CORTICOSTEROID BINDING GLOBULIN:

HIGH RESOLUTION SEPARATION OF PLASMA GLYCAN ISOFORMS FROM PREGNANT AND COMBINED ORAL CONTRACEPTIVE PILL TAKING WOMEN

This thesis is submitted for award of the degree

of Master of Medical Science by

Miss Elizabeth Mitchell

February 2007

CONTENTS

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

DECLARATION

I, Elizabeth Mitchell declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that to my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis.

In addition, I give consent to this copy of my thesis, when deposited in the University Library, being available for photocopying and loan if accepted for the award of Masters of Medical Science.

13th day of February the year 2007

Miss Elizabeth Mitchell

ACKNOWLEDGMENTS

I would like to extend my kind thanks and gratitude to both my supervisors, Associate Professor David J Torpy and Dr Chris J Bagley for all their assistance and encouragement throughout the experimental and writing process. Furthermore, I would like to thank Ian Milne for his assistance with the mass spectrometric analysis of ovalbumin and CBG, and all the laboratory staff who assisted with equipment maintenance and cleaning.

ABSTRACT

Corticosteroid binding globulin (CBG) is the main carrier of cortisol in the circulation. Generally, the glycoprotein bears a mixture of bi- and tri-antennary N-linked glycosylation. During pregnancy, the concentration of CBG increases and the glycosylation converts to more tri-antennary with appearance of a sub-fraction, known as pregnancy-specific CBG, lacking any bi-antennary glycosylation. Previous methods including one dimension SDS-PAGE and crossed immunoelectrophoresis, have not been able to elucidate the full repertoire of CBG glycoforms during pregnancy due to low resolution. Therefore, a high-resolution analytical method, two-dimensional electrophoresis (2D-E), capable of broader examination of CBG glycosylation changes during pregnancy was employed.

It is postulated that glycoforms specific to pregnancy appearing in the first trimester may vary with gestational age. Furthermore, it is postulated that CBG glycosylation may be altered by exogenous oestrogen from the oral contraceptive pill (OCP).

Plasma samples were analysed from five pregnant women at various gestational ages, ten samples from women at 3-16 weeks and forty consecutive samples from ten women at 4 time points between 16-36 weeks. In addition, ten plasma samples were analysed from women taking the OCP, as well as five control samples, from three non-pregnant non-OCP women and two healthy men.

A method developed combining 2D-E and Western blotting detected 9-15 CBG glycoforms, an improvement on the afore mentioned lower resolution, methods. During pregnancy, CBG glycoforms were found to become generally more acidic, presumably as a result of incorporation of increased sialic acid residues.

Experiments using Concanavalin A, which binds bi-antennary but not tri-antennary glycosylated proteins, demonstrated the appearance of a portion, 10-15%, of CBG glycoforms exhibiting solely tri-antennary glycosylation during mid to late pregnancy. This form of CBG, previously termed pregnancy-specific CBG, had a heterogeneous profile on 2D-E that substantially overlapped the CBG profiles from non-con A treated samples.

The OCP 2D-E sample profiles demonstrated overlap with an increased CBG glycoform acidity compared with controls although there was no evidence of the solely tri-antennary-glycosylated glycoforms. These results indicate that there may be additional factors to oestrogen that may be involved in the modulation of CBG glycosylation during pregnancy.

CHAPTER 1:

BACKGROUND

AND

INVESTIGATIVE RATIONALE

1.1 BACKGROUND

1.1.1 INTRODUCTION

Glycosylation is a form of protein posttranslational modification (PTM) important for protein structure and physiological function. The study of glycoproteins has advanced greatly in recent decades with the introduction and improvement of proteomic techniques including one and two-dimensional electrophoresis (1D-E and 2D-E) and immunoblotting techniques including Western blotting. These methods have been used to demonstrate the profiles of various glycoproteins including corticosteroid binding globulin (CBG) under normal conditions and during altered physiological states. CBG is an N-linked glycosylated plasma protein intimately associated with the hypothalamic pituitary adrenal (HPA) axis, and has a high affinity binding site for cortisol. In addition, although lacking protease inhibitory activity, CBG is a member of the serine protease inhibitor (SERPIN) superfamily of proteins.

Alteration of CBG during pregnancy has led to the suggestion of a specific physiological function for CBG during pregnancy. However, there have been no reports of high resolution separation of plasma CBG glycoforms using 2D-E in pregnancy and little is known about the glycosylation/siallylation changes of CBG with the OCP.

1.1.2 GLYCOBIOLOGY: PROTEIN GLYCOSYLATION

The most common and diverse form of covalent PTM is glycosylation, and there are two forms, N-linked and *O-linked. Biological and chemical glycosylation diversity, results from the type of amino acids modified and the structures attached at specific "glycosylation sites" on the newly synthesised protein (Allen & Kisailus, 1992; Kobata, 1992). The varied combination of monosaccharides, which then form oligosaccharides and polysaccharides (glycans), differ not only in sequence and chain length, but anomery (α or β) position of linkages, while branching points gives rise to chemical diversity. Biological diversity arises from the fact that proteins are primary gene products compared with glycans, which are secondary gene products (Lis & Sharon, 1993).

N-glycosylation occurs at a tripeptide and the recognition signal site, Asn-X-Ser/Thr, (where X is an amino acid residue except Pro or Asp) on nascent proteins catalysed by oligosaccharyltransferase in the lumen of the rough endoplasmic reticulum (rER) (Kornfeld & Kornfeld, 1985). An N-glycosidic bond is formed with the protein when the enzyme transfers $Glc_3Man_9GlcNAc_2$ to the Asn amide side chain (Dennis *et al.*, 1999).

*Only N-linked glycosylation will be discussed in this thesis as corticosteroid binding globulin (CBG) is N-linked glycosylated

The resultant glycans are then subjected to extensive modification into glycoproteins which mature and move through the ER via the Golgi complex to their final intra or extracellular destination. In addition, the glycans are heterogeneous due to variable branching of the attached terminal oligosaccharides leading to "high-mannose", "complex" and "hybrid" types, some of which may have other oligosaccharides or fucose added (Figure 1.1) (Charlwood *et al.*, 2001). In some proteins glycans play a pivotal role in folding, oligomerization, quality control, sorting, stability, function and transport (Helenius & Aebi, 2001).

1.1.2a Corticosteroid binding globulin (CBG)

CBG is a 52-56kDa glycoprotein monomer with a single high affinity cortisol binding site. In the circulation approximately 95% of plasma cortisol is bound to CBG, which maintains the solubility of this hydrophobic molecule. The carrier protein role of CBG may conserve glucocorticoid supplies by limiting the amount of unbound steroid in the circulation as well as protecting cortisol from enzymatic degradation (Ballard, 1979; Breuner & Orchinik, 2002; Hammond, 1990). The extent of cortisol plasma binding and the metabolic inactivation rate determines the plasma half-life of cortisol (90 minutes) (Aron *et al.*, 2001). Although there is very minimal sex difference in plasma CBG levels under normal physiological conditions (Brien, 1981), it has been reported that in an elderly population, CBG levels are slightly higher in women compared with men (Stolk *et al.*, 1996).

CBG is cleaved near the C-terminus by neutrophil elastases, reducing its size by 5kDa, resulting in diminished cortisol binding ability by inducing a conformational change, a mechanism proposed to facilitate delivery and release of cortisol at sites of inflammation (Ballard, 1979; Hammond, 1990). Similar cortisol shuttle and delivery mechanisms may operate in other tissues, acting via CBG/cortisol complex which binds to cell membranes and CBG is cleaved, allowing cortisol interaction with tissues on a selective basis (Rosner, 1990). At cell surfaces, cortisol availability to its cytoplasmic receptor may be achieved through putative CBG-cell surface receptor binding (Hryb *et al.*, 1986), as well as cleavage of the C-terminus by cellular elastases during inflammation (Pemberton *et al*, 1988) refer to Figure 1.2. This may provide tissue-specific cortisol availability, complementing the neuroendocrine control of cortisol levels and intracellular cortisol inactivation by the cortisol-cortisone shuttle (Hryb *et al*, 1986).

1.1.2b CBG glycosylation

The six N-linked glycosylation consensus sites for CBG have differing occupancy. Sites I, IV, V and VI are largely limited to bi-antennary oligosaccharides, while those attached to sites II and III are further processed to form multi-branching (tri-antennary) glycans (Avvakumov & Hammond, 1994). The CBG bi-antennary to tri-antennary glycosylation ratio is 3:2 in non-pregnant controls (Strel'chyonok *et al.*, 1982). The resulting microheterogeneity of the whole CBG molecule creates discrete subsets of glycoforms. These glycoforms posses different physical properties - are present in the circulation with a mixture of bi- and tri-antennary glycosylation and have variable MWs – as well as different biochemical properties, both of which result in potential functional diversity (Avvakumov & Hammond, 1994).

CBG glycoforms may provide specificity in cortisol transport – each glycan isoform preferentially binding to certain tissues (Avvakumov & Strel'chyonok, 1988; Misao *et al.*, 1999). Glycosylation at site IV (Asn^{238}) (Figure 1.3) is essential for the acquisition of cortisol binding affinity, perhaps by constituting part of the binding site or by promoting correct protein folding (Avvakumov *et al.*, 1993; Avvakumov & Hammond, 1994). The role of the other glycosylation sites and attachment of carbohydrate chains is poorly understood. Site directed mutagenesis experiments have shown that the other sites do not participate in cortisol binding (Avvakumov, 1991; Avvakumov & Hammond, 1994; Strel'chyonok *et al*, 1982)

1.1.3 THE HYPOTHALAMIC-PITUITARY ADRENAL (HPA) AXIS CORTISOL AND CBG

1.1.3a The HPA axis and Cortisol

Cortisol, the major regulated functional product of the HPA axis, maintains cardiac contractility and peripheral vascular tone in synergy with catecholamines and other vasopressor hormones, as well as modulating immune activity. During stress, which is defined as any threat to homeostasis, the hormone products of the HPA axis and sympathetic nervous system, cortisol and catecholamines, may increase up to tenfold (Torpy & Chrousos, 1997).

There are three main factors regulating the secretion of cortisol, the circadian rhythm, feedback inhibition, and stress. Hypothalamic adrenocorticotropic hormone (ACTH) secretogogues, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), are central modulators of the HPA axis (refer back to Figure 1.4[*]). Cortisol is subject to negative feedback regulation as it acts on the anterior pituitary to inhibit ACTH release and at the hypothalamic paraventricular nuclei and hippocampus to inhibit the release of the hypothalamic ACTH secretogogues (Braunstein, 1980).

