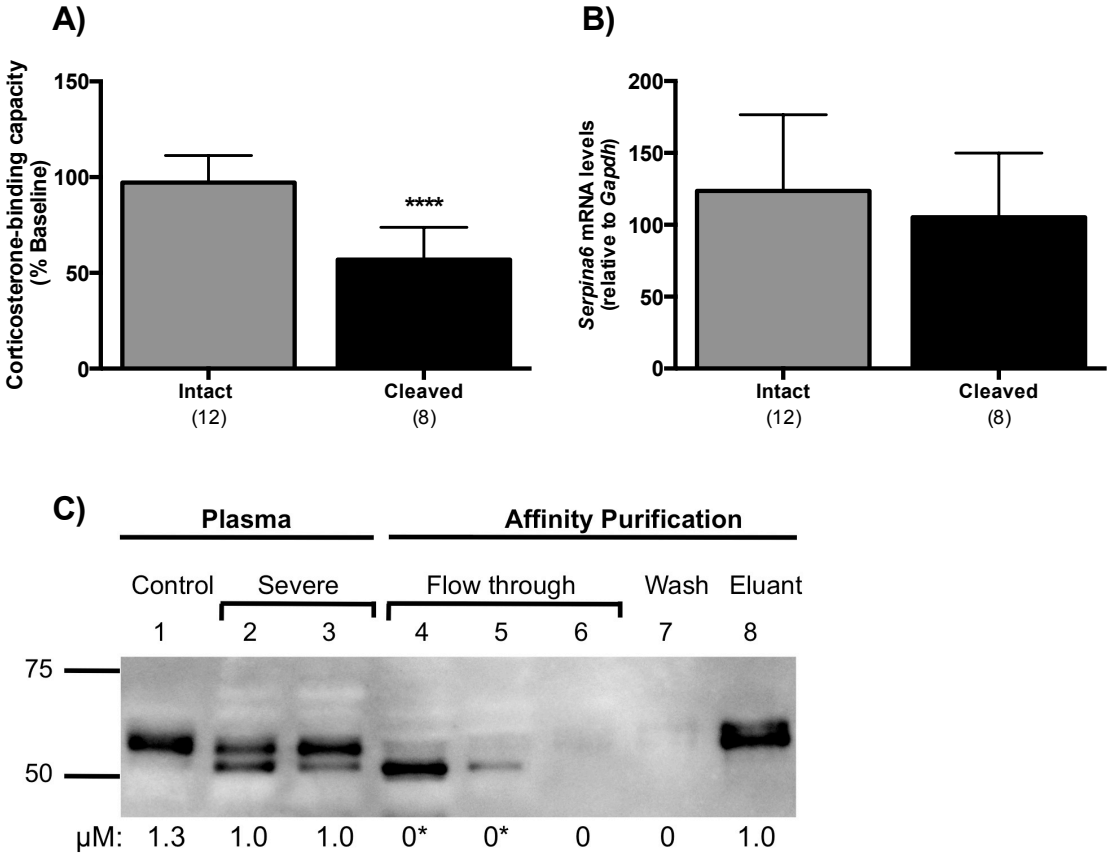

A)
Corticosterone-binding capacity (% Baseline)
150
100
50
0
****
Intact
(12)
Cleaved
(8)
B)
Serpina6 mRNA levels (relative to Gapdh)
200
150
100
50
0
Intact
(12)
Cleaved
(8)
C)
Plasma
Affinity Purification
Control
Severe
Flow through
Wash
Eluant
1
2
3
4
5
6
7
8
75
50
μM:
1.3
1.0
1.0
0*
0*
0
0
1.0

Figure 5.5. Proteolysis of CBG is associated with reduced plasma CBG levels without changes in liver *Serpina6* mRNA levels, and evidence that cleaved CBG in plasma lacks steroid-binding activity. (A) Rats with cleaved CBG had significantly lower plasma CBG values than rats with intact CBG. (B) Liver *Serpina6* mRNA levels were similar irrespective of CBG proteolysis status. In A and B, samples were grouped for analysis based on CBG integrity, as assessed by western blotting (see Figure 5.4C), and classified as being either intact or cleaved. The numbers of animals in each group are shown in parentheses, and data are presented as mean ± SD. **** $P < 0.0001$. (C) Plasma from rats with severe inflammation (*lanes 2 and 3*) were pooled and purified by steroid-affinity chromatography. Cleaved CBG did not bind the steroid-affinity matrix and eluted in the flow through (*lanes 4 and 5*). Intact CBG bound to the steroid-affinity matrix and was eluted with buffer containing excess corticosterone (*lane 8*). CBG-corticosterone binding capacity values (μM) are shown under each lane. There was no detectable CBG steroid-binding activity (*) in the flow through fractions that contained cleaved CBG (*lanes 4 and 5*). Intact CBG (lane *8*) exhibited full steroid-binding activity. An intact control (saline) sample is shown (*lane 1*) for comparison.

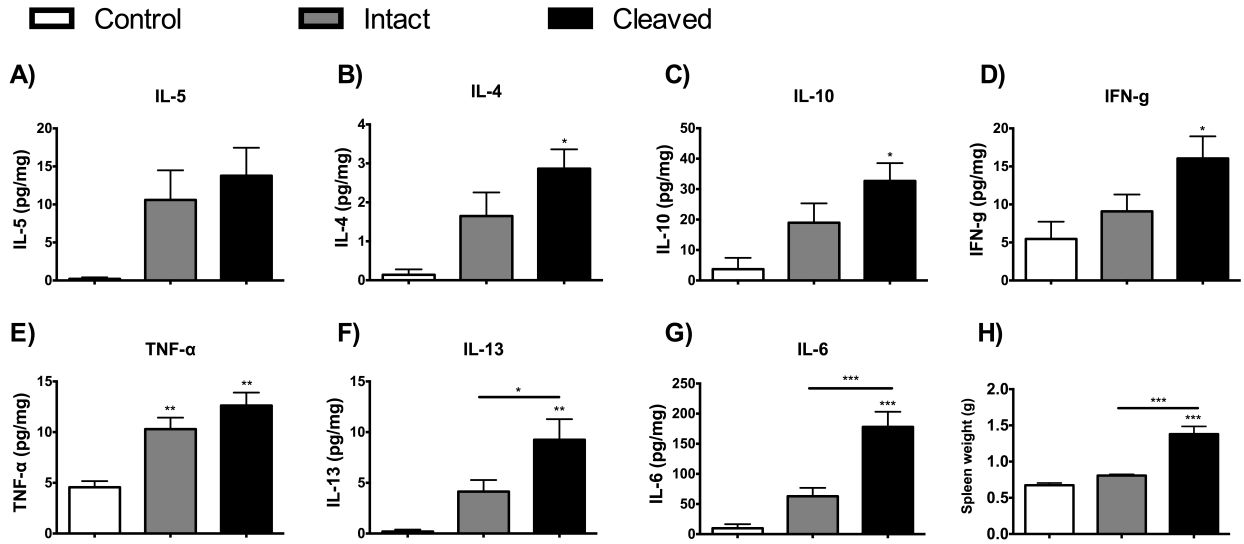


Figure 5.6. Associations between CBG proteolysis, plasma cytokine levels (A-G) and spleen weights (H). As in Figure 5.5, results from adjuvant-treated rats were grouped based on CBG proteolysis status (i.e., cleaved versus intact CBG). When compared to control (saline-treated) rats, all plasma cytokines, apart from IL-5 (A), were significantly increased in rats with evidence of CBG proteolysis, as was spleen weight (H). TNF-α (E, [main effect of CBG proteolysis, $F(2,23) = 7.30$, $p < 0.004$]) showed a treatment effect with significantly increased levels in adjuvant-treated animals (intact and cleaved CBG) when compared to controls. Plasma levels of IL-13 (F, [main effect of CBG proteolysis, $F(2,23) = 6.36$, $p < 0.007$]), IL-6 (G, [main effect of CBG proteolysis, $F(2,23) = 16.50$, $p < 0.001$]) and spleen weight (H, [main effect of CBG proteolysis, $F(2,23) = 30.47$, $p < 0.001$]) were significantly increased in samples with cleaved CBG versus intact CBG. Data (pg/ml) are presented as mean ± SEM. Control, n=4; Intact, n=12; Cleaved, n=8. Fischer's Post hoc: *P < 0.05; **P < 0.01; ***P < 0.001. Data was obtained by Dr. Tamara Bodnar and analyzed by myself.

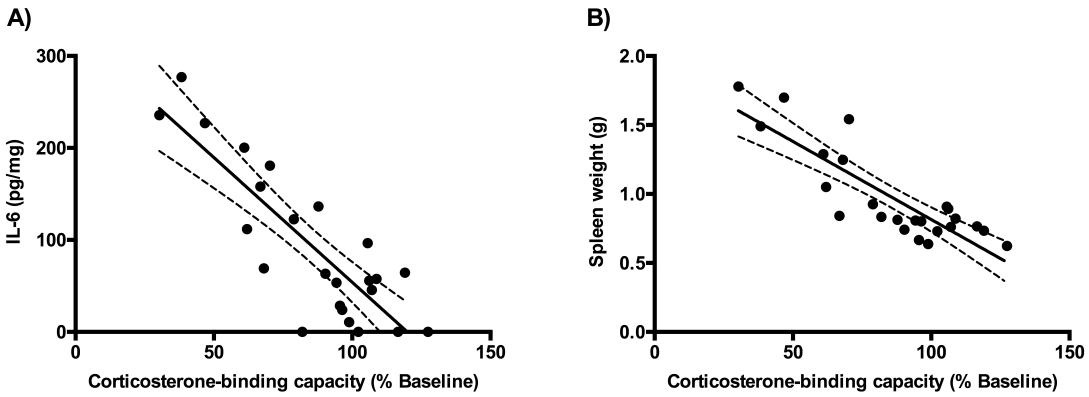


Figure 5.7. Strong negative correlations between plasma CBG and IL-6 levels and CBG and spleen weights. Data were analyzed using linear regression, with plasma CBG levels expressed as a % of pretreatment values. (A) Plasma CBG and IL-6 levels (r^2 =0.71) and (B) plasma CBG and spleen weights (r^2 =0.73), were negatively correlated. The best-fit linear regression line (*solid*) and 95% confidence interval (*dotted*) are also shown.

Chapter 6: Conclusions

In this thesis, I investigated the role of CBG (aka SERPINA6) in health and disease. In particular, I focused on genetic abnormalities and the functional consequences associated with naturally occurring single nucleotide polymorphisms in the *SERPINA6* gene. In addition, I completed both human and animal studies to further elucidate the role that CBG plays in acute inflammatory states. This section summarizes the main findings and their significance and proposes future directions for this area of research.

6.1 Genetic abnormalities

In **chapter 3**, the physiological and biochemical consequences of naturally occurring genetic variants of *SERPINA6* were studied. In the first study, a new *SERPINA6* SNP was identified, resulting in the W371S CBG variant, which lacks cortisol-binding activity. This was followed up by a study where we biochemically characterized non-synonymous SNP listed in various public databases, identifying eight variants with abnormal production and/or function and two variants with altered antibody recognition. The final study of this chapter confirmed that in humans, CBG is the primary determinant of circulating plasma cortisol levels and evidence was provided that discrepancies in CBG values obtained by utilizing parallel ELISAs are likely not due to the presence of cleaved CBG within the circulation.

To date, a clear definition of the clinical consequences of CBG deficiencies has been hampered by the fact that almost all CBG variants with severely compromised production or function occur rarely in genetically diverse populations. Nevertheless, a complete lack of functional CBG does not result in an overt clinical phenotype, yet the number of subjects with CBG deficiencies who have been evaluated clinically is far too limited to permit accurate assessments of their overall pathophysiological significance. As seen in **chapter 3** and in other

studies (16, 101), both the concentration and steroid-binding properties of CBG influence the plasma distribution of cortisol, leading to elevations in the percentage of free cortisol within the circulation. Therefore, functional alterations in CBG alter the bioavailability of glucocorticoids, thereby affecting their biological activities, metabolism and clearance. In CBG deficient patients, this may be particularly important for modulating the expression of glucocorticoid-regulated genes, where transient increases in glucocorticoids are likely to exceed the buffering capacity of CBG. Further, abnormal glucocorticoid levels found in individuals with inherited CBG deficits may be linked to altered physiological states through epigenetic modifications or alterations in the maturation of organ systems *in utero*.

Identifying patients with CBG deficiencies presents a diagnostic challenge, as guidelines for the diagnosis of defective CBG production and/or function are not well defined, due to the fact that CBG variants are not always associated with non-specific signs of adrenal insufficiency. Based on our findings, a CBG deficiency should be suspected in patients presenting with low total plasma cortisol who have normal free cortisol levels, salivary cortisol or urinary cortisol, along with a lower than normal increase in total plasma cortisol after exogenous glucocorticoid administration or ACTH stimulation test. The identification of variants with a loss of recognition by immunoassays together with the growing number of variants identified with abnormal steroid-binding activity illustrates the limitations of using steroid-binding capacity assays or immunoassays alone in clinical evaluations and indicates that both assays should be used when CBG deficiencies are suspected. To identify individuals with CBG variants, the direct measurement of total and free cortisol levels is recommended in conjunction with serum CBG measurements by both immunoassay and cortisol-binding capacity assays. Due to inherent issues with monoclonal antibody derived immunoassays and the ability of cortisol-binding capacity

assays to provide an accurate assessment of the amount of functional CBG, binding assays should be considered the gold standard for the determination of plasma CBG levels. A lack of functional information provided by immunoassays also limits there usefulness in calculations of serum free cortisol values and the measurement of functional CBG by cortisol-binding capacity assay is recommended for these purposes.

Recently, a number of studies have been completed with parallel ELISAs that utilize specific monoclonal antibodies in order to discriminate between CBG with an intact versus cleaved RCL. Despite reports that cleaved CBG makes up 25-30% of total CBG in the circulation (217), direct evidence of CBG RCL cleavage has never been presented. In **chapter 3**, it was demonstrated that a lack of glycosylation at Asn347 in the T349A variant resulted in altered 9G12 antibody recognition. In addition, the strongest correlation of CBG levels in the GWAS study was found between CBG-cortisol binding capacity and 12G2 ELISA values, with a lack of correlations observed with the 9G12 ELISA. Based on our results, we suspect that differential *N*-glycosylation of Asn347 alters 9G12 antibody recognition and is responsible for the discrepancy seen between CBG-cortisol binding capacity, 12G2 and 9G12 ELISA values in some samples. Once again, this highlights the limitations of solely relying on immunoassays to assess plasma CBG levels and suggests that caution should be taken when interpreting the meaning of differences in CBG values obtained using the parallel ELISAs.

6.2 Disease states

In **chapter 4 and 5**, studies were completed to further define the role of CBG in acute inflammation, in humans and rodents, respectively. In **chapter 4**, various bacterial strains were screened for their ability to cleave the human CBG RCL and *Pseudomonas aeruginosa* elastase was found to proteolytically cleave the CBG RCL after Asn347. Building on these findings,

when comparing ICU patient plasma to normal healthy controls, α-2-macroglobulin was shown to have an enhanced ability to specifically inhibit CBG RCL cleavage by *P. aeruginosa*. In addition, significantly decreased plasma CBG levels were found in various ICU patient populations, with particularly low values observed in individuals that presented with a clinical profile that included severe inflammation. Similar findings were made in **chapter 5**, where significant decreases in the CBG-corticosterone binding capacity were found in Sprague Dawley rats that developed severe inflammation. Interestingly, Harlan SD rats, which were found to have 30-50% lower baseline plasma CBG levels than Charles River SD rats, had a greater incidence and severity of inflammation. Using a time course study to explore the mechanisms responsible for changes in plasma CBG levels over the course of inflammation in Charles River SD rats, we demonstrated that CBG RCL cleavage occurs prior to reductions in hepatic *Serpina6* gene expression. We also showed that decreases in plasma CBG levels occur before clinical signs of inflammation, suggesting that changes in CBG may be a pre-symptomatic biomarker of inflammation.

In **chapter 4**, we discovered that PAE, a protease secreted by *P. aeruginosa*, specifically cleaves the human CBG RCL at novel sites to that of neutrophil elastase (8, 9) and chymotrypsin (120), resulting in a loss of CBG-cortisol binding activity. In addition, I demonstrated that α-2-macroglobulin inhibits CBG RCL cleavage by PAE secreted by *P. aeruginosa*. In infectious and inflammatory diseases of tissues like the lung and skin, where *P. aeruginosa* infections are common, the cleavage of CBG by PAE may augment the actions of neutrophil elastase, promoting the localized release of cortisol. Increases in free cortisol levels at these sites will have a broad range of actions, including modifying the production of cytokines, free radicals, prostaglandins and chemotactic factors, by various cell types involved in the inflammatory

response, as well as controlling the activity of infiltrating immune cells (256). Understanding the relative contribution of PAE and neutrophil elastase, as well as their inhibitors α2-macroglobulin and α1-antitrypsin, respectively, to these processes is likely to be relevant in optimizing the dosage and route of glucocorticoid administration to treat inflammation associated with severe *P. aeruginosa* infections.

When comparing Harlan and Charles River SD rats, lower baseline plasma CBG levels in Harlan rats suggests a lower corticosterone reservoir, which could play a key role in the observed increased susceptibility of Harlan rats to the immune challenge. Thus, the larger corticosteroid reservoir in Charles River SD rats may function to more effectively deliver corticosterone to sites of inflammation and result in reduced incidence and severity of inflammation in these rats. In the severe inflammation state, when CBG levels are significantly lower and total corticosterone levels are significantly higher, there is a substantial increase in free corticosterone, which would also be available at target tissues.

In both rodent studies (**chapter 5**), significant decreases in the corticosterone-binding capacity of plasma CBG occurred in rats that developed inflammation, with the magnitude of the decline matching the clinical severity. Notably, in the time course study, large decreases in plasma CBG levels occurred prior to clinical signs of inflammation. These significant decreases in the corticosterone-binding capacity of CBG coincided with CBG proteolysis that causes a loss of steroid-binding activity. Notably, in humans, a size reduction of CBG consistent with RCL cleavage has never been observed, and it has been hypothesized that RCL cleaved human CBG is rapidly removed from the blood circulation (233), but further studies are needed to confirm this hypothesis. By contrast, cleaved CBG is readily observed in rats with severe inflammation and this suggests a species difference in the plasma clearance of CBG after proteolysis.

Significant increases in pro- and anti-inflammatory plasma cytokine levels, as well as increased spleen weights, were all associated with evidence of CBG proteolysis and suggest that CBG proteolysis is a marker of active inflammation and, perhaps even more importantly, is a prognostic indicator of inflammation onset. In addition, the finding that rats with evidence of plasma CBG proteolysis had similar liver *Serpina6* mRNA levels to rats with intact CBG, suggests that cleavage of plasma CBG occurs before any reductions in hepatic CBG production provides evidence for a long hypothesized theory of the mechanisms leading to plasma CBG reductions during inflammation. Overall, the rodent data suggests that changes in CBG, particularly CBG proteolysis, is an early, pre-symptomatic, marker of inflammation and a useful biomarker of inflammation onset and severity.

Similar to the results in **chapter 5**, significant reductions in plasma CBG levels were observed in various human ICU patient populations. Notably, differences exist based on the clinical etiology, suggesting a differential role of altered plasma CBG levels depending on the underlying pathophysiology. Nonetheless, together, the results from our studies completed in both human and rodents support the notion that CBG plays an important role in inflammatory reactions.

Further support for the notion that discrepancies in plasma CBG values obtained by the parallel ELISAs do not represent the presence of proteolytically cleaved CBG within the circulation were provided by measurements of CBG levels in ICU samples (**chapter 4.3**). Similar to that seen in the GWAS samples (**chapter 3.4**), the strongest correlation was observed between CBG-cortisol binding capacity and 12G2 ELISA values. Seeing as the binding capacity assay measures the amount of functional CBG and it is known that CBG with a cleaved RCL is unable to bind steroid with high enough affinity to detect in our steroid-binding assays, a strong

correlation between the binding assay and 12G2 ELISA suggest that, despite lower 9G12 ELISA values, RCL cleaved CBG is not present. Once again, although further studies are needed to confirm the cause of discrepant 9G12 ELISA values, a likely candidate is differential glycosylation of the *N*-glycan site, particularly within the CBG RCL.

6.3 Future directions

Given the expanded list of *SERPINA6* SNP characterized in these studies, it will now be important to examine the effects of these CBG variant proteins in both normal physiological and clinical contexts, particularly in relation to specific disease groups. In this regard, it may be helpful to focus on specific ethnic populations, in which CBG variants, such as A51V (Han Chinese (143)), D367N (Mediterranean/Middle Eastern (16)) and L93H (European (14, 140)), have been identified at higher frequencies. In addition, further studies will need to be completed to characterize additional *SERPINA6* SNP identified in genome-sequencing projects completed after the publication of our study to keep the list of functionally significant CBG variants up to date.

While measurements of CBG by immunoassay may have utility in monitoring changes in CBG levels in response to changes in physiological state or disease, it is becoming increasingly evident that CBG measurements by immunoassays should not be used reliably in mathematical calculations of serum free cortisol levels (213). Given the inconsistencies seen in the measurement of plasma CBG using immunoassays, the identification of variants with a loss of recognition by immunoassays and the growing number of variants identified with abnormal steroid-binding activity, there is an urgent need to develop robust clinical chemical assays to measure both the levels and steroid-binding activity of CBG, in conjunction with direct measurements of plasma free cortisol levels.