[*] Reference for Figure 1.4 (Kirk, Jr. *et al.*, 2000)

1.1.3b The HPA axis, pregnancy and CBG

The placenta does not directly produce oestrogen and progesterone as it lacks the relevant steroidogenic enzymes. Placental oestrogen and progesterone production occurs in the syncytiotrophoblast and is dependent upon precursors of both maternal and foetal origin, leading to the concept of the *materno-foetal-placental unit* (Braunstein, 1980; Pepe & Albrecht, 1995). Furthermore, steroidogenesis increase throughout pregnancy in line with increased trophoblast mass (Braunstein, 1980).

Pregnancy and oral oestrogen treatment produce a hyperoestrogenemic state in the maternal circulation with concurrent doubling of maternal plasma CBG due to increased hepatic production (Braunstein, 1980). In combination, with increased maternal CBG, increased hormone levels lead to a decreased metabolic clearance rate (MCR) of cortisol as a large proportion is bound to the circulating CBG. Despite elevated CBG, and increased cortisol binding to this increased CBG, the levels of serum "free" cortisol remain high due to increased cortisol production rates, (O'Connell & Welsh, 1969).

During pregnancy circulating cortisol follows a diurnal rhythm in the same way as ACTH. The levels of both hormones increase in response to adrenal cortex hyper-responsiveness to ACTH stimulation, which in turn drives enhanced cortisol production (Carr *et al*, 1981; Magiakou *et al.*, 1996). Furthermore, the active or free cortisol MCR is lower during pregnancy and with oestrogen treatment compared with non-pregnant non-oral contraceptive pill (non-OCP) taking control women as a result of increased CBG with associated increased cortisol binding. Total plasma cortisol increases to reach a plateau by week 26, then increases again at the onset of labour (Carr *et al.*, 1981; Doe *et al.*, 1964).

There is insufficient information regarding the primary role of CBG during pregnancy including the environmental ligands and binding properties. However, it has been suggested that CBG provides protection to circulating cortisol from catabolism; as well as providing biochemical buffering to cortisol during late pregnancy to prevent premature labour (Challis *et al*, 1993).

CBG produced during pregnancy or pregnancy related CBG, provides putative cortisol transport to maternal and foetal target cells (i.e. uterus, lung, brain, kidneys as well as others organs and tissues), possibly explaining a lower cortisol MCR during pregnancy (Benassayag *et al.*, 2001). In addition, these interactions result in the activation of membrane signal transduction pathways and the production of a second messenger (i.e. $_{c}$AMP), internalisation of the CBG/cortisol complex, or cleavage of CBG by proteases with the local release of cortisol, (Demey-Ponsart *et al.*, 1982; Hammond *et al.*, 1987; Hammond *et al.*, 1990; Scrocchi *et al.*, 1993) are all events postulated to be mediated by the interaction of the CBG/cortisol complex with plasma membrane receptors, especially on human decidua (Avvakumov, 1995; Rosner, 1990) and syncytiotrophoblast (Avvakumov & Strel'chyonok, 1988). CBG therefore appears to play a subtle role in the modulation of steroid signals, especially at the fetomaternal interface (Benassayag *et al.*, 2001).

1.1.3c CBG genetics and biosynthesis

The 19kb CBG gene has been cloned and localised to chromosome 14 in region q31-q32.1, and is part of a cluster of five genes which encode for the serine protease inhibitors or SERPIN protein superfamily (Hammond *et al*, 1987; Seralini *et al.*, 1990; Underhill & Hammond, 1989). CBG is most closely related to α_1-protease inhibitor (A1-PI) and α_1-anti-chymotrypsin (ACT) (Rabin *et al.*, 1986; Schroeder *et al.*, 1985).

Three rare mutations of the CBG gene (Leuven, Lyon and Null) have been observed, two of which reduce CBG/cortisol binding affinity and the third, CBG Null, leads to an absence of plasma CBG (Hammond *et al*, 1990; Torpy *et al.*, 2001; Van Baelen *et al.*, 1982). In some of the cases analysed, CBG Null affected individuals were observed after presentation with fatigue and hypotension, although chronic fatigue syndrome does not appear to be associated with these mutations (Lewis *et al.*, 2005).

The liver is the principal site of CBG biosynthesis under normal physiological conditions with relatively abundant amounts of CBG mRNA have been found in this organ (Brien, 1981). Furthermore, CBG mRNA has been found in the plasma membranes of lung and kidney tissue (Hammond *et al*, 1987), possibly residual from foetal use of CBG by these organs (Benassayag *et al.*, 2001); as well as a variety of reproductive organs (including the testes, prostate (Hryb *et al*, 1986), and uterus (Misao *et al.*, 1995)); especially tissues which develop or increase in mass during pregnancy, including amniotic fluid (Challis & Bennett, 1977). In addition, the protein was found in the pregnancy related tissues, uterine endometrium and placental syncytiotrophoblast (Avvakumov & Strel'chyonok, 1988).

1.1.4 INTRINSIC AND EXTRINSIC PHYSIOLOGICAL FACTORS INFLUENCING CBG LEVELS AND/OR GLYCOSYLATION

1.1.4a Pregnancy induced CBG changes

The levels of many plasma proteins increase during pregnancy with the appearance of some new pregnancy-associated foeto-placental proteins (Westphal, 1971). Indeed, not only do CBG levels increase during pregnancy, there is a significant alteration in the glycosylation profile of this protein (Strel'chyonok *et al.*, 1984). Several proteins of maternal origin exhibit decreased Concanavalin A (Con A) binding or become more acidic during pregnancy. For instance, serum α1 glycoprotein (AGP) exhibits increased tri-antennary N-linked glycosylation evident by its lack of binding to Concanavalin A (Con A) during pregnancy (Raynes, 1982). Transferins (de Jong & et al, 1992), CBG and related thyroid-hormone binding globulin (TBG) (Strel'chyonok *et al.*, 1984) exhibit similar changes. Investigation of retroplacental serum using crossed-immunoelecrophoresis (using CBG-lectin, Con A as the lectin) demonstrated the existence of a variant form of CBG specific to pregnancy (Benassayag *et al.*, 2001).

Con A, a lectin tetrameric metalloprotein isolated from *Canavalia ensiformis* (jack bean), only binds bi-antennary oligosaccharides and not tri-antennary glycosylated glycoproteins (Strel'chyonok *et al*, 1984). Hence coupled to sepharose, Con A is an invaluable means of investigating the presence of the pregnancy associated CBG variants in plasma.

Experiments successfully demonstrated a CBG variant "specific" to pregnancy which was Con A non-binding CBG (CAN-CBG), and the variant demonstrated tri-antennary glycosylation in five of the six N-linked glycosylation sites (Strel'chyonok *et al*, 1984). In addition, the fraction of CBG which bound to the Con A and later eluted; the Con A binding or CAB-CBG variant possessed the normal 3:2, bi-antennary to tri-antennary glycosylation ratio. The CAN-CBG variant was detected in the serum of some pregnant women from the beginning of the first lunar month with most women producing it by the second trimester and all by the third trimester of pregnancy. However, it was not detected on screening of blood from normal non-pregnant donors (Avvakumov & Strel'chyonok, 1987) and the relative proportions of CAN versus CAB-CBG remain unknown (Benassayag *et al*, 2001). There may be other CAB-CBG type glycoforms, of mixed bi- and tri-antennary glycosylation present in pregnancy plasma that have as yet, not been detected.

While the physiological function of altered CBG glycosylation during pregnancy has not been elucidated to date, the physiological functions of other glycosylated human proteins have been documented. For instance, glycosylation of human protein C, an important regulator of the blood coagulation system is essential for efficient secretion and activation of the zymogen (Lis & Sharon, 1993).

1.1.4b Physiology and possible function(s) of CAN-CBG

The basic physico- and immuno-chemical properties of CAN-CBG were found to be similar to those of normal CBG. The amino acid composition of the CAB-CBG variant is identical to that of the normal and CAN-CBG variants, as the N- and C-terminal sequence was found to be common to both, although their carbohydrate moieties are different (Avvakumov & Strel'chyonok, 1988). The carbohydrate moieties are not involved in CBG steroid (cortisol) binding (Avvakumov & Hammond, 1994; Strel'chyonok *et al*, 1984).

The biosynthesis of many pregnancy associated proteins is due to activation of specific genes, whereas, CAN-CBG results from post-translational modification of the CBG polypeptide chain (Strel'chyonok & Avvakumov, 1990; Strel'chyonok & Avvakumov, 1991). A physiological characteristic unique to CAN-CBG, is that the level of this subset of CBG in the maternal blood at term has previously been observed to be similar to that in retroplacental serum and accounted for 7-14% of total plasma CBG levels (Strel'chyonok & Avvakumov, 1991). At term 20-50% of foetal cortisol is of maternal origin, despite the action of 11-β hydroxysteroid dehydrogenase type 2, converting cortisol to inactive cortisone in the placenta (Beitins *et al.*, 1973). CAN-CBG may have a primary role in placental regulation, wherein cortisol stimulates the release of placental corticotropin-releasing hormone CRH, which in turn stimulates maternal ACTH secretion. Functionally, CAN-CBG may provide a transport mechanism to facilitate materno-foetal cortisol transport (Beitins *et al*, 1973).

Early in gestation, higher proportions of foetal cortisol may be of maternal origin, as the foetal HPA axis has not developed complete function. The development of many tissues including the lungs requires a certain level of circulating foetal cortisol (de Fenci & Tulchinsky, 1975). However, high foetal cortisol levels may influence development by causing retarded growth perhaps increasing the risk of adult onset metabolic syndrome (Clark *et al.*, 1996; Economides *et al.*, 1988).

Total serum CBG levels have been found to rapidly decrease to values characteristic of normal serum, while the level of CAN-CBG remains relatively high up to day 40 after parturition, even persisting for up to 8 weeks postpartum (Potter *et al.*, 1987; Strel'chyonok & Avvakumov, 1991). These data suggest that the half-life of the CAN-CBG variant in the circulation is significantly longer than that of normal CBG (approximately 5 days) (Sandberg *et al.*, 1964), or it may be that factors influencing its biosynthesis were still existing in the mother after delivery (Strel'chyonok & Avvakumov, 1991). Furthermore, in support of previous findings, some prolongation of the circulatory half-life of the CAN-CBG variant with more branched and more sialylated sugar chains have been reported (Ain & Refetoff, 1988), and may relate to the rate of CBG degradation postpartum not the site of synthesis. CBG was not detected in umbilical cord serum or placental extracts, indicating a maternal rather than foeto-placental origin of the CAN-CBG variant (Avvakumov & Strel'chyonok, 1987).