The immunolocalization of CBG within cells remains a topic of interest. Previous studies have identified immunoreactive CBG within various cell types, but the validity of such results is challenged by a number of methodological limitations, including the lack of negative controls and the use of non-specific polyclonal antibodies. In addition, there is no direct evidence for the intracellular uptake of CBG. Some of the strongest evidence for the presence of CBG within the cell has recently been shown using *Serpina6* knockout mice (283), where the presence of low levels of *Serpina6* transcripts as well as immunoreactive CBG were detected in the mouse brain. Further studies will need to be completed to further investigate the role of intracellular CBG, but its expression in various brain regions could be important for negative feedback regulation of the HPA axis, as well as alterations in behavior.

Although progress was made in characterizing and elucidating the mechanisms responsible for the changes in plasma CBG levels that occur during inflammation, more work still needs to be done. Our studies investigating the mechanisms responsible for CBG changes during inflammation were completed in a rodent model and will therefore need to be completed in humans. In addition, the role of CBG in the recovery process and the timing of the normalization of plasma CBG levels during inflammation is largely unknown and is of interest, as alterations in CBG levels likely plays a role in mediating how glucocorticoids act to restore the normal homeostatic balance. Finally, with mounting evidence of the importance of CBG in the inflammatory reaction, studies examining the treatment potential of plasma CBG supplementation are of interest.

References

1. **Daughaday WH** 1956 Binding of corticosteroids by plasma proteins. I. dialysis equilibrium and renal clearance studies. J Clin Invest 35(12):1428-1433
2. **Dunn JF, Nisula BC, Rodbard D** 1981 Transport of steroid hormones: Binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 53(1):58-68
3. **Westphal U** 1986 Steroid-protein interactions II. In: Monographs on Endocrinology. Berlin, Germany: Springer Verlag; 59-83
4. **Westphal U** 1983 Steroid-protein interactions I. In: Monographs on Endocrinology. Berlin, Germany: Springer Verlag; 164-350
5. **Henley DE, Lightman SL** 2011 New insights into corticosteroid-binding globulin and glucocorticoid delivery. Neuroscience 180:1-8
6. **Hammond GL, Smith CL, Goping IS, Underhill DA, Harley MJ, Reventos J, Musto NA, Gunsalus GL, Bardin CW** 1987 Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs, exhibits homology with serine protease inhibitors. Proc Natl Acad Sci U S A 84(15):5153-5157
7. **Avvakumov GV, Hammond GL** 1994 Glycosylation of human corticosteroid-binding globulin. differential processing and significance of carbohydrate chains at individual sites. Biochemistry 33(19):5759-5765
8. **Pemberton PA, Stein PE, Pepys MB, Potter JM, Carrell RW** 1988 Hormone binding globulins undergo serpin conformational change in inflammation. Nature 336(6196):257-258

9. **Hammond GL, Smith CL, Paterson NA, Sibbald WJ** 1990 A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. J Clin Endocrinol Metab 71(1):34-39

10. **Hammond GL, Smith CL, Underhill DA** 1991 Molecular studies of corticosteroid binding globulin structure, biosynthesis and function. J Steroid Biochem Mol Biol 40(4-6):755-762

11. **Klieber MA, Underhill C, Hammond GL, Muller YA** 2007 Corticosteroid-binding globulin, a structural basis for steroid transport and proteinase-triggered release. J Biol Chem 282(40):29594-29603

12. **Zhou A, Wei Z, Stanley PL, Read RJ, Stein PE, Carrell RW** 2008 The S-to-R transition of corticosteroid-binding globulin and the mechanism of hormone release. J Mol Biol 380(1):244-251

13. **Gardill BR, Vogl MR, Lin HY, Hammond GL, Muller YA** 2012 Corticosteroid-binding globulin: Structure-function implications from species differences. PLoS One 7(12):e52759

14. **Smith CL, Power SG, Hammond GL** 1992 A leu----his substitution at residue 93 in human corticosteroid binding globulin results in reduced affinity for cortisol. J Steroid Biochem Mol Biol 42(7):671-676

15. **Torpy DJ, Bachmann AW, Grice JE, Fitzgerald SP, Phillips PJ, Whitworth JA, Jackson RV** 2001 Familial corticosteroid-binding globulin deficiency due to a novel null mutation: Association with fatigue and relative hypotension. J Clin Endocrinol Metab 86(8):3692-3700

16. **Emptoz-Bonneton A, Cousin P, Seguchi K, Avvakumov GV, Bully C, Hammond GL, Pugeat M** 2000 Novel human corticosteroid-binding globulin variant with low cortisol-binding affinity. J Clin Endocrinol Metab 85(1):361-367

17. **Gagliardi L, Ho JT, Torpy DJ** 2010 Corticosteroid-binding globulin: The clinical significance of altered levels and heritable mutations. Mol Cell Endocrinol 316(1):24-34

18. **Scrocchi LA, Orava M, Smith CL, Han VK, Hammond GL** 1993 Spatial and temporal distribution of corticosteroid-binding globulin and its messenger ribonucleic acid in embryonic and fetal mice. Endocrinology 132(2):903-909

19. **Benassayag C, Souski I, Mignot TM, Robert B, Hassid J, Duc-Goiran P, Mondon F, Rebourcet R, Dehennin L, Nunez EA, Ferre F** 2001 Corticosteroid-binding globulin status at the fetomaternal interface during human term pregnancy. Biol Reprod 64(3):812-821

20. **Sivukhina EV, Jirikowski GF, Bernstein HG, Lewis JG, Herbert Z** 2006 Expression of corticosteroid-binding protein in the human hypothalamus, co-localization with oxytocin and vasopressin. Horm Metab Res 38(4):253-259

21. **Hammond GL** 2016 Plasma steroid-binding proteins: Primary gatekeepers of steroid hormone action. J Endocrinol 230(1):R13-25

22. **Scrocchi LA, Hearn SA, Han VK, Hammond GL** 1993 Corticosteroid-binding globulin biosynthesis in the mouse liver and kidney during postnatal development. Endocrinology 132(2):910-916

23. **Bernier J, Jobin N, Emptoz-Bonneton A, Pugeat MM, Garrel DR** 1998 Decreased corticosteroid-binding globulin in burn patients: Relationship with interleukin-6 and fat in nutritional support. Crit Care Med 26(3):452-460

24. **Tinnikov AA, Legan MV, Sheveluk NA, Cvetovskaya GA, Naumenko SE, Sidelnikov SG** 1996 Corticosteroid and immune responses to cardiac surgery. Steroids 61(7):411-415

25. **Ho JT, Al-Musalhi H, Chapman MJ, Quach T, Thomas PD, Bagley CJ, Lewis JG, Torpy DJ** 2006 Septic shock and sepsis: A comparison of total and free plasma cortisol levels. J Clin Endocrinol Metab 91(1):105-114

26. **Kadmiel M, Cidlowski JA** 2013 Glucocorticoid receptor signaling in health and disease. Trends Pharmacol Sci 34(9):518-530

27. **Mendel CM** 1989 The free hormone hypothesis: A physiologically based mathematical model. Endocr Rev 10(3):232-274

28. **Tsai MJ, O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451-486

29. **Oakley RH, Cidlowski JA** 2011 Cellular processing of the glucocorticoid receptor gene and protein: New mechanisms for generating tissue-specific actions of glucocorticoids. J Biol Chem 286(5):3177-3184

30. **Htun H, Barsony J, Renyi I, Gould DL, Hager GL** 1996 Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. Proc Natl Acad Sci U S A 93(10):4845-4850

31. **Rhen T, Cidlowski JA** 2005 Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. N Engl J Med 353(16):1711-1723

32. **Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR** 2009 DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324(5925):407-410

33. **Lonard DM, O'malley BW** 2007 Nuclear receptor coregulators: Judges, juries, and executioners of cellular regulation. Mol Cell 27(5):691-700

34. **Sacta MA, Chinenov Y, Rogatsky I** 2016 Glucocorticoid signaling: An update from a genomic perspective. Annu Rev Physiol 78:155-180

35. **Anbalagan M, Huderson B, Murphy L, Rowan BG** 2012 Post-translational modifications of nuclear receptors and human disease. Nucl Recept Signal 10:e001

36. **Siiteri PK, Murai JT, Hammond GL, Nisker JA, Raymoure WJ, Kuhn RW** 1982 The serum transport of steroid hormones. Recent Prog Horm Res 38:457-510

37. **Lin HY, Muller YA, Hammond GL** 2010 Molecular and structural basis of steroid hormone binding and release from corticosteroid-binding globulin. Mol Cell Endocrinol 316(1):3-12

38. **Hunt LT, Dayhoff MO** 1980 A surprising new protein superfamily containing ovalbumin, antithrombin-III, and alpha 1-proteinase inhibitor. Biochem Biophys Res Commun 95(2):864-871

39. **Huntington JA, Read RJ, Carrell RW** 2000 Structure of a serpin-protease complex shows inhibition by deformation. Nature 407(6806):923-926

40. **Billingsley GD, Walter MA, Hammond GL, Cox DW** 1993 Physical mapping of four serpin genes: Alpha 1-antitrypsin, alpha 1-antichymotrypsin, corticosteroid-binding globulin, and protein C inhibitor, within a 280-kb region on chromosome I4q32.1. Am J Hum Genet 52(2):343-353

41. **Namciu SJ, Friedman RD, Marsden MD, Sarausad LM, Jasoni CL, Fournier RE** 2004 Sequence organization and matrix attachment regions of the human serine protease inhibitor gene cluster at 14q32.1. Mammalian genome : official journal of the International Mammalian Genome Society 15(3):162-78

42. **Zhao H, Friedman RD, Fournier RE** 2007 The locus control region activates serpin gene expression through recruitment of liver-specific transcription factors and RNA polymerase II. Mol Cell Biol 27(15):5286-5295

43. **Underhill DA, Hammond GL** 1989 Organization of the human corticosteroid binding globulin gene and analysis of its 5'-flanking region. Mol Endocrinol 3(9):1448-1454

44. **Avvakumov GV, Warmels-Rodenhiser S, Hammond GL** 1993 Glycosylation of human corticosteroid-binding globulin at aspargine 238 is necessary for steroid binding. J Biol Chem 268(2):862-866

45. **Smith CL, Hammond GL** 1989 Rat corticosteroid binding globulin: Primary structure and messenger ribonucleic acid levels in the liver under different physiological conditions. Mol Endocrinol 3(2):420-426

46. **Avvakumov GV, Hammond GL** 1994 Substitutions of tryptophan residues in human corticosteroid-binding globulin: Impact on steroid binding and glycosylation. J Steroid Biochem Mol Biol 49(2-3):191-194