1.1.4c CAN-CBG binding

In the 7-12th week of gestation, the placenta is a glucocorticoid target tissue (Avvakumov & Strel'chyonok, 1988) and in human serum glucocorticoids are mainly bound to CBG (Westphal, 1983). CAN-CBG was demonstrated to have a 60-fold increased affinity for syncytiotrophoblast plasma membranes (Avvakumov & Hammond, 1994; Avvakumov & Strel'chyonok, 1988). In general, the CBG/cortisol complex appears to preferentially bind to two classes of specific CBG binding sites and CAN-CBG membrane receptors on the syncytiotrophoblast (Avvakumov & Strel'chyonok, 1988; Strel'chyonok & Avvakumov, 1991). The precise mechanism of enhanced syncytiotrophoblast membrane binding has not been determined, although it may be related to the site-specific addition of tri-antennary oligosaccharides to CBG. There is limited knowledge regarding the CBG hormone-binding site (Hryb *et al*, 1986) as the putative CBG receptor has not been cloned

1.1.5 THE ORAL CONTRACEPTIVE PILL (OCP) AND CBG

The combined oral contraceptive pill OCP is a popular form of contraception employing oestrogen and progesterone to suppress gonadotrophins hence ovulation, while allowing relative timing and quantity of menstrual loss. A variety of hormones (synthetic or intrinsic) as well as prophylactic and or therapeutic drugs has been reported to modify the concentration of plasma CBG. Synthetic oestrogens (in OCP preparations) and intrinsically produced placental oestrogen cause an increase (Heyns & Coolens, 1988; Wiegratz *et al.*, 2003), while dexamethasone or prednisolone (Schwartz *et al.*, 1983), cause decreased CBG levels. I

However, only the effect(s) of the oral contraceptive pill (OCP) on CBG glycosylation were investigated for this thesis. The oestrogenic effect of OCPs was evaluated by the measurement of serum levels of CBG, which increased dose-dependently with ethinylestradiol (EE) ingestion irrespective of the progestrogen component (Moore *et al.*, 1978). This indicates a strong influence of EE and no effect of the progestrogenic component on CBG concentrations (Wiegratz *et al*, 2003). There is very little in the literature about studies examining possible molecular changes and an increase in CBG concentration that may occur with consumption of the OCP. Therefore, the current study sought to determine if the pregnancy related CBG glycosylation changes also occur in women taking the OCP, which would suggest that the glycosylation as well as the enhanced CBG synthesis of pregnancy are both oestrogen driven.

1.2 INVESTIGATIVE RATIONALE

1.2.1 GLYCOPROTEIN CHARACTERISATION METHODS AND APPLICATIONS TO CBG ANALYSIS

1.2.1a General glycoprotein analytical methods

The definition of branching, linkages and configuration as well as identification of sugar isomers are all required for the full structural characterisation of glycans. Furthermore, glycoform analysis generally consists of the separation of intact glycoprotein glycoforms, structural characterisation of the released glycan pool, glycosylation site determination and glycoform distribution (Dell & Morris, 2001; Liu *et al.*, 2001). Mass spectrometry (MS) in conjunction with searching protein or nucleotide sequence databases has developed into the analytical method of choice for protein structure identification. Extensive, purification and preparation including several chromatographic steps is required to ensure correct name assignment and protein characterisation (Kuster *et al.*, 2001). Common MS methods include "soft ionisation" methods of fast atom bombardment (FAB), electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI) (Dell & Morris, 2001; Kuster *et al*, 2001).

Analysis of intact glycoproteins in complex or purified mixtures is possible using, one dimensional SDS-PAGE (1D-E), isoelectric focussing (IEF) (Avvakumov & Hammond, 1994; Hammond *et al*, 1990; Kato *et al.*, 1988; Mickelson *et al.*, 1982; Strel'chyonok *et al*, 1984), capillary electrophoresis (which in recent years has begun to include ES-MS), liquid chromatography, and lectin affinity electrophoresis (Bergstrom *et al.*, 2004; Liu *et al*, 2001; Lopez-Soto-Yarritu *et al.*, 2002; Zeng *et al.*, 1999).

1.2.1b CBG analysis:SDS-PAGE and IEF

CBG was first described by (Daughaday, 1958) and later purified (Le Gaillard *et al.*, 1975; Seal & Doe, 1962). SDS-PAGE analysis revealed two distinct electrophoretic molecular variants of CBG purified from fresh, stored and pooled pregnancy plasma which appear as a doublet (Mickelson *et al*, 1982).

The variants were shown in the presence and absence of SDS and mercaptoethanol, and storage or freezing did not appear to affect their presence. Furthermore, it was suggested that the variants arose from a difference in deamidation and were described as being indistinguishable by amino acid and carbohydrate analysis, immunologically identical, and bound cortisol equally at one steroid binding site per molecule (Westphal, 1983b).

The nature of CBG glycosylation was proposed as the reason for the appearance of the doublet when CBG is analysed by SDS-PAGE, that is, the bi- and tri-antennary glycosylation, resulting in variable branching, thence differences in the retardation in the polyacrylamide gel (Strel'chyonok *et al*, 1984).

The abundance of negatively charged sialic acid residues on the carbohydrates on the CBG molecule accounts for the acidic isoelectric point (pI) of some glycoforms during pregnancy (Bernutz *et al.*, 1979). The carbohydrate component was postulated to contribute to the heterogeneity in CBG electrophoretic mobility and pI (Kato *et al*, 1988). In addition, the pI was reported to be between 3.8 and 4.5 for a normal individual and upon IEF produced approximately 8 bands (Hammond, 1990).

1.2.1c Two- dimension electrophoresis (2D-E)

Two dimensional electrophoresis (2D-E) separates proteins in both a molecular weight (MW) and pI gradient, usually involving IEF and SDS-PAGE for the first and second dimension (respectively). This method is especially useful for the separation of glycosylated proteins as the glycoforms form discrete spots appearing as ladders spanning the MW and pI gradients. The separated proteins may then be analysed directly in polyacrylamide gels by staining (usually Coomassie blue) or indirectly by electroblotting and radiological or immunological probing (Packer *et al.*, 1997) with acquisition of a digital following gel or film scanning

The lectin, Con A, is a useful resource in the study of glycoproteins as they may either completely or partially interact with the substance based on their state of glycosylation, mixed bi- and tri-antennary or tri-antennary only (respectively) (Wells *et al.*, 1981). Con A is usually used in conjunction with electrophoresis and IEF to compare and contrast the fractions obtained from affinity chromatography.

Crossed immunoelectrophoresis (CIE) is a complex form of 2D-E usually involving an electrophoretic step in the first dimension (e.g. SDS-PAGE) for MW gradient separation, then electrophoresis in the second dimension for pI separation of the separated proteins into an antibody (Wells *et al*, 1981) or lectin containing gel (Nakamura *et al.*, 1960).

Pregnancy and oestrogen treatment both induce alteration in the carbohydrate moiety structure of several proteins including A1-PI, ACT and CBG, which may be detected using CIE and standard 2D-E (Benassayag *et al*, 2001; Raynes, 1982; Wells *et al*, 1981). Although CBG has been analysed extensively using CIE, standard SDS-PAGE and IEF, there have, to date been no studies documenting the analysis of this glycoprotein using a combination of SDS-PAGE/IEF (known collectively as 2 dimension electrophoresis, 2D-E), in human serum. In rodents, hamsters, however, the pregnancy profile of CBG has previously been demonstrated in serum. It was determined that the CBG like protein detected was the only one and was identical to that found in human proestrous serum. Furthermore, a higher titre of the CBG like protein was found in hamster pregnancy serum; as is the case for humans (O'Gray *et al*, 1987). Thus based on these results the use of 2D-E is expected to better resolve CBG glycoforms in human plasma, including those associated with pregnancy.

1.2.1d 2D-E analysis of plasma CBG during pregnancy and OCP use

Knowledge regarding CBG glycosylation changes during pregnancy and with use of the OCP is incomplete in several important respects. It is uncertain if the solely tri-antennary glycosylated CAN-CBG variant, said to comprise 10-20% of total CBG present during pregnancy, represents the sole CBG glycosylation alteration or whether the changes are more general, i.e. if less specific changes made to the remaining fractions of CBG during pregnancy. There is uncertainty too as to whether the global CBG glycosylation changes observed in pregnancy serum relate to gestational age or if similar changes nay be induced by oestrogen treatment alone in non-pregnant women. For instance, would it be possible to induce the same changes in CBG from women taking the OCP.

The functional effects of altered CBG glycosylation observed during pregnancy have not been studied to any great degree. Consequently, this thesis addresses the first three issues presented, while the fourth issue, regarding CBG tissue binding is not addressed as it was beyond the scope of the investigation and would have required purification of the protein from plasma along with a series of experiments to investigate the tissue binding. Furthermore, to ascertain the effects of CBG glycosylation, it would be necessary to conduct experiments to investigate the molecular structure of the separated glycoforms and this would require analysis by MS.

1.3 HYPOTHESES

The following hypotheses were proposed based on the issues raised in 1.2.3.

1. 2D-E will resolve isoforms of CBG in a more complete manner than any previously published methods.
2. A specific set of isoforms of CBG arises during pregnancy and becomes more abundant at mid to late pregnancy.
3. Pregnancy induced CBG isoforms are anticipated to occur in other hyperoestrogenic conditions such as women taking the OCP.

1.4 AIMS

To:

1. To determine the glycoform profile of CBG during and across pregnancy using 2D-E and western blotting
2. To use Concanavalin A affinity chromatography, 2D-E and western blotting to demonstrate the difference between CAN-CBG and CAB-CBG based on glycosylation in mid to late pregnancy
3. To determine and compare the glycoform profile of CBG from the plasma of women pregnant, women taking the OCP with and control using 2D-E and western blotting

CHAPTER 2

SUBJECTS

MATERIALS AND METHODS

2.1 SUBJECTS

A pilot study investigating CBG glycoforms during pregnancy using plasma samples collected from individual women at different stages of gestation was conducted. The samples were analysed using two dimensional electrophoresis (2D-E) and Western blotting, and demonstrated qualitatively that there was a difference between the CBG glycoforms occurring during pregnancy compared with controls.

In order to further investigate these findings, and determine if the initial observations represented a global or local change in CBG 2D-E glycoform profiles during pregnancy; plasma samples were collected from individual women through normal pregnancy. Prof Gus Dekker generously donated the samples from ten pregnant women enrolled in his clinical study through the Lyell McEwin Hospital, Adelaide. The women were a subset of a much larger cohort of subjects recruited for Prof Dekker's observational prospective study directed at identifying factors that may relate to pathogenesis of pre-eclampsia.

Exclusion criteria included previous pregnancy within 12 months, current major illness, or the use of medications that may influence the HPA axis, including glucocorticoids within 12 months (inhaled or topical preparations), psychotropic drug use within 12 months, or oral contraceptive use within one month of pregnancy. Ethical approval was obtained from the University of Adelaide as well as written informed consent from all pregnant women.

Gestational ages were determined from the woman's last reported menstrual cycle and ultrasound scanning. All samples used for the study were from women who had normal term pregnancies with normal vaginal or caesarean section deliveries.

The other samples collected for my study were from 3 non-pregnant women taking the oral contraceptive pill (OCP) and 5 mixed sex controls (3 non-pregnant non-OCP taking women and 2 healthy males). Blood was collected from each subject and prepared as per section 2.3.1.

2.2 MATERIALS

2.2.1 CHEMICALS AND APPARATUS

Standard stock solutions (eg TrisHCl, SDS running and transfer buffer, phosphate-buffered saline (PBS) and staining solutions) were prepared from analytical grade reagents.

2.2.1a Isoelectric Focussing (IEF):

Isoelectric focussing was performed with IEF apparatus (IPGphor) supplied by GE Healthcare formerly Amersham USA. Other reagents for IEF are listed below:

1. Immobilised pH gradient (IPG) strips:

Table 2.1.

	Description	**Supplier**
CA 1	Immobiline dry strip pH4 to 7	GE Healthcare (Upsala, Sweden)
CA 2	Ready strip pH 3 to 6	BioRad laboratories Inc (Australia)

2. IEF carrier ampholytes:

Table 2.2.

	Description	**Supplier**
IPG 1	IPG buffer pH 4 to 7	GE Healthcare (Upsala, Sweden
IPG 2	IPG buffer pH 3.5 to 5:	GE Healthcare (Upsala, Sweden
IPG 3	Biolyte ampholytes for isoelectric focussing pH 5 to 7	BioRad laboratories Inc (Australia)

3. IEF focussing/ rehydration buffers:

Table 2.3.

	IEF buffer	
Reagent	**2**	**3**
Urea	7M	9M
Thiourea	2M	-
Triton X-100	2%	2%
Bromophenol blue	Trace amounts	Trace amounts

4. IEF equilibration buffer:

Table 2.4.

Reagent	Volume
Urea	6M
TrisHCl pH 8.8	50mM
Glycerol	30%
DTT	20mM
SDS	2%
Bromophenol blue	Trace amounts

2.2.1b SDS-PAGE:

1. Gel apparatus, composition and casting

In order to ensure batch-to-batch reproducibility of SDS-PAGE gels, a gel-casting chamber (Hoefer, GE Healthcare, USA) was used for casting the second dimension SDS-PAGE gels. A Hoefer Mini-VE electrophoresis unit was used to run the SDS-PAGE gels (GE Healthcare, USA) and 1D-E, 4% acrylamide stacking gels were used.

The gels had the following acrylamide compositions:

1. 10% polyacrylamide gels using: a 29:1 acrylamide to Bis-acrylamide cross linker ratio (Pilot study)
2. 10% polyacrylamide gels using a: 37.5:1 acrylamide to Bis-acrylamide cross linker ratio (larger main study)

2. Gel buffers and sealing agarose

Table 2.5: SDS-PAGE gel buffers

	Reagent (Volume)		
	TrisHCl	**pH**	**SDS**
***Stacking gel buffer**	62.5µM	6.8	0.1%
***Resolving gel buffer**	375mM	8.8	0.1%

**10% ammonium persulphate (APS) and 1% TEMED were added to the gel casting mixtures and the gels allowed to polymerise at room temperature for 1 hour then left overnight at 4 C before use.*

Table 2.6: SDS-PAGE sample load buffer (makes 10mL)

Reagent	**Volume**
TrisHCl pH 6.8 (1M stock)	2mL
Glycerol	2.5mL
DTT	2µl
SDS-PAGE (4% stock)	2mL
Bromophenol blue (1% stock)	0.4mL

Table 2.7 SDS-PAGE and Electroblot working buffers

	Reagent (to make 1L)			
	TrisHCl	**Glycine**	**SDS**	**Methanol**
Running buffer	60g	288g	10g	-
Transfer buffer	2.4g	11.25g	1g	20%

**When running the 2nd dimension gels, 7.5 µl of Pre-stained Benchmark Protein standard markers (Invitrogen) was soaked into a small piece of filter paper approximately 0.5 X 0.75cm in area. A ladder of coloured MW markers then appeared on the gels following electrophoresis, which served a dual purpose: (a) to track the progress of the protein electroblot to NC and (b) give a rough guide of the MW of the CBG signal on the western blots.*

Table 2.8: Sealing agarose preparation for 2D-E second dimension

Reagent	**Volume**
Running buffer (see Table 5)	100mL
*Agarose (low melting point)	0.5%
Bromophenol blue	Trace amounts

** The mixture was heated in a microwave oven to melt the agarose then thoroughly agitated to mix. When required the mixture was reheated in the microwave oven to liquefy for overlaying on IPG strip and marker soaked filter paper.*

2.2.1c Western blotting:

Electroblotting

- BioRad semidry transfer cell (BioRad Australia)
- GE Healthcare semidry transfer apparatus (Upsala, Sweden)
- Nitrocellulose NC (Pall Corporation, USA)

Blot development: Antibodies and reagents

1. Primary antibody: Rabbit anti-human CBG (Medos Company, Australia, from Nordic Laboratories, Tilburg, Denmark) – 1 in 5 or 10 000 dilution
2. Secondary antibody: Goat anti-Rabbit IgG, horseradish peroxidase (HRP) coupled (Pierce, USA) – 1 in 20 000 dilution
3. Detection reagents: Enhance Chemiluminescence (ECL) Western Detection reagents (GE Healthcare, USA)
4. Film: Fuji Medical X-Ray film, (Fuji, Japan)

Table 2.9: 0.05% v/v Tween 20 Phosphate buffered saline (PBS-Tween)

Reagent	Concentration
Potassium phosphate	1.5mM
Sodium phosphate	12.3mM
Sodium chloride	138mM
Potassium chloride	2.7mM

Film digitisation and data acquisition

- GS-800 densitometric scanner (BioRad, Australia)
- PD-Quest version 7.1 (BioRad, Australia)
- Image Quant version 5.2
- Microsoft Office Excel 2000 (Microsoft, Redmond, Washington, USA) spread sheet extinction coefficient

  Extinction, http://www.imvs.sa.gov.au/immunology/research/cjbpage.htm

- GraphPad: Prism 4 (USA)

2.2.1d Affinity chromatography: Concanavalin A:

Table 2.10: Con A affinity chromatography buffers

	Reagent concentration	
Reagent	**Binding**	**Elution**
TrisHCl buffer (pH 7)	20mM	20mM
NaCl	150mM	150mM
$CaCl_2$, $MgCl_2$ and $MnCl_2$ of each	10mM	10mM
α-D- methylmannopyranoside	-	20mM

2.3 METHODS

2.3.1 PLASMA PREPARATION

Plasma collection and storage was the same for both the pilot and main studies. Whole blood samples collected from patients were centrifuged at 1800rpm for 10 minutes at 4°C in a Heraeus Megafuge 1.0 centrifuge (Hanau, Germany). The plasma was aspirated into clean (1. 5mL) plastic tubes, and as much fibrin was removed from the samples as was possible by further centrifugation at 13000rpm for 5 minutes in an Eppendorf microfuge 5415D (Hamburg, Germany). The "clean" supernatant was then transferred to autoclaved 1.5mL labelled plastic tubes in 50µl aliquots and stored at-20°C for future use. The samples were used within a few days to a week of storage, thus there was a reduced risk of any significant changes to the structure and composition of plasma proteins especially sialylation. In addition, some sample degradation is likely to occur regardless of the storage conditions chosen and may be a side-effect of the conditions of initial collection and processing

2.3.2 TWO DIMENSIONAL ELECTROPHORESIS (2D-E): SAMPLE PREPARATION, IEF AND SDS-PAGE

2.3.2a TCA precipitation: Pilot samples

Sample preparation for IEF involves several steps for removal of contaminants and concentration of proteins. In addition, the steps are generally designed for cell preparations and therefore required adjustment to accommodate the analysis of plasma. Trichloroacetic acid (TCA) precipitation was used in combination with acetone washing to remove lipids and other contaminants as well as concentrate the protein pellets from the plasma samples. Although, TCA is a widely used sample cleanup reagent and may not denature CBG, a potential problem with it, as it is possible that some CBG glycosylation might be lost during processing (BioRad, *2-D Electrophoresis for Proteomic: A Methods and Product Manual*); which may be unlikely to be significant, with regard to the experiments, as demonstrated by the results in chapter 3.

On the day of use the plasma samples were allowed to thaw at room temperature, and prepared for IEF by TCA precipitation by direct pipetting of 0.25µl of crude plasma into a clean plastic tube and addition of 100µl of 10% TCA. The tubes were then placed in a –20 C freezer for 30 minutes. The tubes were centrifuged at 13000rpm in a microfuge at 4°C for 15 minutes, and the supernatant was aspirated before the addition of 200µl of 100% ice-cold acetone followed by another round of centrifugation under the latter mentioned conditions. The washing step was repeated twice to ensure proper washing of the protein pellet.

2.3.2b TCA precipitation: direct 2D-E plasma samples

The procedure for TCA precipitation for the serial samples was similar to 2.3.2b, The samples were diluted 1:10 in MilliQ (MQ) water and a small aliquot of the diluted plasma (10µl) transferred to a clean 1.5mL plastic tube with 10µl of a 20% TCA solution followed by the same incubation and washing steps as for 2.3.2b.

2.3.2c Affinity chromatography protocol

1. The resin was washed 3 times with 1mL of binding buffer and equilibrated for 30mins or overnight.
2. A fresh 400µl volume of binding buffer was added to 75µl* of prepared resin along with 10µl pregnancy plasma and the tube placed on an end-on-end rotator for 30 minutes at room temperature.
3. The tubes were then centrifuged and the supernatant, the unbound fraction, carefully removed and kept for analysis. The con A was then three times with MQ water and centrifugation with careful pipette removal of the water each time.
4. 400µl of elution buffer, which was the binding buffer with the addition of 20mM α-D- methylmannopyranoside, was added to the washed resin and allowed to mix for 30 minutes on an end-on-end-rotator. The supernatant, the bound fraction, was removed and kept for analysis.
5. The chromatographic fractions were then prepared for 1D-E using the TCA method outlined in 2.3.2d below. In addition, to ensure complete dissolution, of the protein pellets, an SDS-PAGE load buffer containing 6M urea was used and tubes were left at room temperature overnight then centrifuged the next day at room temperature for 1 minute at 13000 rpm in a microfuge before loading onto the gels.

*Refer to Chapter 4 section 4.3.3 for more details about the capacity of the con A and concentration of CBG in the plasma used.