47. **Grenot C, Blachere T, Rolland de Ravel M, Mappus E, Cuilleron CY** 1994 Identification of trp-371 as the main site of specific photoaffinity labeling of corticosteroid binding globulin using delta 6 derivatives of cortisol, corticosterone, and progesterone as unsubstituted photoreagents. Biochemistry 33(30):8969-8981

48. **Underhill DA, Hammond GL** 1995 Cis-regulatory elements within the proximal promoter of the rat gene encoding corticosteroid-binding globulin. Gene 162(2):205-211

49. **Zhao XF, Underhill DA, Hammond GL** 1997 Hepatic nuclear proteins that bind cis-regulatory elements in the proximal promoter of the rat corticosteroid-binding globulin gene. Mol Cell Endocrinol 126(2):203-212

50. **Li Y, Wu L, Lei J, Zhu C, Wang H, Yu X, Lin H** 2012 Single nucleotide polymorphisms in the human corticosteroid-binding globulin promoter alter transcriptional activity. Sci China Life Sci 55(8):699-708

51. **Schlechte JA, Hamilton D** 1987 The effect of glucocorticoids on corticosteroid binding globulin. Clin Endocrinol (Oxf) 27(2):197-203

52. **Deak T, Meriwether JL, Fleshner M, Spencer RL, Abouhamze A, Moldawer LL, Grahn RE, Watkins LR, Maier SF** 1997 Evidence that brief stress may induce the acute phase response in rats. Am J Physiol 273(6 Pt 2):R1998-2004

53. **Tinnikov AA** 1999 Responses of serum corticosterone and corticosteroid-binding globulin to acute and prolonged stress in the rat. Endocrine 11(2):145-150

54. **Feldman D, Mondon CE, Horner JA, Weiser JN** 1979 Glucocorticoid and estrogen regulation of corticosteroid-binding globulin production by rat liver. Am J Physiol 237(6):E493-9

55. **Yamamoto S, Ohsawa N** 1976 Effects of dexamethasone on the levels of plasma corticosteroid binding globulin in rats and monkeys. Biochem Biophys Res Commun 72(2):489-498

56. **Smith CL, Hammond GL** 1992 Hormonal regulation of corticosteroid-binding globulin biosynthesis in the male rat. Endocrinology 130(4):2245-2251

57. **Challis JR, Huhtanen D, Sprague C, Mitchell BF, Lye SJ** 1985 Modulation by cortisol of adrenocorticotropin-induced activation of adrenal function in fetal sheep. Endocrinology 116(6):2267-2272

58. **Berdusco ET, Hammond GL, Jacobs RA, Grolla A, Akagi K, Langlois D, Challis JR** 1993 Glucocorticoid-induced increase in plasma corticosteroid-binding globulin levels in fetal

sheep is associated with increased biosynthesis and alterations in glycosylation. Endocrinology 132(5):2001-2008

59. **Pepe GJ, Jury HH, Hammond GL, Albrecht ED** 1996 Developmental regulation of corticosteroid-binding globulin biosynthesis in the baboon fetus. Endocrinology 137(8):3323-3328

60. **Zha Q, Wang Y, Fan Y, Zhu MY** 2011 Dexamethasone-induced up-regulation of the human norepinephrine transporter involves the glucocorticoid receptor and increased binding of C/EBP-beta to the proximal promoter of norepinephrine transporter. J Neurochem 119(3):654-663

61. **Wang JC, Stromstedt PE, Sugiyama T, Granner DK** 1999 The phosphoenolpyruvate carboxykinase gene glucocorticoid response unit: Identification of the functional domains of accessory factors HNF3 beta (hepatic nuclear factor-3 beta) and HNF4 and the necessity of proper alignment of their cognate binding sites. Mol Endocrinol 13(4):604-618

62. **Verhoog N, Allie-Reid F, Vanden Berghe W, Smith C, Haegeman G, Hapgood J, Louw A** 2014 Inhibition of corticosteroid-binding globulin gene expression by glucocorticoids involves C/EBPbeta. PLoS One 9(10):e110702

63. **Brien TG** 1981 Human corticosteroid binding globulin. Clin Endocrinol (Oxf) 14(2):193-212

64. **Perogamvros I, Ray DW, Trainer PJ** 2012 Regulation of cortisol bioavailability--effects on hormone measurement and action. Nat Rev Endocrinol 8(12):717-727

65. **Durber SM, Lawson J, Daly JR** 1976 The effect of oral contraceptives on plasma cortisol and cortisol binding capacity throughout the menstrual cycle in normal women. Br J Obstet Gynaecol 83(10):814-818

66. **van Seters AP, Moolenaar AJ** 1991 Mitotane increases the blood levels of hormone-binding proteins. Acta Endocrinol (Copenh) 124(5):526-533

67. **Nader N, Raverot G, Emptoz-Bonneton A, Dechaud H, Bonnay M, Baudin E, Pugeat M** 2006 Mitotane has an estrogenic effect on sex hormone-binding globulin and corticosteroid-binding globulin in humans. J Clin Endocrinol Metab 91(6):2165-2170

68. **Gala RR, Westphal U** 1965 Corticosteroid-binding globulin in the rat: Studies on the sex difference. Endocrinology 77(5):841-851

69. **Jansson JO, Oscarsson J, Mode A, Ritzen EM** 1989 Plasma growth hormone pattern and androgens influence the levels of corticosteroid-binding globulin in rat serum. J Endocrinol 122(3):725-732

70. **Van Baelen H, Adam-Heylen M, Vandoren G, De Moor P** 1977 Neonatal imprinting of serum transcortin levels in the rat. J Steroid Biochem 8(7):735-736

71. **Jansson JO, Ekberg S, Isaksson O, Mode A, Gustafsson JA** 1985 Imprinting of growth hormone secretion, body growth, and hepatic steroid metabolism by neonatal testosterone. Endocrinology 117(5):1881-1889

72. **Yoon JB, Towle HC, Seelig S** 1987 Growth hormone induces two mRNA species of the serine protease inhibitor gene family in rat liver. J Biol Chem 262(9):4284-4289

73. **Clark RG, Jansson JO, Isaksson O, Robinson IC** 1985 Intravenous growth hormone: Growth responses to patterned infusions in hypophysectomized rats. J Endocrinol 104(1):53-61

74. **Tschop M, Lahner H, Feldmeier H, Grasberger H, Morrison KM, Janssen OE, Attanasio AF, Strasburger CJ** 2000 Effects of growth hormone replacement therapy on

levels of cortisol and cortisol-binding globulin in hypopituitary adults. Eur J Endocrinol 143(6):769-773

75. **Crave JC, Lejeune H, Brebant C, Baret C, Pugeat M** 1995 Differential effects of insulin and insulin-like growth factor I on the production of plasma steroid-binding globulins by human hepatoblastoma-derived (hep G2) cells. J Clin Endocrinol Metab 80(4):1283-1289
76. **Berryman DE, Glad CA, List EO, Johannsson G** 2013 The GH/IGF-1 axis in obesity: Pathophysiology and therapeutic considerations. Nat Rev Endocrinol 9(6):346-356
77. **Emptoz-Bonneton A, Crave JC, LeJeune H, Brebant C, Pugeat M** 1997 Corticosteroid-binding globulin synthesis regulation by cytokines and glucocorticoids in human hepatoblastoma-derived (HepG2) cells. J Clin Endocrinol Metab 82(11):3758-3762
78. **Bartalena L, Hammond GL, Farsetti A, Flink IL, Robbins J** 1993 Interleukin-6 inhibits corticosteroid-binding globulin synthesis by human hepatoblastoma-derived (hep G2) cells. Endocrinology 133(1):291-296
79. **Tsigos C, Kyrou I, Chrousos GP, Papanicolaou DA** 1998 Prolonged suppression of corticosteroid-binding globulin by recombinant human interleukin-6 in man. J Clin Endocrinol Metab 83(9):3379
80. **Fleshner M, Silbert L, Deak T, Goehler LE, Martin D, Watkins LR, Maier SF** 1997 TNF-alpha-induced corticosterone elevation but not serum protein or corticosteroid binding globulin reduction is vagally mediated. Brain Res Bull 44(6):701-706
81. **Weiser JN, Do YS, Feldman D** 1979 Synthesis and secretion of corticosteroid-binding globulin by rat liver. A source of heterogeneity of hepatic corticosteroid-binders. J Clin Invest 63(3):461-467

82. **Perrot-Applanat M, Milgrom E** 1979 Messenger ribonucleic acid for corticosteroid-binding globulin. translation and preliminary characterization. Biochemistry 18(25):5732-5737

83. **Perrot-Applanat M, David-Ferreira JF, David-Ferreira KL** 1981 Immunocytochemical localization of corticosteroid-binding globulin (CBG) in guinea pig hepatocytes. Endocrinology 109(5):1625-1633

84. **Wolf G, Armstrong EG, Rosner W** 1981 Synthesis in vitro of corticosteroid-binding globulin from rat liver messenger ribonucleic acid. Endocrinology 108(3):805-811

85. **Sueda K, Seo H, Matsui N** 1985 Human transcortin synthesis by a cell-free translation of hepatic mRNA. Endocrinol Jpn 32(2):295-303

86. **Khan MS, Aden D, Rosner W** 1984 Human corticosteroid binding globulin is secreted by a hepatoma-derived cell line. J Steroid Biochem 20(2):677-678

87. **Lin GX, Selcer KW, Beale EG, Gray GO, Leavitt WW** 1990 Characterization of the corticosteroid-binding globulin messenger ribonucleic acid response in the pregnant hamster. Endocrinology 127(4):1934-1940

88. **Waite C, Mejia R, Ascoli M** 2016 Gq/11-dependent changes in the murine ovarian transcriptome at the end of gestation. Biol Reprod 94(3):62

89. **Seralini GE, Smith CL, Hammond GL** 1990 Rabbit corticosteroid-binding globulin: Primary structure and biosynthesis during pregnancy. Mol Endocrinol 4(8):1166-1172

90. **Caldwell JD, Jirikowski GF** 2013 An active role for steroid-binding globulins: An update. Horm Metab Res 45(7):477-484

91. **Schafer HH, Gebhart VM, Hertel K, Jirikowski GF** 2015 Expression of corticosteroid-binding globulin CBG in the human heart. Horm Metab Res 47(8):596-599

92. **Misao R, Iwagaki S, Sun WS, Fujimoto J, Saio M, Takami T, Tamaya T** 1999 Evidence for the synthesis of corticosteroid-binding globulin in human placenta. Horm Res 51(4):162-167

93. **Misao R, Nakanishi Y, Fujimoto J, Iwagaki S, Tamaya T** 1999 Levels of sex hormone-binding globulin and corticosteroid-binding globulin mRNAs in corpus luteum of human subjects: Correlation with serum steroid hormone levels. Gynecol Endocrinol 13(2):82-88