2.3.2d TCA Precipitation: Affinity chromatography samples

The affinity chromatography procedure involved large dilutions of the samples, thus a "carrier solution" was required in the TCA precipitation step to enable visualisation of the protein pellet. 0.025% triton X100 (a detergent found in the rehydration buffer for IEF) and with 0.025% sodium dodecylsulphate (SDS-PAGE) were added to the precipitation volume before the TCA was added to make up a final concentration which was 10% of the total volume. The tubes were then incubated in ice and the pellets subsequently washed as per step 2.3.2b above.

2.3.2e 2D-E First Dimension: Strip rehydration SDS-PAGE

Rehydration buffer, used for IEF was prepared on the day of use by addition of 0.75% carrier ampholytes (CA) 1 and 20mM DTT. The protein pellets prepared in sections 2.3.2b, c and f were rehydrated and focussed on 7 cm pH 4-7 IPG strips using IEF protocol 1 (Table 2.11). The results of these experiments led to revisions of this method and the revisions produced more consistent results and better resolution of the CBG glycoforms.

2.3.2f[*] 2D-E first Dimension: Strip rehydration SDS-PAGE revised protocol

The rehydration buffer was prepared on the day of use by the addition of 0.5% CA (a combination of CA 2 and 3; Table 2.1, section 2.2.1a), and 20mM DTT. The prepared buffer was used to rehydrate and appropriately dilute the sample pellets from sections 2.3.2b, c and f. Initially 80µl of IEF rehydration buffer 2 (Table 2.3, section 2.2.1a) was added to each pellet and incubated at room temperature for 30 minutes to ensure complete dissolution. The tubes were then centrifuged at 13000rpm in a microfuge to pellet any undissolved protein. Ovalbumin (see chapter 3 for more detail) was diluted with Milli Q water to a concentration of 1µg/µl and 6µl of this mixture was added to a clean 1.5mL plastic tube along with 20µl of the rehydrated precipitated sample solution.

The total load volume was made up to just over 125µl with the prepared rehydration buffer. The samples were pipetted into the sample strip holders starting near the basic end and the IPG 2 stirps overlaid. The acrylamide IPG stirps on the plastic backing strips was allowed to reswell by incubation for 30 minutes prior to the addition of the overlaying mineral oil and strip holder lids. The IEF protocol involved a further 12 hours of electrically assisted rehydration then the four isoelectric focussing steps outlined in protocol 2, Table 2.11, all at 20 C.

[*]Refer to Appendix 1 for complete names of abbreviations

Table 2.11: IEF protocols

	Settings	
Protocol ID	**Voltage**	**Vhr**
***1**	100	200
	500	250
	1000	500
	8000	8000
$^{\Phi}$2	500	250
	8000	6400
	8000	8 hours
	8000	8000

*Final voltage and total expected VHrs for protocol 1 was 2000 and 8500 (respectively)

$^{\Phi}$Final voltage and total expected VHrs for protocol 2 was 3-4000 and 15-17000 (respectively)

Following IEF the IPG strips were then equilibrated for 30 minutes in 3-5mL of SDS containing IEF equilibration buffer in clean plastic tubes that were allowed to rotate on an end on end mixer, in preparation for the SDS-PAGE, the second dimension.

2.3.2g 2D-E Second Dimension: SDS-PAGE

The equilibrated IPG strips were mounted on to 8 by 8cm SDS-PAGE gels and the strips sealed with molten sealing agarose. When loading an equilibrated IPG strip, the strip was brought into close contact with the top of the SDS gel by application of gentle pressure from above using a metal spatula; molten sealing agarose was then overlayed to seal the strip to the SDS gel. The gels were then run for 2-2.5 hours with limits set at 130V and 25mA per gel until the bromophenol blue dye front had run off the bottom of the gels.

2.3.3 WESTERN BLOTTING

Western blotting is a method used to visualise proteins on porous membranes following their transfer from acrylamide gels using an electrical current, electroblot. A sandwich arrangement of primary and secondary antibodies with chemiluminescence was used to illuminate the protein(s) of interest on x-ray film.

2.3.3a Electroblotting

The SDS-PAGE gels were electroblotted for 1.5 hours with the voltage set for a maximum of 25V and 0.8mA per cm^2 of 0.45µm NC membrane at room temperature.

2.3.3b Blot development

The NC membranes were stained in 0.1% Ponceau S Red (Sigma, Deisenhofen, Germany) reversible stain (in 5% acetic acid), which was ideal for this purpose as it did not interfere with the immunological analysis of the Western blotting procedure. The stain was used to detect an internal standard, ovalbumin, which was then marked using a HB pencil before gentle washing with MQ water to remove the stain.

Non-specific binding sites on the blot membrane were blocked for 1 hour at room temperature by immersion in a PBS-Tween solution containing 2% skimmed milk powder. The skimmed milk powder was useful in reducing background on the final Western blots. The membranes were then incubated with the primary antibody (rabbit anti-human CBG) diluted 1:10 000 in the same blocking buffer for 1 hour.

The membranes were washed for 10 minutes then three times again for 5 minutes each with the PBS-Tween solution (without skimmed milk powder); then incubated in the blocking buffer containing the secondary antibody (anti-rabbit-IgG HRP conjugated) diluted 1:20 000 for 1 hour. The washing step repeated and the final step involved immersion of the membranes in a small volume of ECL detection solution. The membranes were then exposed to x-ray film for 3-5 minutes and the films developed in an x-ray film developer.

The ECL detection method was chosen for its sensitivity, and so as to run a minimal amount of total protein on the IPG strips as too much would have led to CBG profile distortions, since it is not an abundant plasma protein.

2.3.4 OVALBUMIN MASS SPECTROMETRY (MS) ANALYSIS

Ovalbumin was used as an internal standard after it was found from the pilot data analysis that CBG glycoform identification would be necessary for enhanced qualitative and quantitative analysis. In order to understand the properties OVA mass spectrometry analysis was conducted by Dr CJ Bagley. In summary, the OVA was dephosphorylated using alkaline phosphatase for a total of 24 hours, desalted by reverse phase HPLC to remove the dephosphorylation buffer then analysed using a mass spectrometer. A complete report of the data was not documented in this thesis as it was beyond the scope of the study.

2.3.5 DATA ANALYSIS: MAIN STUDY DATA

2D-E gels are complex to standardise and analysis of the pilot study data was completed without an internal standard, however, the MW markers provided sufficient alignment. Western blots were scanned using a densitometric scanner and the images processed using PD Quest software. The images were then exported to Microsoft Photoshop Editor (2000) for annotation and alignment. There was no other analysis conducted on these images.

2.3.6 DATA ANALYSIS: PILOT STUDY DATA

Analysis of the pilot data highlighted the need for an internal standard for more accurate image and data analysis. The western blots for these experiments were initially digitised and analysed as per section 2.3.5, with the addition of an internal standard, ovalbumin and several stages to the analytical process. The images were edited with PDQuest and exported as 16 bit Tagged Image File Format (TIFF) files to Image Quant (IQ); and this program was then used to complete editing with annotation and volumetric data analysis of the images.

The IQ drawing tools were used to draw ellipses that were of the correct size and orientation to completely encompass the CBG spots (see figure 2.1). The IQ analysis involved drawing an ellipse around the largest spot then using a copy/paste function, copying this ellipse for the rest of the spots on the image in order to ensure consistency. One ellipse was always placed in a blank area to act as background subtraction.

The ellipse set was then used for all four images in each set; as each set of 2D-E gels was run on the same day ensuring consistent set to set analysis. The IQ program then produced the volumetric data by summarising the height of all the pixels in the sample ellipse, and subtracting the sum of pixel intensities from the control ellipse. The volumetric data were then transferred to MS Office Excel for the calculation of what percentage each CBG spot (or glycoform) was of the total CBG spot volume of the image. The data were then transferred to another program, GraphPad Prism 4 for further graphical and statistical analyses. Chapter 3, section 3.3 contains a more detailed description of the image analysis process

CHAPTER 3

PHYSIOLOGICAL EFFECTS ON CBG GLYCOFORMS DURING PREGNANCY AND WITH THE ORAL CONTRACEPTIVE PILL

3.1 INTRODUCTION

Two-dimensional electrophoresis (2D-E) is currently the method of choice for the investigation of complex protein mixtures such as plasma. The technique is particularly useful for analysis of posttranslational modifications (PTMs), including glycosylation and phosphorylation. One of the advantages of the technique is that it separates proteins by both isoelectric point (pI) in the first dimension and molecular weight (MW) in the second dimension. There are, however, several well known and documented disadvantages associated with this method.

The pilot 2D-E experiments were designed to investigate the glycosylation profile of human CBG during pregnancy and individual plasma samples from women at different gestational ages were analysed (see chapter 3 section 3.4). 2D-E gels are generally stained with Coomassie brilliant blue, however, the complex mixture of proteins in crude plasma would have resulted in CBG being "lost" among the numerous other spot clusters on the gels. Major interference would have arisen from proteins with similar pI and MW, particularly human serum albumin (HSA) and apolipoprotein A1 (AP-A1).

The method chosen in this study was an alternative for visualisation of CBG on the gels was 2D-E and Western blotting with chemiluminescence and x-ray film. The specificity of the Western blotting antibodies binding to the CBG on the NC selected the CBG from among the other plasma proteins "unlabelled". This enabled the CBG labelled with the secondary HRP conjugated antibody to be activated with ECL and the luminescent signal recorded on x-ray film. The CBG glycoform 2D-E spot profiles were then analysed using image analysis software.

3.2 CBG HETEROGENEITY

CBG heterogeneity – variability of glycosylation between glycoforms - has been demonstrated in terms of pI and MW using SDS-PAGE and IEF, (Avvakumov & Hammond, 1994; Kato *et al.*, 1988; Strel'chyonok *et al.*, 1984). Variable glycosylation may contribute to some CBG heterogeneity since the peptide backbone possesses a single *N*- and *C*-terminus (Avvakumov & Strel'chyonok, 1987), and there have been no reports of any other forms of CBG post translational modifications (PTMs). While on the other hand, in other species, such as the rat it has been suggested that there may be PTMs other than N-glycosylation in CBG as three bands appear on SDS-PAGE for the rat (Ali & Bassett, 1995).

The combination of variable occupancy of the six potential N-linked glycosylation sites on CBG (Figure 3.1), a mixture of bi- and tri-antennary glycosylation and sub-stoichiometric addition of sialic acid to the sugar termini (Avvakumov & Hammond, 1994), all contribute to CBG microheterogeneity - variable glycosylation within each glycoform - as well as account for the broad range of pI and MW observed.

The extent of CBG glycosylation increases during pregnancy with the appearance of solely tri-antennary glycosylated glycoforms characterised by their inability to bind Concanavalin A (con A), (Avvakumov & Strel'chyonok, 1987). This con A non-binding CBG or CAN-CBG was further described by (Benassayag *et al.*, 2001) who used a crossed affinoimmunoelectrophoresis method (see 1.2.3 for details). The experiments demonstrated that CBG glycoforms undergo a generalized shift to predominantly tri-antennary glycosylation during pregnancy. However, these published methods provide limited data about the molecular detail of CBG sialylation status, which requires high-resolution separation of CBG glycoforms.

3.3 RESULTS PART A: PILOT EXPERIMENTS

3.3.1 EXPERIMENTAL RATIONALE

The lack of previous CBG glycoform resolution led to the use of IEF and SDS-PAGE, known as two-dimensional electrophoresis (2D-E), to provide enhanced CBG glycoform resolution. In addition, 2D-E would enable visualisation of any CBG profile or relative glycoform abundance changes in conditions such as pregnancy.

Pilot experiments were designed to resolve CBG glycoforms in random pregnancy plasma samples from individual women at different stages of gestation compared with normal plasma. The main aims were to:

i Demonstrate that CBG glycoforms could be resolved with 2D-E and visualised using Western blotting.

ii Investigate whether there were any changes in these glycoforms during pregnancy visible using 2D-E.

3.3.2 EXPERIMENTAL PROTOCOL

Refer to chapter 2 for details of subjects, materials, and methods. In summary five random plasma samples from individual women at 8, 12, 17, 33 and 37 weeks gestation and a female non-pregnant control were collected and analysed individually. Previous testing demonstrated that 20ng of CBG is present in 0.25µl of crude plasma; therefore, this volume was the amount chosen for testing. A narrow pH range, 4-7, was chosen for the IPG strips (Tables 2.1 and 2.2) as the IEF bands for CAN-CBG are known to shift towards lower pH values with respect to normal CBG with SDS-PAGE (Avvakumov & Strel'chyonok, 1987).

SDS-PAGE with 10% polyacrylamide gels was used to resolve the CBG glycoproteins in the second dimension (MW direction). This choice was based on previous investigations which demonstrated that during electrophoresis in low porosity gels, CAN-CBG (solely tri-antennary glycosylated variant) migrated as a single band while normal CBG migrated as two electrophoretic bands (possibly explained by mixed bi- and tri-antennary glycosylation) (Avvakumov & Strel'chyonok, 1987).

CBG is a minor component of plasma, therefore indirect detection by Western blotting with a primary polyclonal rabbit anti-CBG antibody and a secondary horse-radish-peroxidase-conjugated goat anti-rabbit IgG antibody with enhanced chemiluminescence (see section 2.3.3 for more detail) was required for the CBG in the crude plasma samples to be visualised. This was the best technique available to visualise the immobilised CBG among all the other plasma proteins on the nitrocellulose after electroblotting.

3.3.2a Data analysis

The Western blot images recorded on film were digitised using a densitometric scanner, and the images refined using PD Quest, image analysis software. The images were rotated, cropped to size, and aligned using the MW markers (the 62.3kDa marker was used as the landmark marker as it was pink on the original filters and its position was easily marked on the x-ray films for a permanent record). The images were aligned using the pH 4 electrode, however, a more refined method of analysis was used later for the larger study, see section 3.3.3 for details.

3.3.3 HIGH RESOLUTION SEPARATION OF CBG GLYCOFORMS IN PREGNANCY PLASMA

These data represent the first application of two-dimensional electrophoresis (2D-E) and Western blotting to the analysis of CBG glycoforms in human pregnancy plasma. (These novel findings were published in 2004, see Appendix 2). The technique enabled a greater degree of CBG glycoform resolution and charge heterogeneity analysis than was previously possible using IEF under native conditions (Avvakumov & Strel'chyonok, 1987; Benassayag *et al.*, 2001). Furthermore, the data demonstrated a difference between the pregnancy and control plasma CBG glycoform 2D-E profiles.

Figure 3.2 illustrates six Western blot images analysed, 5 pregnancy and 1 control sample. In each panel, 8-10 elongated spots are visible in a pI range of 4 to 5 with a MW spread of 60 to 80kDa.

In the late pregnancy panels (e and f), the spots appear slightly closer to the pH 4 electrode than the control as well as having a higher apparent MW than control or early pregnancy. Although, slightly different experimental methods compared with those outlined in section 3.1 above were used for these experiments, the observations concur with the previous descriptions (see section 3.4.2 for more details).

3.2.4 LIMITATIONS OF EXPERIMENTAL METHODS AND ANALYSIS

Despite the high-resolution separation of CBG glycoforms achieved using 2D-E and Western blotting, both techniques have limitations. Western blotting is an inherently non-linear process as chemiluminescent signals appearing on the detection medium (x-ray film) are produced by the emission of light from the "activated" substrate. The x-ray film may, in some cases become saturated beyond a certain level, thus further increases in signal may not be detected. Other variables associated with Western blotting may include the activity of the conjugated enzyme, antibody titre, substrate concentration, chemical reactivity, and ambient room temperature (Conrad *et al.*, 2001). However, for the current experiments, one of the most critical variables was the amount of CBG on the blot, and since only 0.25µl of plasma was TCA precipitated, the possibility of pellet mishandling was greatly increased, resulting in a reduced concentration of CBG on the blot, thereby reducing image intensity.

Signal analysis of 2D-E images is a cumbersome process even with imaging programs such as PD Quest (PDQ) and Image Quant (IQ). These programs are designed to analyse conventional 2D-E Western blot images - which usually have round spots unlike the ovoid spots obtained for the CBG blots- and SDS-PAGE stained gels. CBG has a unique 2D profile and data regarding analysis of this protein were not readily available to assist with the analyses.

Pilot data image analysis was initially conducted with MW markers, however this proved unreliable. The SDS-PAGE gels were of unequal size as they were poured in individual gel cassettes and run on different days, resulting in different run rates and non-uniform protein MW marker ladders for alignment. Although, the "pink" 62.3kDa marker was used as the "standard" to align the gels, there was a lack of other reference points for more accurate gel alignment.

The samples analysed for the pilot study were from *individual* women at *different stages* of gestation, therefore, it was difficult to assess whether the observed changes in the CBG glycoform profiles were unique to each individual woman, or were general "global" changes occurring among all women during gestation. This led to the investigation of women through pregnancy, outlined in section 3.4.

3.3.5 EXPERIMENTAL DESIGN IMPROVEMENTS

The limitations evident from the pilot experiments prompted methodological and technical revisions. A larger sample volume was used to ensure a relatively consistent amount of CBG was loaded onto each gel, in order to obtain similar Western blot signal intensities. The composition of the rehydration buffer was altered to include 2M thiourea, to ensure that the TCA precipitated pellets dissolved more readily (section 2.2.1a, point 4, Table 2.3). In addition, new pH 3-6 IPG strips were used to improve the pI separation of the CBG glycoforms (see section 2.2.1a, point 1 Table 2.1).

The second dimension gels were poured in a casting chamber to ensure gel size and consistency, which was important to ensure that the proteins in each gel ran at the same rate.

To facilitate analysis an internal standard (IS) was included allowing more accurate glycoform identity assignment. There are several commercially available standards for 2D-E, however, it was not possible to use any of them for Western blotting, as they were not pre-stained. A possible explanation for the lack of pre-staining is that the dye molecules in the stain may interact with and neutralise the proteins in the standard solution. Furthermore, the quoted pI of the markers may not always be accurate due to uncertainty as to their complete covalent structure and commercial manufacturers of IPG strips conduct the experiments for determination of pI in the absence of urea. The experiments for this thesis were conducted under conditions of urea, which significantly alters the pI of proteins.

3.3 REVISED DATA ANALYSIS

3.3.1 INTERNAL STANDARDS (IS)

3.3.1a Rationale

The selection of an internal standard (IS) enabled more accurate alignment of the Western blot CBG images for the comparison and contrast of the pregnancy and control samples. A suitable IS requires a pI value, or series of values, close to but not overlapping the CBG glycoform pI values (4-5.5) or MW range, thus allowing visualisation on Western blots. Ideally, the pI of the IS would need to be known, or calculable from its sequence and its post-translational modifications (PTMs) in order to have an absolute reference point. A non-glycosylated serum protein of known pI may have been used as an IS for ease of sample handling. However, no suitable serum protein was found with Coomassie brilliant blue staining of the 2D gels. Hence, a sufficient amount of protein detectable by a weak reversible stain of the nitrocellulose was used as an IS.

3.3.1b Internal standard: 14.3.3

The 27kDa protein 14.3.3, readily available in the laboratory, was initially tested as a potential IS. However, 1D-E testing revealed a contaminant in the recombinant 14.3.3 sample, which ran at the same MW as the CBG, rendering this protein unsuitable.

3.3.1c Internal standard: ovalbumin

Ovalbumin (OVA) was tested next as the PTMs of this protein result in the formation of variations with several pI values, some within the range of CBG. In addition, the MW (43kDa) was ideal for SDS-PAGE as this ensured that OVA ran ahead of CBG. OVA was inexpensive, readily available, and could be dosed in microgram amounts during sample preparation. The presence of OVA enabled the "labelling" of the CBG glycoforms, which in turn assisted in the tracking and validation of any changes, or "shifts" that may have been observed under different conditions, including pregnancy (see section 3.2) and oral contraceptive pill (OCP) use.

Ponceau S red (in 5% acetic acid) was used to reversibly stain the nitrocellulose after electroblotting to visualise the OVA spots. A permanent mark able to withstand the Western blot process (HB pencil) was then made on the filters where the spots appeared. The position of the OVA spots on the nitrocellulose was transferred to the chemiluminescent film image using a permanent marker and overlaying of the film onto the nitrocellulose allowed the CBG spot positions relative to OVA to be visualised and assessed.

3.3.1d Ovalbumin properties

Information regarding the structural properties and molecular composition of OVA made it possible to understand the migration of OVA during 2D-E. OVA was a glycoprotein of unknown function secreted in the oviduct and found in large quantities in avian egg white. OVA is a member of the serine protease inhibitor (SERPIN) superfamily, and like CBG lacks any protease inhibitory activity (Huntington & Stein, 2001).

The amino terminus of the protein is acetylated and the glycoprotein is N glycosylated at a single site (Asn^{292}) in the secreted form (Huntington & Stein, 2001). There is a second potential glycosylation site at residue 311, which, although not glycosylated in the secreted form, has been observed to be transiently glycosylated in the oviduct. Furthermore, the *di-glycosylated* form of OVA, *CHO-Asn-292/CHO-Asn-311,* may be proof read by peptide N-glycanase (PNGase) to the mono-N-glycosylated form, *CHO-Asn-292/-Asp-311,* before secretion and may be critical in the secretory process and/or for the correct function of the protein (Suzuki *et al.*, 1997). Two genetic polymorphisms have been reported, a Glu 289 →Gln (Ishihara *et al.*, 1981) and an Asn →Asp 311 (Wiseman *et al.*, 1972).