94. **Werthamer S, Samuels AJ, Amaral L** 1973 Identification and partial purification of "transcortin"-like protein within human lymphocytes. J Biol Chem 248(18):6398-6407

95. **Grasa MM, Cabot C, Balada F, Virgili J, Sanchis D, Monserrat C, Fernandez-Lopez JA, Remesar X, Alemany M** 1998 Corticosterone binding to tissues of adrenalectomized lean and obese zucker rats. Horm Metab Res 30(12):699-704

96. **Perrot-Applanat M, Racadot O, Milgrom E** 1984 Specific localization of plasma corticosteroid-binding globulin immunoreactivity in pituitary corticotrophs. Endocrinology 115(2):559-569

97. **de Kloet ER, Voorhuis TA, Leunissen JL, Koch B** 1984 Intracellular CBG-like molecules in the rat pituitary. J Steroid Biochem 20(1):367-371

98. **Mopert B, Herbert Z, Caldwell JD, Jirikowski GF** 2006 Expression of corticosterone-binding globulin in the rat hypothalamus. Horm Metab Res 38(4):246-252

99. **Predine J, Brailly S, Delaporte P, Milgrom E** 1984 Protein binding of cortisol in human cerebrospinal fluid. J Clin Endocrinol Metab 58(1):6-11

100. **Lightman SL** 2008 The neuroendocrinology of stress: A never ending story. J Neuroendocrinol 20(6):880-884

101. **Perogamvros I, Underhill C, Henley DE, Hadfield KD, Newman WG, Ray DW, Lightman SL, Hammond GL, Trainer PJ** 2010 Novel corticosteroid-binding globulin variant that lacks steroid binding activity. J Clin Endocrinol Metab 95(10):E142-50

102. **Petersen HH, Andreassen TK, Breiderhoff T, Brasen JH, Schulz H, Gross V, Grone HJ, Nykjaer A, Willnow TE** 2006 Hyporesponsiveness to glucocorticoids in mice genetically deficient for the corticosteroid binding globulin. Mol Cell Biol 26(19):7236-7245

103. **Robinson PA, Hammond GL** 1985 Identification and characterization of a human corticosteroid binding globulin variant with a reduced affinity for cortisol. J Endocrinol 104(2):269-277

104. **Smith CL, Hammond GL** 1991 An amino acid substitution in biobreeding rat corticosteroid binding globulin results in reduced steroid binding affinity. J Biol Chem 266(28):18555-18559

105. **Mickelson KE, Forsthoefel J, Westphal U** 1981 Steroid-protein interactions. human corticosteroid binding globulin: Some physicochemical properties and binding specificity. Biochemistry 20(21):6211-6218

106. **Hammond GL** 1990 Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. Endocr Rev 11(1):65-79

107. **Cizza G, Rother KI** 2012 Cortisol binding globulin: More than just a carrier? J Clin Endocrinol Metab 97(1):77-80

108. **Song HK, Lee KN, Kwon KS, Yu MH, Suh SW** 1995 Crystal structure of an uncleaved alpha 1-antitrypsin reveals the conformation of its inhibitory reactive loop. FEBS Lett 377(2):150-154

109. **Baumann U, Huber R, Bode W, Grosse D, Lesjak M, Laurell CB** 1991 Crystal structure of cleaved human alpha 1-antichymotrypsin at 2.7 A resolution and its comparison with other serpins. J Mol Biol 218(3):595-606

110. **Marver D, Chiu W, Wolff ME, Edelman IS** 1976 Photoaffinity site-specific covalent labeling of human corticosteroid-binding globulin. Proc Natl Acad Sci U S A 73(12):4462-4466

111. **Vashchenko G, Das S, Moon KM, Rogalski JC, Taves MD, Soma KK, Van Petegem F, Foster LJ, Hammond GL** 2016 Identification of avian corticosteroid-binding globulin (SerpinA6) reveals the molecular basis of evolutionary adaptations in SerpinA6 structure and function as a steroid-binding protein. J Biol Chem 291(21):11300-11312

112. **Breuner CW, Orchinik M** 2002 Plasma binding proteins as mediators of corticosteroid action in vertebrates. J Endocrinol 175(1):99-112

113. **Jin L, Abrahams JP, Skinner R, Petitou M, Pike RN, Carrell RW** 1997 The anticoagulant activation of antithrombin by heparin. Proc Natl Acad Sci U S A 94(26):14683-14688

114. **Baglin TP, Carrell RW, Church FC, Esmon CT, Huntington JA** 2002 Crystal structures of native and thrombin-complexed heparin cofactor II reveal a multistep allosteric mechanism. Proc Natl Acad Sci U S A 99(17):11079-11084

115. **Lin HY, Underhill C, Gardill BR, Muller YA, Hammond GL** 2009 Residues in the human corticosteroid-binding globulin reactive center loop that influence steroid binding before and after elastase cleavage. J Biol Chem 284(2):884-896

116. **Irving JA, Pike RN, Lesk AM, Whisstock JC** 2000 Phylogeny of the serpin superfamily: Implications of patterns of amino acid conservation for structure and function. Genome Res 10(12):1845-1864

117. **Graur D, Li WH** 1988 Evolution of protein inhibitors of serine proteinases: Positive darwinian selection or compositional effects? J Mol Evol 28(1-2):131-135

118. **Gettins PG** 2002 Serpin structure, mechanism, and function. Chemical reviews 102(12):4751-804

119. **Hill RE, Hastie ND** 1987 Accelerated evolution in the reactive centre regions of serine protease inhibitors. Nature 326(6108):96-99

120. **Lewis JG, Elder PA** 2014 The reactive centre loop of corticosteroid-binding globulin (CBG) is a protease target for cortisol release. Mol Cell Endocrinol 384(1-2):96-101

121. **Hopkins PC, Carrell RW, Stone SR** 1993 Effects of mutations in the hinge region of serpins. Biochemistry 32(30):7650-7657

122. **Mitra N, Sinha S, Ramya TN, Surolia A** 2006 N-linked oligosaccharides as outfitters for glycoprotein folding, form and function. Trends Biochem Sci 31(3):156-163

123. **Hebert DN, Lamriben L, Powers ET, Kelly JW** 2014 The intrinsic and extrinsic effects of N-linked glycans on glycoproteostasis. Nat Chem Biol 10(11):902-910

124. **Aebi M** 2013 N-linked protein glycosylation in the ER. Biochim Biophys Acta 1833(11):2430-2437

125. **Hossner KL, Billiar RB** 1981 Plasma clearance and organ distribution of native and desialylated rat and human transcortin: Species specificity. Endocrinology 108(5):1780-1786

126. **Seralini GE, Underhill CM, Smith CL, Nguyen VT, Hammond GL** 1989 Biological half-life and transfer of maternal corticosteroid-binding globulin to amniotic fluid in the rabbit. Endocrinology 125(3):1321-1325

127. **Lewis JG, Saunders K, Dyer A, Elder PA** 2015 The half-lives of intact and elastase cleaved human corticosteroid-binding globulin (CBG) are identical in the rabbit. J Steroid Biochem Mol Biol 149:53-57

128. **Kato EA, Hsu BR, Kuhn RW** 1988 Comparative structural analyses of corticosteroid binding globulin. J Steroid Biochem 29(2):213-220

129. **Mickelson KE, Harding GB, Forsthoefel M, Westphal U** 1982 Steroid-protein interactions. human corticosteroid-binding globulin: Characterization of dimer and electrophoretic variants. Biochemistry 21(4):654-660

130. **Robinson PA, Langley MS, Hammond GL** 1985 A solid-phase radioimmunoassay for human corticosteroid binding globulin. J Endocrinol 104(2):259-267

131. **Nyberg L, Marekov LN, Jones I, Lundquist G, Jornvall H** 1990 Characterization of the murine corticosteroid binding globulin: Variations between mammalian forms. J Steroid Biochem 35(1):61-65

132. **Ali S, Bassett JR** 1995 Studies on the role of glycosylation in the origin of the electrophoretic variants for rat corticosteroid-binding globulin. Steroids 60(11):743-752

133. **Sumer-Bayraktar Z, Kolarich D, Campbell MP, Ali S, Packer NH, Thaysen-Andersen M** 2011 N-glycans modulate the function of human corticosteroid-binding globulin. Molecular & cellular proteomics : MCP 10(8):M111 009100

134. **Sumer-Bayraktar Z, Grant OC, Venkatakrishnan V, Woods RJ, Packer NH, Thaysen-Andersen M** 2016 Asn347 glycosylation of corticosteroid-binding globulin fine-tunes the

host immune response by modulating proteolysis by pseudomonas aeruginosa and neutrophil elastase. J Biol Chem 291(34):17727-17742

135. **Mitchell E, Torpy DJ, Bagley CJ** 2004 Pregnancy-associated corticosteroid-binding globulin: High resolution separation of glycan isoforms. Horm Metab Res 36(6):357-359

136. **Mihrshahi R, Lewis JG, Ali SO** 2006 Hormonal effects on the secretion and glycoform profile of corticosteroid-binding globulin. J Steroid Biochem Mol Biol 101(4-5):275-285

137. **Strel'chyonok OA, Avvakumov GV** 1991 Interaction of human CBG with cell membranes. J Steroid Biochem Mol Biol 40(4-6):795-803

138. **Van Baelen H, Brepoels R, De Moor P** 1982 Transcortin leuven: A variant of human corticosteroid-binding globulin with decreased cortisol-binding affinity. J Biol Chem 257(7):3397-3400

139. **Roitman A, Bruchis S, Bauman B, Kaufman H, Laron Z** 1984 Total deficiency of corticosteroid-binding globulin. Clin Endocrinol (Oxf) 21(5):541-548

140. **Van Baelen H, Power SG, Hammond GL** 1993 Decreased cortisol-binding affinity of transcortin leuven is associated with an amino acid substitution at residue-93. Steroids 58(6):275-277

141. **Torpy DJ, Lundgren BA, Ho JT, Lewis JG, Scott HS, Mericq V** 2012 CBG santiago: A novel CBG mutation. J Clin Endocrinol Metab 97(1):E151-5

142. **Cizza G, Bernardi L, Smirne N, Maletta R, Tomaino C, Costanzo A, Gallo M, Lewis JG, Geracitano S, Grasso MB, Potenza G, Monteleone C, Brancati G, Ho JT, Torpy DJ, Bruni AC** 2011 Clinical manifestations of highly prevalent corticosteroid-binding globulin mutations in a village in southern italy. J Clin Endocrinol Metab 96(10):E1684-93

143. **Lin HY, Underhill C, Lei JH, Helander-Claesson A, Lee HY, Gardill BR, Muller YA, Wang H, Hammond GL** 2012 High frequency of SERPINA6 polymorphisms that reduce plasma corticosteroid-binding globulin activity in chinese subjects. J Clin Endocrinol Metab 97(4):E678-86

144. **Torpy DJ, Bachmann AW, Gartside M, Grice JE, Harris JM, Clifton P, Easteal S, Jackson RV, Whitworth JA** 2004 Association between chronic fatigue syndrome and the corticosteroid-binding globulin gene ALA SER224 polymorphism. Endocr Res 30(3):417-429

145. **Zhao XF** 1996 Regulation of corticosteroid-binding globulin gene expression. The University of Western Ontario

146. **Orava M, Zhao XF, Leiter E, Hammond GL** 1994 Structure and chromosomal location of the gene encoding mouse corticosteroid-binding globulin: Strain differences in coding sequence and steroid-binding activity. Gene 144(2):259-264

147. **Guyonnet-Duperat V, Geverink N, Plastow GS, Evans G, Ousova O, Croisetiere C, Foury A, Richard E, Mormede P, Moisan MP** 2006 Functional implication of an Arg307Gly substitution in corticosteroid-binding globulin, a candidate gene for a quantitative trait locus associated with cortisol variability and obesity in pig. Genetics 173(4):2143-2149

148. **Jung C, Ho JT, Torpy DJ, Rogers A, Doogue M, Lewis JG, Czajko RJ, Inder WJ** 2011 A longitudinal study of plasma and urinary cortisol in pregnancy and postpartum. J Clin Endocrinol Metab 96(5):1533-1540

149. **Ho JT, Lewis JG, O'Loughlin P, Bagley CJ, Romero R, Dekker GA, Torpy DJ** 2007 Reduced maternal corticosteroid-binding globulin and cortisol levels in pre-eclampsia and gamete recipient pregnancies. Clin Endocrinol (Oxf) 66(6):869-877

150. **Strel'chyonok OA, Avvakumov GV** 1990 Specific steroid-binding glycoproteins of human blood plasma: Novel data on their structure and function. J Steroid Biochem 35(5):519-534

151. **Smith CL, Hammond GL** 1991 Ontogeny of corticosteroid-binding globulin biosynthesis in the rat. Endocrinology 128(2):983-988

152. **O'Leary P, Boyne P, Flett P, Beilby J, James I** 1991 Longitudinal assessment of changes in reproductive hormones during normal pregnancy. Clin Chem 37(5):667-672

153. **Lei J, Yang XK, Peng S, Li Y, Underhill C, Zhu C, Lin HY, Wang H, Hammond GL** 2015 Impact of corticosteroid-binding globulin deficiency on pregnancy and neonatal sex. J Clin Endocrinol Metab

154. **Navara KJ** 2010 Programming of offspring sex ratios by maternal stress in humans: Assessment of physiological mechanisms using a comparative approach. J Comp Physiol B 180(6):785-796

155. **Rosenfeld CS, Roberts RM** 2004 Maternal diet and other factors affecting offspring sex ratio: A review. Biol Reprod 71(4):1063-1070

156. **Scott SM, Wells L** 1995 Corticosteroid-binding globulin in preterm infants in an intensive care unit. Horm Res 44(5):218-221

157. **Hadjian AJ, Chedin M, Cochet C, Chambaz EM** 1975 Cortisol binding to proteins in plasma in the human neonate and infant. Pediatric Research (9):40-45

158. **Gala RR, Westphal U** 1967 Corticosteroid-binding activity in serum of mouse, rabbit and guinea pig during pregnancy and lactation: Possible involvement in the initiation of lactation. Acta Endocrinol (Copenh) 55(1):47-61

159. **McCormick CM, Linkroum W, Sallinen BJ, Miller NW** 2002 Peripheral and central sex steroids have differential effects on the HPA axis of male and female rats. Stress 5(4):235-247

160. **Kuhn RW, Green AL, Raymoure WJ, Siiteri PK** 1986 Immunocytochemical localization of corticosteroid-binding globulin in rat tissues. J Endocrinol 108(1):31-36

161. **Challis JR, Berdusco ET, Jeffray TM, Yang K, Hammond GL** 1995 Corticosteroid-binding globulin (CBG) in fetal development. J Steroid Biochem Mol Biol 53(1-6):523-527

162. **Haddad JJ, Saade NE, Safieh-Garabedian B** 2002 Cytokines and neuro-immune-endocrine interactions: A role for the hypothalamic-pituitary-adrenal revolving axis. J Neuroimmunol 133(1-2):1-19

163. **Bumiller A, Gotz F, Rohde W, Dorner G** 1999 Effects of repeated injections of interleukin 1beta or lipopolysaccharide on the HPA axis in the newborn rat. Cytokine 11(3):225-230

164. **Brattsand R, Linden M** 1996 Cytokine modulation by glucocorticoids: Mechanisms and actions in cellular studies. Aliment Pharmacol Ther 10 Suppl 2:81-90; discussion 91-2

165. **Simon HU** 2003 Neutrophil apoptosis pathways and their modifications in inflammation. Immunol Rev 193:101-110

166. **Sapolsky RM, Romero LM, Munck AU** 2000 How do glucocorticoids influence stress responses? integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21(1):55-89

167. **Chan WL, Carrell RW, Zhou A, Read RJ** 2013 How changes in affinity of corticosteroid-binding globulin modulate free cortisol concentration. J Clin Endocrinol Metab 98(8):3315-3322

168. **Cameron A, Henley D, Carrell R, Zhou A, Clarke A, Lightman S** 2010 Temperature-responsive release of cortisol from its binding globulin: A protein thermocouple. J Clin Endocrinol Metab 95(10):4689-4695

169. **Beishuizen A, Thijs LG, Vermes I** 2001 Patterns of corticosteroid-binding globulin and the free cortisol index during septic shock and multitrauma. Intensive Care Med 27(10):1584-1591

170. **Muller CA, Belyaev O, Vogeser M, Weyhe D, Gloor B, Strobel O, Werner J, Borgstrom A, Buchler MW, Uhl W** 2007 Corticosteroid-binding globulin: A possible early predictor of infection in acute necrotizing pancreatitis. Scand J Gastroenterol 42(11):1354-1361

171. **Pugeat M, Bonneton A, Perrot D, Rocle-Nicolas B, Lejeune H, Grenot C, Dechaud H, Brebant C, Motin J, Cuilleron CY** 1989 Decreased immunoreactivity and binding activity of corticosteroid-binding globulin in serum in septic shock. Clin Chem 35(8):1675-1679

172. **Bendel S, Karlsson S, Pettila V, Loisa P, Varpula M, Ruokonen E, Finnsepsis Study Group** 2008 Free cortisol in sepsis and septic shock. Anesth Analg 106(6):1813-1819

173. **Savu L, Zouaghi H, Carli A, Nunez EA** 1981 Serum depletion of corticosteroid binding activities, an early marker of human septic shock. Biochem Biophys Res Commun 102(1):411-419

174. **Zouaghi H, Savu L, Kleinknecht D, Nunez E** 1983 Decrease in the binding activity of transcortin (CBG) in the serum of chronic hemodialysis patients. Ann Biol Clin (Paris) 41(4):285-286

175. **Zouaghi H, Savu L, Coulon A, Nunez EA, Guerot C, Gryman R, Amamou R, Miled A** 1984 Corticosteroid-binding globulin in various diseases. Clin Chem 30(2):332-333

176. **Garrel DR, Zhang L, Zhao XF, Hammond GL** 1993 Effect of burn injury on corticosteroid-binding globulin levels in plasma and wound fluid. Wound Repair Regen 1(1):10-14

177. **D'Elia M, Patenaude J, Hamelin C, Garrel DR, Bernier J** 2005 Corticosterone binding globulin regulation and thymus changes after thermal injury in mice. Am J Physiol Endocrinol Metab 288(5):E852-60

178. **Carroll JA, Gaines AM, Spencer JD, Allee GL, Kattesh HG, Roberts MP, Zannelli ME** 2003 Effect of menhaden fish oil supplementation and lipopolysaccharide exposure on nursery pigs. I. effects on the immune axis when fed diets containing spray-dried plasma. Domest Anim Endocrinol 24(4):341-351

179. **Dhabhar FS, McEwen BS, Spencer RL** 1993 Stress response, adrenal steroid receptor levels and corticosteroid-binding globulin levels--a comparison between sprague-dawley, fischer 344 and lewis rats. Brain Res 616(1-2):89-98

180. **Joe B, Griffiths MM, Remmers EF, Wilder RL** 1999 Animal models of rheumatoid arthritis and related inflammation. Curr Rheumatol Rep 1(2):139-148

181. **Turnbull AV, Rivier CL** 1999 Sprague-dawley rats obtained from different vendors exhibit distinct adrenocorticotropin responses to inflammatory stimuli. Neuroendocrinology 70(3):186-195

182. **Libert C, Wielockx B, Hammond GL, Brouckaert P, Fiers W, Elliott RW** 1999 Identification of a locus on distal mouse chromosome 12 that controls resistance to tumor necrosis factor-induced lethal shock. Genomics 55(3):284-289

183. **Jones BC, Sarrieau A, Reed CL, Azar MR, Mormede P** 1998 Contribution of sex and genetics to neuroendocrine adaptation to stress in mice. Psychoneuroendocrinology 23(5):505-517

184. **Rose AJ, Herzig S** 2013 Metabolic control through glucocorticoid hormones: An update. Mol Cell Endocrinol 380(1-2):65-78

185. **Incollingo Rodriguez AC, Epel ES, White ML, Standen EC, Seckl JR, Tomiyama AJ** 2015 Hypothalamic-pituitary-adrenal axis dysregulation and cortisol activity in obesity: A systematic review. Psychoneuroendocrinology 62:301-318

186. **Fernandez-Real JM, Grasa M, Casamitjana R, Pugeat M, Barret C, Ricart W** 1999 Plasma total and glycosylated corticosteroid-binding globulin levels are associated with insulin secretion. J Clin Endocrinol Metab 84(9):3192-3196

187. **Fernandez-Real JM, Grasa M, Casamitjana R, Ricart W** 2000 The insulin resistance syndrome and the binding capacity of cortisol binding globulin (CBG) in men and women. Clin Endocrinol (Oxf) 52(1):93-99

188. **Schafer HH, de Villiers JD, Sivukhina E, Lewis J, Wande D, Perembe B, Jirikowski G** 2013 Altered homeostasis of systemic glucocorticoids as related to obesity, glucose tolerance, and smoking. Horm Metab Res 45(3):245-251

189. **Holt HB, Wild SH, Postle AD, Zhang J, Koster G, Umpleby M, Shojaee-Moradie F, Dewbury K, Wood PJ, Phillips DI, Byrne CD** 2007 Cortisol clearance and associations with insulin sensitivity, body fat and fatty liver in middle-aged men. Diabetologia 50(5):1024-1032