Two potential phosphorylation sites for OVA were reported at Ser69 and Ser345 (Huntington & Stein, 2001), and two additional phosphorylation sites were described at Ser237 and Ser241 using multidimensional liquid chromatography and tandem mass spectrometry (MacCoss *et al.*, 2002). An approximate ratio of 8:2:1, for two, one and zero phosphate groups per OVA molecule (respectively) has previously been reported (Perlman, 1952), and the different degrees of phosphorylation at the previously named sites result in heterogeneity in the electrophoretic behaviour displayed by OVA (Milstein, 1968; Nisbet *et al.*, 1981). Glycosylation is not thought to contribute to the pI variations of OVA isoforms as only neutral sugars are attached at the Asn^{292} glycosylation site (eg mannose and GlcNAc), while on the other hand, the phosphorylation(s) and possibly other as yet unidentified PTMs may result in up to six isoforms (see section 3.3.2b, Table 3.2).

3.3.2 ISOELECTRIC POINT (PI) CALCULATION AND ESTIMATION: OVA ISOFORM PI

3.3.2a Isoelectric point (pI)

Mass spectrometric analyses of the OVA used as an IS were conducted by Dr Bagley. A complete report of the data and analyses has not been included as this is beyond the scope of the current study. The data verified the phosphorylation, *N*-terminal acetylation and glycosylation of OVA, which confirmed the origin of the PTMs for 4 of the 6 possible spots. However, there were 2 extra spots on Ponceau S staining that this information did not explain.

The pI of CBG is the pH at which it bears no net charge; the positive charge on basic groups (*N*-terminus, side-chains of histidine, lysine and arginine) balances the negative charge on the acidic groups (C-terminus, side-chains of aspartic acid and glutamic acid & sialic acid). The combination of the pKa or ionisability of these groups determines the charge on CBG at any given pH. The OVA pI calculations were completed using a Microsoft Office Excel worksheet and based on phosphate and amino acid residue content.

Calculation of the theoretical pI range of CBG and OVA was completed with the aid of an Excel spreadsheet:

Extinction, http://www.imvs.sa.gov.au/immunology/research/cjbpage.htm

The spreadsheet enabled prediction of the position of CBG and OVA after electrophoresis (eg pI, MW) based on the extent of sialylation or phosphorylation using the protein sequences and standard measured pKa values for the amino acid, sialic acid and phosphate residues under the IEF conditions used in the current experiments.

The extinction spreadsheet calculated pI in two steps. Firstly, it calculated the sum of the positive and negative charges on all ionisable groups at the pH set in a cell in the work sheet. The second step involved a command that instructed the macro to set the net charge to zero by changing the pH value in the same cell, by successive approximation. The CBG sequence entry from SWISSPROT was used to calculate the theoretical pI and MW values for CBG. The theoretical MW of CBG was calculated from the sum of the amino acid residue MWs for all its residues and PTMs plus the equivalent of one water molecule.

Referring to Figure 3.3, the scatter plot demonstrates the relative alignment of CBG and OVA spots following 2D-E based on the theoretical pI values calculated using Extinction. Reading left to right the first CBG glycoform corresponds to the first OVA spot, which is equivalent in pI value to that obtained when 5 sialic acids are added to the CBG pI calculation formula (Table 3.1). Information from the spreadsheet calculations indicate that the CBG profiles observed in the Western blot images is probably the result of 5 to 18 sialic acid additions. However, some blots appeared to have spots that may have had *more* than 18 sialic acid residues, as they were more acidic than the most acidic control spots (see section 3.4). A possible explanation for this observation is that the spreadsheet values may not correspond to the values observed in reality on the 2D-E images or like OVA, there may be other undocumented CBG PTMs contributing to the appearance of these highly acidic spots.

3.3.2b OVA Isoelectric point (pI) estimation

The mass spectrometric data and an MS Excel extinction coefficient spread sheet (derived using information about the OVA sequence from the SWISSPROT, OVAL_CHICK entry), into which the number of phosphorylations and *N*-terminal acetylation were entered, was used to calculate the theoretical pI values of OVA (Figure 3.3).

Referring to Table 3.2, OVA species A and D are composed of a single species of OVA with PTM descriptions (a) and (f). While species B and C represent OVA species composed of a mixture of PTMs (b) and (c); and (d) and (e), respectively. In addition, the species which are di-phosphorylated C and D would be the most abundant, that is, appeared more prominently than species A and B with ponceau staining.

TABLE 3.2 *Summary of OVA PTMs modifications (Note: all OVA species are N-terminally acetylated, however, species (a) is thought only to be N-terminally acetylated). For ease of discussion the species have been labelled A to D.*

PTM modification summary		**pI**	**Species**
a)	*N*-terminal acetylation only	5.13	A
b)	Asn → Asp substitution, no phosphorylation	5.07	B
c)	1 phosphorylation	5.06	
d)	Asn →Asp substitution, 1 phosphorylation	5.01	C
e)	**2 phosphorylations**	5.00	
f)	**Asn →Asp substitution, 2 phosphorylations**	4.95	D

Although Table 3.2 illustrates the calculated pI values of up to six OVA isoforms (pI column) based on the possible PTMs; isoform pI overlap results in Table 3.2 only adequately explain the presence of four OVA isoforms. This discrepancy was practically demonstrated when up to 6 spots were visualised in the OVA region following Ponceau staining of the 2D-E nitrocellulose and the presence of these spots could not be explained with the MS data, spreadsheet and other calculations alone.

There was nitrocellulose (N/C) blot to blot variation in the number of spots observed at the approximate known MW and pI for OVA. Referring to Figure 3.4, the most common number of spots observed was four, with spots 2 and 3 being the most abundant (or darkest), probably representing the di-phosphorylated OVA species (C and D Table 3.2). In addition, OVA 2 and 3 where the *landmark* spots and were always numbered accordingly, with the neighbouring spots numbered, 0 and 1 for spots to the left of OVA 2 and 4 and 5 for spots to the right of OVA 3.

The information in Table 3.2 explains the pI values of the four most commonly seen OVA isoforms with ponceau staining after 2D-E (spots 1 – 4), although it does not explain the presence of the two extra spots, 0 and 5, which were intermittently observed. *N*-terminal acetylation is thought to be constitutive in OVA; however, it is unclear which type of PTM would result in a more basic spot such as spot 0 which is postulated to have a pI of 5.20. A mutation that may result in altered pI may be a glutamic acid to glutamine substitution polymorphism reported by (Ishihara *et al*, 1981). While, spot 5 which appeared at the lowest pI for the OVA used, may have been due to tri-phosphorylation and the OVA heterogeneity referred to previously may assist in explaining the presence of spots 0 and 5. The information regarding OVA PTMs is incomplete and leads one to speculate that there may be other as yet unidentified OVA PTMs.

The inclusion of an IS greatly improved the qualitative description of the 2D-E images (Figure 3.5) and together with the image analysis software programs, PD Quest and Image Quant enabled quantitative analysis of the images. Furthermore, partial separation of CBG was achieved using affinity chromatography (concanavalin A, see chapter 4) and the data collected from these experiments further characterised the CBG glycoform changes that occur during pregnancy.

Therefore, the qualitative and quantitative description of the CBG profiles and con A data, enabled a more detailed description of the changes that occur during pregnancy.

3.3.3 2-D IMAGE DIGITISATION AND COMPUTER ANALYSIS

3.3.3a Summary

In order to establish a method for analysis of the digitised Western blot pilot images, PD-Quest 7.1 (PD-Q) was used to refine the images, and several new stages were added to the process described in section 3.2.2a. The analysed files were exported as 16 bit Tag Image File Format (TIFF) files to another image analysis program, Image Quant (IQ). IQ was used to complete the analysis, which included annotation (qualitative analysis) and spot volume calculation (quantitative analysis). Figure 3.6 is a summary of the annotations added to the images using IQ. The data were then transferred to MS Office Excel and the individual spot pixel volume percentages calculated by IQ were calculated as a percentage of the total spot pixel volume in all the ellipses in the image. A statistical analysis program, GraphPad Prism 4, was used to produce Histograms, and Gaussian distribution curves with a test of the difference of the means calculated.

3.3.3b 2-D image computer analysis: qualitative analysis

In order to follow the progress of CBG glycoforms during pregnancy it was important to label each spot using a consistent system. An alphabetical system, easily converted to a numerical scale for later calculations and discussion was used to identify the CBG spots (see Figure 3.6). These spot identities were assigned according to their relative alignment with the OVA spots as seen in Figure 3.4, where, the CBG spot above OVA4 or slightly more acidic was designated CBG D. Spot CBG D was the landmark spot as this spot was present in all gels, while CBG A was the most basic CBG spot seen only on some gels.

The ECL detection system is very sensitive and was chosen for this analysis as it was the best system available for the purposes of this study. One of the most weaknesses of this system is that the NC must be manually exposed to the photographic film,, thus the resulting exposures of each of the films were not optimal for analysis with PDQuest and it is unfortunately too late to rectify. However, the normalisation against total spot intensity assisted in correction for overexposure, for instance Figures 3.14.1 and 3.9.7 (24 weeks). . There were difficulties too associated with the alignment of OVA spots with the CBG, and as well as the fact that the pI shifts detected in this study during pregnancy were modest, had they been more the question of alignment would be less of an issue.

3.3.3c 2-D image computer analysis: quantitative analysis

The Image Quant (IQ) drawing tools were used to draw shapes around the CBG spots, which best matched their ellipsoid shape, as illustrated in Figure 2.1 section 2.3.3e. IQ was ideal as it was possible to rotate the ellipses enabling flexibility and increased accuracy of the volumetric data calculations. In addition, since the ellipses drawn around the CBG spots included some background, a "background" ellipse was drawn somewhere on the image away from the CBG and OVA spots (see Figures 3.161 and 2 for more detail), and this enabled background subtraction from the spot pixel volume calculation giving a "true" spot volume.

The spot volume data were transferred to MS Office Excel, normalised, and subsequently transferred to GraphPad Prism for final analysis, which included histogram and Gaussian curve construction. For each group mean, a calculated mean glycoform (CMG) was derived from the Gaussian curves for comparative purposes and the p values were calculated for the curve fits of the Gaussian curves in order to test the difference of these means. Flow diagram 3.1 summaries the process.