190. **Lewis JG, Shand BI, Elder PA, Scott RS** 2004 Plasma sex hormone-binding globulin rather than corticosteroid-binding globulin is a marker of insulin resistance in obese adult males. Diabetes Obes Metab 6(4):259-263

191. **Fernandez-Real JM, Pugeat M, Emptoz-Bonneton A, Ricart W** 2001 Study of the effect of changing glucose, insulin, and insulin-like growth factor-I levels on serum corticosteroid binding globulin in lean, obese, and obese subjects with glucose intolerance. Metabolism 50(10):1248-1252

192. **Fernandez-Real JM, Pugeat M, Grasa M, Broch M, Vendrell J, Brun J, Ricart W** 2002 Serum corticosteroid-binding globulin concentration and insulin resistance syndrome: A population study. J Clin Endocrinol Metab 87(10):4686-4690

193. **Joyner JM, Hutley LJ, Bachmann AW, Torpy DJ, Prins JB** 2003 Greater replication and differentiation of preadipocytes in inherited corticosteroid-binding globulin deficiency. Am J Physiol Endocrinol Metab 284(5):E1049-54

194. **Yanovski JA, Yanovski SZ, Gold PW, Chrousos GP** 1997 Differences in corticotropin-releasing hormone-stimulated adrenocorticotropin and cortisol before and after weight loss. J Clin Endocrinol Metab 82(6):1874-1878

195. **Manco M, Fernandez-Real JM, Valera-Mora ME, Dechaud H, Nanni G, Tondolo V, Calvani M, Castagneto M, Pugeat M, Mingrone G** 2007 Massive weight loss decreases corticosteroid-binding globulin levels and increases free cortisol in healthy obese patients: An adaptive phenomenon? Diabetes Care 30(6):1494-1500

196. **Moisan MP, Castanon N** 2016 Emerging role of corticosteroid-binding globulin in glucocorticoid-driven metabolic disorders. Front Endocrinol (Lausanne) 7:160

197. **Richard EM, Helbling JC, Tridon C, Desmedt A, Minni AM, Cador M, Pourtau L, Konsman JP, Mormede P, Moisan MP** 2010 Plasma transcortin influences endocrine and behavioral stress responses in mice. Endocrinology 151(2):649-659

198. **Minni AM, de Medeiros GF, Helbling JC, Duittoz A, Marissal-Arvy N, Foury A, De Smedt-Peyrusse V, Pallet V, Moisan MP** 2014 Role of corticosteroid binding globulin in emotional reactivity sex differences in mice. Psychoneuroendocrinology 50:252-263

199. **Vogeser M, Halser B, Baron A, Jacob K, Demant T** 2000 Corticosteroid-binding globulin and unbound serum cortisol in women with polycystic ovary syndrome. Clin Biochem 33(2):157-159

200. **Vogeser M, Briegel J, Zachoval R** 2002 Dialyzable free cortisol after stimulation with synacthen. Clin Biochem 35(7):539-543

201. **Zhang X, Lan N, Bach P, Nordstokke D, Yu W, Ellis L, Meadows GG, Weinberg J** 2012 Prenatal alcohol exposure alters the course and severity of adjuvant-induced arthritis in female rats. Brain Behav Immun 26(3):439-450

202. **Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, Sirotkin K** 2001 dbSNP: The NCBI database of genetic variation. Nucleic Acids Res 29(1):308-311

203. **Tennessen JA, Bigham AW, O'Connor TD, Fu W, Kenny EE, Gravel S, McGee S, Do R, Liu X, Jun G, Kang HM, Jordan D, Leal SM, Gabriel S, Rieder MJ, Abecasis G, Altshuler D, Nickerson DA, Boerwinkle E, Sunyaev S, Bustamante CD, Bamshad MJ, Akey JM, Broad GO, Seattle GO, NHLBI Exome Sequencing Project** 2012 Evolution and functional impact of rare coding variation from deep sequencing of human exomes. Science 337(6090):64-69

204. **1000 Genomes Project Consortium, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, Handsaker RE, Kang HM, Marth GT, McVean GA** 2012 An integrated map of genetic variation from 1,092 human genomes. Nature 491(7422):56-65

205. **Hammond GL, Lahteenmaki PL** 1983 A versatile method for the determination of serum cortisol binding globulin and sex hormone binding globulin binding capacities. Clin Chim Acta 132(1):101-110

206. **Lebrun I, Marques-Porto R, Pereira AS, Pereira A, Perpetuo EA** 2009 Bacterial toxins: An overview on bacterial proteases and their action as virulence factors. Mini Rev Med Chem 9(7):820-828

207. **Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenthner D, Bovee D, Olson MV, Manoil C** 2003 Comprehensive transposon mutant library of pseudomonas aeruginosa. Proc Natl Acad Sci U S A 100(24):14339-14344

208. **Lewis JG, Lewis MG, Elder PA** 2003 An enzyme-linked immunosorbent assay for corticosteroid-binding globulin using monoclonal and polyclonal antibodies: Decline in CBG following synthetic ACTH. Clin Chim Acta 328(1-2):121-128

209. **Lewis JG, Elder PA** 2011 Corticosteroid-binding globulin reactive centre loop antibodies recognise only the intact natured protein: Elastase cleaved and uncleaved CBG may coexist in circulation. J Steroid Biochem Mol Biol 127(3-5):289-294

210. **Dai Y, Whittal RM, Li L** 1999 Two-layer sample preparation: A method for MALDI-MS analysis of complex peptide and protein mixtures. Anal Chem 71(5):1087-1091

211. **Keller BO, Li L** 2000 Discerning matrix-cluster peaks in matrix-assisted laser desorption/ionization time-of-flight mass spectra of dilute peptide mixtures. J Am Soc Mass Spectrom 11(1):88-93

212. **Keller BO, Sui J, Young AB, Whittal RM** 2008 Interferences and contaminants encountered in modern mass spectrometry. Anal Chim Acta 627(1):71-81

213. **Coolens JL, Van Baelen H, Heyns W** 1987 Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroid-binding globulin. J Steroid Biochem 26(2):197-202

214. **Boksa P** 1997 Early developmental profiles of plasma corticosterone are altered by birth condition in the rat: A comparison of vaginal birth, cesarean section, and cesarean section with added anoxia. Pediatr Res 41(1):34-43

215. **Perrin FM, Forest MG** 1975 Time course of the effect of adrenalectomy on transcortin binding characteristics: Appraisal of different methods of calculation. Endocrinology 96(4):869-878

216. **Brunner E, Baima J, Vieira TC, Vieira JG, Abucham J** 2003 Hereditary corticosteroid-binding globulin deficiency due to a missense mutation (Asp367Asn, CBG lyon) in a brazilian kindred. Clin Endocrinol (Oxf) 58(6):756-762

217. **Lewis JG, Elder PA** 2013 Intact or "active" corticosteroid-binding globulin (CBG) and total CBG in plasma: Determination by parallel ELISAs using monoclonal antibodies. Clin Chim Acta 416:26-30

218. **Stavreva DA, Wiench M, John S, Conway-Campbell BL, McKenna MA, Pooley JR, Johnson TA, Voss TC, Lightman SL, Hager GL** 2009 Ultradian hormone stimulation induces glucocorticoid receptor-mediated pulses of gene transcription. Nat Cell Biol 11(9):1093-1102

219. **Cottrell EC, Seckl JR** 2009 Prenatal stress, glucocorticoids and the programming of adult disease. Front Behav Neurosci 3:19

220. **Fowden AL, Forhead AJ** 2009 Endocrine regulation of feto-placental growth. Horm Res 72(5):257-265

221. **Inglis GC, Ingram MC, Holloway CD, Swan L, Birnie D, Hillis WS, Davies E, Fraser R, Connell JM** 1999 Familial pattern of corticosteroids and their metabolism in adult human subjects--the scottish adult twin study. J Clin Endocrinol Metab 84(11):4132-4137

222. **Bartels M, de Geus EJ, Kirschbaum C, Sluyter F, Boomsma DI** 2003 Heritability of daytime cortisol levels in children. Behav Genet 33(4):421-433

223. **Mormede P, Foury A, Barat P, Corcuff JB, Terenina E, Marissal-Arvy N, Moisan MP** 2011 Molecular genetics of hypothalamic-pituitary-adrenal axis activity and function. Ann N Y Acad Sci 1220:127-136

224. **Walker BR** 2007 Glucocorticoids and cardiovascular disease. Eur J Endocrinol 157(5):545-559

225. **Phillips DI, Barker DJ, Fall CH, Seckl JR, Whorwood CB, Wood PJ, Walker BR** 1998 Elevated plasma cortisol concentrations: A link between low birth weight and the insulin resistance syndrome? J Clin Endocrinol Metab 83(3):757-760

226. **Reynolds RM, Walker BR, Syddall HE, Andrew R, Wood PJ, Whorwood CB, Phillips DI** 2001 Altered control of cortisol secretion in adult men with low birth weight and cardiovascular risk factors. J Clin Endocrinol Metab 86(1):245-250

227. **Filipovsky J, Ducimetiere P, Eschwege E, Richard JL, Rosselin G, Claude JR** 1996 The relationship of blood pressure with glucose, insulin, heart rate, free fatty acids and plasma cortisol levels according to degree of obesity in middle-aged men. J Hypertens 14(2):229-235

228. **Fraser R, Ingram MC, Anderson NH, Morrison C, Davies E, Connell JM** 1999 Cortisol effects on body mass, blood pressure, and cholesterol in the general population. Hypertension 33(6):1364-1368

229. **Ball TM** 2006 Cortisol circadian rhythms and stress responses in infants at risk of allergic disease. Neuroimmunomodulation 13(5-6):294-300

230. **Lupien SJ, de Leon M, de Santi S, Convit A, Tarshish C, Nair NP, Thakur M, McEwen BS, Hauger RL, Meaney MJ** 1998 Cortisol levels during human aging predict hippocampal atrophy and memory deficits. Nat Neurosci 1(1):69-73

231. **Holsboer F** 2001 Stress, hypercortisolism and corticosteroid receptors in depression: Implications for therapy. J Affect Disord 62(1-2):77-91

232. **Seckl JR, Meaney MJ** 2006 Glucocorticoid "programming" and PTSD risk. Ann N Y Acad Sci 1071:351-378

233. **Mast AE, Enghild JJ, Pizzo SV, Salvesen G** 1991 Analysis of the plasma elimination kinetics and conformational stabilities of native, proteinase-complexed, and reactive site cleaved serpins: Comparison of alpha 1-proteinase inhibitor, alpha 1-antichymotrypsin, antithrombin III, alpha 2-antiplasmin, angiotensinogen, and ovalbumin. Biochemistry 30(6):1723-1730

234. **Arnold K, Bordoli L, Kopp J, Schwede T** 2006 The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. Bioinformatics 22(2):195-201

235. **Hill RE, Shaw PH, Boyd PA, Baumann H, Hastie ND** 1984 Plasma protease inhibitors in mouse and man: Divergence within the reactive centre regions. Nature 311(5982):175-177

236. **Rapala-Kozik M, Potempa J, Nelson D, Kozik A, Travis J** 1999 Comparative cleavage sites within the reactive-site loop of native and oxidized alpha1-proteinase inhibitor by selected bacterial proteinases. Biological chemistry 380(10):1211-6

237. **Engel LS, Hill JM, Caballero AR, Green LC, O'Callaghan RJ** 1998 Protease IV, a unique extracellular protease and virulence factor from pseudomonas aeruginosa. J Biol Chem 273(27):16792-16797

238. **Bleves S, Viarre V, Salacha R, Michel GP, Filloux A, Voulhoux R** 2010 Protein secretion systems in pseudomonas aeruginosa: A wealth of pathogenic weapons. Int J Med Microbiol 300(8):534-543

239. **Morihara K** 1995 Pseudolysin and other pathogen endopeptidases of thermolysin family. Methods Enzymol 248:242-253

240. **Cathcart GR, Quinn D, Greer B, Harriott P, Lynas JF, Gilmore BF, Walker B** 2011 Novel inhibitors of the pseudomonas aeruginosa virulence factor LasB: A potential therapeutic approach for the attenuation of virulence mechanisms in pseudomonal infection. Antimicrobial agents and chemotherapy 55(6):2670-8

241. **Azghani AO, Bedinghaus T, Klein R** 2000 Detection of elastase from pseudomonas aeruginosa in sputum and its potential role in epithelial cell permeability. Lung 178(3):181-9

242. **Leduc D, Beaufort N, de Bentzmann S, Rousselle JC, Namane A, Chignard M, Pidard D** 2007 The pseudomonas aeruginosa LasB metalloproteinase regulates the human urokinase-type plasminogen activator receptor through domain-specific endoproteolysis. Infect Immun 75(8):3848-3858

243. **Fullagar JL, Garner AL, Struss AK, Day JA, Martin DP, Yu J, Cai X, Janda KD, Cohen SM** 2013 Antagonism of a zinc metalloprotease using a unique metal-chelating scaffold: Tropolones as inhibitors of P. aeruginosa elastase. Chem Commun (Camb) 49(31):3197-9

244. **Miyoshi S, Shinoda S** 2000 Microbial metalloproteases and pathogenesis. Microbes Infect 2(1):91-98

245. **Armstrong PB, Quigley JP** 1999 Alpha2-macroglobulin: An evolutionarily conserved arm of the innate immune system. Dev Comp Immunol 23(4-5):375-390

246. **Sharpe JR, Booth S, Jubin K, Jordan NR, Lawrence-Watt DJ, Dheansa BS** 2012 Progression of wound pH during the course of healing in burns. Journal of burn care & research : official publication of the American Burn Association

247. **McShane D, Davies JC, Davies MG, Bush A, Geddes DM, Alton EW** 2003 Airway surface pH in subjects with cystic fibrosis. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology 21(1):37-42

248. **Bjarnsholt T, Kirketerp-Moller K, Jensen PO, Madsen KG, Phipps R, Krogfelt K, Hoiby N, Givskov M** 2008 Why chronic wounds will not heal: A novel hypothesis. Wound Repair Regen 16(1):2-10

249. **Nichols DP, Caceres S, Caverly L, Fratelli C, Kim SH, Malcolm K, Poch KR, Saavedra M, Solomon G, Taylor-Cousar J, Moskowitz S, Nick JA** 2013 Effects of azithromycin in pseudomonas aeruginosa burn wound infection. J Surg Res 183(2):767-776

250. **van Delden C** 2007 Pseudomonas aeruginosa bloodstream infections: How should we treat them? Int J Antimicrob Agents 30 Suppl 1:S71-5

251. **Roux D, Ricard JD** 2011 Novel therapies for pseudomonas aeruginosa pneumonia. Infect Disord Drug Targets 11(4):389-394

252. **Ciofu O, Hansen CR, Hoiby N** 2013 Respiratory bacterial infections in cystic fibrosis. Curr Opin Pulm Med 19(3):251-258

253. **Gellatly SL, Hancock RE** 2013 Pseudomonas aeruginosa: New insights into pathogenesis and host defenses. Pathog Dis 67(3):159-173

254. **Morihara K, Tsuzuki H, Harada M, Iwata T** 1984 Purification of human plasma alpha 1-proteinase inhibitor and its inactivation by pseudomonas aeruginosa elastase. J Biochem 95(3):795-804

255. **Taggart C, Cervantes-Laurean D, Kim G, McElvaney NG, Wehr N, Moss J, Levine RL** 2000 Oxidation of either methionine 351 or methionine 358 in alpha 1-antitrypsin causes loss of anti-neutrophil elastase activity. J Biol Chem 275(35):27258-27265

256. **Annane D, Cavaillon JM** 2003 Corticosteroids in sepsis: From bench to bedside? Shock 20(3):197-207

257. **Nenke MA, Lewis JG, Rankin W, Torpy DJ** 2016 Evidence of reduced CBG cleavage in abdominal obesity: A potential factor in development of the metabolic syndrome. Horm Metab Res 48(8):523-528

258. **Nenke MA, Holmes M, Rankin W, Lewis JG, Torpy DJ** 2016 Corticosteroid-binding globulin cleavage is paradoxically reduced in alpha-1 antitrypsin deficiency: Implications for cortisol homeostasis. Clin Chim Acta 452:27-31

259. **Nenke MA, Lewis JG, Rankin W, McWilliams L, Metcalf RG, Proudman SM, Torpy DJ** 2016 Reduced corticosteroid-binding globulin cleavage in active rheumatoid arthritis. Clin Endocrinol (Oxf) 85(3):369-377

260. **Nenke MA, Rankin W, Chapman MJ, Stevens NE, Diener KR, Hayball JD, Lewis JG, Torpy DJ** 2014 Depletion of high-affinity corticosteroid-binding globulin corresponds to illness severity in sepsis and septic shock; clinical implications. Clin Endocrinol (Oxf)

261. **Rombouts Y, Jonasdottir HS, Hipgrave Ederveen AL, Reiding KR, Jansen BC, Freysdottir J, Hardardottir I, Ioan-Facsinay A, Giera M, Wuhrer M** 2016 Acute phase inflammation is characterized by rapid changes in plasma/peritoneal fluid N-glycosylation in mice. Glycoconj J 33(3):457-470

262. **Gornik O, Lauc G** 2008 Glycosylation of serum proteins in inflammatory diseases. Dis Markers 25(4-5):267-278

263. **Sottrup-Jensen L, Sand O, Kristensen L, Fey GH** 1989 The alpha-macroglobulin bait region. sequence diversity and localization of cleavage sites for proteinases in five mammalian alpha-macroglobulins. J Biol Chem 264(27):15781-15789

264. **Armstrong PB** 2006 Proteases and protease inhibitors: A balance of activities in host-pathogen interaction. Immunobiology 211(4):263-281

265. **Borth W** 1992 Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics. FASEB J 6(15):3345-3353

266. **Schaefer U, Brucker B, Elbers A, Neugebauer E** 2004 The capacity of alpha2-macroglobulin to inhibit an exogenous protease is significantly increased in critically ill and septic patients. Shock 22(1):16-22

267. **Khan MM, Shibuya Y, Nakagaki T, Kambara T, Yamamoto T** 1994 Alpha-2-macroglobulin as the major defence in acute pseudomonal septic shock in the guinea-pig model. Int J Exp Pathol 75(4):285-293

268. **Fuller DD, Baker TL, Behan M, Mitchell GS** 2001 Expression of hypoglossal long-term facilitation differs between substrains of sprague-dawley rat. Physiol Genomics 4(3):175-181

269. **Yoon YW, Lee DH, Lee BH, Chung K, Chung JM** 1999 Different strains and substrains of rats show different levels of neuropathic pain behaviors. Exp Brain Res 129(2):167-171

270. **Billiau A, Matthys P** 2001 Modes of action of freund's adjuvants in experimental models of autoimmune diseases. J Leukoc Biol 70(6):849-860

271. **Ratkay LG** 1994 Investigation of an adjuvant-enhanced model of murine arthritis and its therapeutic application. The University of British Columbia, Department of Oral Biology

272. **Sarlis NJ, Chowdrey HS, Stephanou A, Lightman SL** 1992 Chronic activation of the hypothalamo-pituitary-adrenal axis and loss of circadian rhythm during adjuvant-induced arthritis in the rat. Endocrinology 130(4):1775-1779

273. **Harbuz MS, Conde GL, Marti O, Lightman SL, Jessop DS** 1997 The hypothalamic-pituitary-adrenal axis in autoimmunity. Ann N Y Acad Sci 823:214-224

274. **Harbuz MS, Rooney C, Jones M, Ingram CD** 1999 Hypothalamo-pituitary-adrenal axis responses to lipopolysaccharide in male and female rats with adjuvant-induced arthritis. Brain Behav Immun 13(4):335-347

275. **Webster EL, Barrientos RM, Contoreggi C, Isaac MG, Ligier S, Gabry KE, Chrousos GP, McCarthy EF, Rice KC, Gold PW, Sternberg EM** 2002 Corticotropin releasing hormone (CRH) antagonist attenuates adjuvant induced arthritis: Role of CRH in peripheral inflammation. J Rheumatol 29(6):1252-1261

276. **Poli V** 1998 The role of C/EBP isoforms in the control of inflammatory and native immunity functions. J Biol Chem 273(45):29279-29282

277. **Perera F, Herbstman J** 2011 Prenatal environmental exposures, epigenetics, and disease. Reprod Toxicol 31(3):363-373

278. **Champagne FA, Curley JP** 2009 Epigenetic mechanisms mediating the long-term effects of maternal care on development. Neurosci Biobehav Rev 33(4):593-600

279. **Simard M, Hill LA, Lewis JG, Hammond GL** 2015 Naturally occurring mutations of human corticosteroid-binding globulin. J Clin Endocrinol Metab 100(1):E129-39

280. **Fonseca JE, Santos MJ, Canhao H, Choy E** 2009 Interleukin-6 as a key player in systemic inflammation and joint destruction. Autoimmun Rev 8(7):538-542

281. **Choy EH, Panayi GS** 2001 Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med 344(12):907-916

282. **Ramadori G, Armbrust T** 2001 Cytokines in the liver. Eur J Gastroenterol Hepatol 13(7):777-784

283. **Sivukhina E, Helbling JC, Minni AM, Schafer HH, Pallet V, Jirikowski GF, Moisan MP** 2013 Intrinsic expression of transcortin in neural cells of the mouse brain: A histochemical and molecular study. J Exp Biol 216(Pt 2):245-252