In most cases the CMG corresponded to the most abundant glycoform in the 2D-E profile and was calculated from the Gaussian curves, as it was not possible to determine from visual inspection alone. The profiles (for example, Figure 3.2) show a gaussian like distribution in spot intensity and (higher pI spots are lighter, becoming progressively darker to a maximum in the mid range pI values with the higher pI spots lighter). Thus this value represented the most abundant glycoform for each group.

The theoretically estimated sialic acids per CBG molecule or SAC is obtained from an Excel spread sheet (introduced in section 3.3.2) which was able to predict the number of sialic acids molecules per CBG molecule. Therefore, the acidity of each glycoform was dependent upon the glycosylation of the CBG molecules within each glycoform. The most acidic glycoforms, CBG O, P and Q, may have possessed more tri-antennary and perhaps tetra-antennary glycosylation thus allowing more sialic acid to bind. Furthermore, these very acidic glycoforms may have possessed branched sialylation, which is present in other proteins, for instance, major histocompatibility complex (MHC) glycoproteins (Swiedler *et al*,1985) and $\alpha 3\beta 1$ integrin N-glycans (Yamamoto *et al*, 2001).

One of the major findings reported in this thesis (in agreement with the available literature) was that pregnancy induces an increase in the acidity of CBG. This increase in acidity was demonstrated with Gaussian curves, which demonstrated a peak height shift of the curve toward the more acidic. For instance the control CMG may have been CBG G and pregnancy CMG CBG I, thus representing an increase in acidity by one glycoform.

The CMG values were correlated with the SAC and corresponding IS related alphabetical glycoform label. Table 3.1 illustrates this correlation between the estimated SAC compared with the alphabetical and numerical glycoform identifiers and both CBG and the IS estimated pI. Figure 3.3 demonstrates derivation of the CBG glycoform alphabetical identifiers.

3.4 RESULTS PART B: SERIAL SAMPLE DATA ANALYSIS

3.4.1 2D-E ANALYSIS OF CBG IN PREGNANCY PLASMA

3.4.1a Experimental Rationale

The pilot experiments successfully demonstrated separation of CBG glycoforms with high resolution as well as differences between pregnancy and control plasma CBG. However, as mentioned in section 3.3.2, it was unclear whether the CBG glycoform changes are specific to individual women or occur as a generalised pattern ongoing through pregnancy. Therefore, the hypothesis to be tested for these experiments was: *that CBG glycoforms specific to pregnancy vary in relation to gestational age*.

In order to investigate this hypothesis, plasma samples were collected from 3 different subject groups:

1. 10 sets of serial pregnancy samples at four gestational time points, 16, 24, 32 and 36 weeks (total of 40 samples)
2. 10 random pregnancy samples from women in early gestation, at or near 3 to 15 weeks gestation
3. 3 non-pregnant women not taking the OCP and 2 healthy men as control samples

The samples were analysed using 2D-E and Western blotting as per chapter 2.

3.4.1b Sample preparation

5-10 ml blood samples were collected and samples prepared as per section 2.3.1-2. The methodological and technical revisions outlined in section 3.2.4 above were applied to the sample preparation for the 2D-E experiments. In summary, the revisions included a larger TCA precipitation volume using MQ water for plasma dilution with subsequent loading of a 0.25µl volume equivalent of plasma (see section 2.3.2a for detail). The samples were then dissolved in IEF buffer 2 and IEF was conducted as per section 2.3.2f-h on pH 3-6 IPG strips using the revised protocol (protocol 2, Table 2.11). The IPG strips were then equilibrated in SDS buffer and transferred to the second dimension 10% SDS-PAGE gels. Following SDS-PAGE the plasma proteins were electroblotted onto nitrocellulose (see section 2.3.3a), and probed for CBG with enhanced chemiluminescence (Western blotting). The images produced by chemiluminescence on nitrocellulose were recorded on film, digitised, and analysed as per section 3.3.3b and c.

3.4.2 THE CBG 2D-E PROFILE IS ALTERED IN PREGNANCY COMPARED WITH CONTROL

The CBG profiles of 10 random early pregnancy samples are demonstrated in Figures 3.8.1-10, while Figures 3.9.1-10 demonstrate the 10 serial pregnancy sample sets (4 time points are shown for each), and Figures 3.10.1-5 demonstrate 3 non-pregnant non-OCP females and 2 male controls. The profiles of the CBG spots in Figures 3.8.1 to 3.10.5 are very similar to those shown in Figure 3.2 as they demonstrate elongated ellipsoid spots in a diagonal pattern. However, there appears to be more spots in Figures 3.8.1-3.10.5; 9 to 13 compared with 3.10.8 to 10 and Figure 3.2, and this may have been due to greater resolution using the pH 3-6 (section 3.4 samples) strips compared with the pH 4-7 strips (section 3.2 samples). The new pH range (3-6) more closely approximated the predicted pI of CBG, which was calculated to be approximately 4.3 to 5.2 (Figure 3.5) hence it was postulated that the strips may have provided a more optimal pH environment for the protein's migration. Furthermore the spot CBG A was only seen in the male control samples and this may be a characteristic of male CBG in general, however, more samples would have to be run in order to determine is this were the case.

In order to utilise the information from the CMGs calculated from the Gaussian curves quantitively, a *deduced SAC (DedSAC)* was calculated (Table 3.3). The DedSAC was calculated using the relationship between the theoretically estimated CBG glycoform SAC and the corresponding numerical glycoform value. The difference between these two values was 4 - eg estimated SAC of 5 corresponded to the numerical glycoform value of 1 – hence, this arbitrary value, 4, was added to the group CMG which corresponded to the numerical glycoform value (Table 3.1 contains values used). Furthermore, the difference between the control and each group, DedSAC, was then used as a comparator to measure any shifts in SAC; $p<0.05$ was considered statistically significant.

In the normal state CBG has been reported to have a SAC of 10.0 which has been measured to increase to 12.7 (Avvakumov & Strel'chyonok, 1987) and up to 13.6 (Blithe *et al.*, 1992) during pregnancy. The difference between normal and pregnancy SAC, denoted here as ΔSAC, is that in the former study SAC was found to be 2.7 which equates to the addition of almost three sialic acid residues during pregnancy and these may have been associated with an increase in the ratio of tri-antennary glycosylated glycoforms. Based on the proposed structure of CBG and the 6 known glycosylation sites it is possible therefore that there could be increased sialylation without an increase in glycosylation, for instance if all six sites were occupied instead of only five. There was no SAC difference available for the Blithe *et al* (1992) study as no analysis of normal plasma was reported.

Previously, pregnancy induced CBG glycosylation changes were defined as a shift to solely tri-antennary glycosylation which may be associated with increased sialylation (acidity) (Avvakumov & Strel'chyonok, 1987; Strel'chyonok *et al*, 1984). Therefore, in this thesis a change in glycosylation was defined as a shift in the CBG profile towards greater acidity (represented by the SAC) and/or the appearance of any new highly acidic glycoforms not present in the control samples.

3.4.2a CBG 2D profile alteration in early and mid to late pregnancy

The early pregnancy histogram bars (Figure 3.11.1) demonstrate a disappearance of the most basic glycoform, CBG A, when compared with control, and no appearance of any new highly acidic glycoforms, that is, both profiles end at CBG N. Assessment of the early pregnancy SAC, (Figure 3.11.2 and Table 3.3), demonstrates that it is slightly greater than control. Therefore, early in pregnancy (3-15 weeks), there is loss of the most basic CBG glycoform (CBG A) compared with control.

Visual inspection of the serial pregnancy profiles revealed little difference between the histogram bar profiles for each of the 4 time points (Figure 3.12.1). Hence, the data were collated and the serial pregnancy data treated as one group, collated serial pregnancy (CSP), and these data represented mid to late pregnancy (Figure 12.2).

The CSP ΔSAC was 1.7 and the discrepancy with the previous studies (section 3.3.3c (Avvakumov & Strel'chyonok, 1987)) may be explained by the fact that different source materials and methods were used, as well as a lack of CBG purified from the plasma in the current experiments.

Comparison of the SACs (Figure 3.12.3 and Table 3.3) demonstrated a significant difference between CSP and controls, $p<0.0001$, and together with the appearance of new glycoforms, it is reasonable to conclude that there was a shift toward increased sialylation in mid to late pregnancy.

This shift may have been associated with increased tri-antennary glycosylation as has been previously postulated (Avvakumov & Hammond, 1994; Avvakumov & Strel'chyonok, 1987; Strel'chyonok *et al*, 1984).

Comparison of the two pregnancy groups, early pregnancy and CSP (refer to figure 3.13), demonstrates that the most basic glycoform, CBG A, disappears in both groups and only the CSP samples demonstrate a shift to a relatively acidic glycoform profile with appearance of two highly acidic (possibly high MW) glycoforms, CBG O and P. Therefore, the 2D-E analysis of the pregnancy plasma when compared with control indicates that pregnancy induces a significant increase in glycoform acidity, evident as increased SAC (Table 3.3), which becomes more apparent in mid to late pregnancy (CSP), indicating a trend towards increased sialylation with possible associated increased tri-antennary glycosylation.

Hence, the increases in CBG glycoform acidity induced during pregnancy may result from an increased ratio of tri-antennary to bi-antennary in the normal CBG glycosylation state previously suggested to be 2:3 normally. The ratio may instead be as high as 4:1, 5:1 or even 6:0 during pregnancy- (Avvakumov & Hammond, 1994; Avvakumov & Strel'chyonok, 1987). Tetra-antennary glycosylation suggested by Avvakumov & Strel'chyonok, 1985; Blithe *et al*, 1992, may be another plausible explanation for the increase in pregnancy related increase in CBG acidity observed in this study.

3.4.3 COMPARISON OF CBG 2D-E PROFILES FROM OCP WITH CONTROL SAMPLES

3.4.3a Experimental Rationale

In order to investigate the hypothesis that *the OCP may induce a "pregnancy like" change in the 2D-E CBG profiles of non-pregnant women compared with controls*; the CBG 2D-E profiles of 3 non-pregnant women taking the OCP and 2 healthy men were compared with the same control group as in section 3.4.1. The samples were prepared for IEF and SDS-PAGE as well as Western blot analysis as per section 3.4.1b.

3.4.3b The OCP causes a change in plasma CBG visible using 2D-E and Western blotting

The 2D-E Western images of the OCP samples are illustrated in Figures 3.14.1-10, and are very similar to the other 2D-E images demonstrated in Figures 3.8.1 to 3.10.10 and 3.2. There are 10-15 elongated ellipsoid spots with a diagonal profile. In the same way as subtle differences between the two pregnancy groups and the control images were not readily detectable by visual inspection alone, the OCP images required computer image analysis in order for volumetric data to be obtained and evaluated.

Figure 3.15 demonstrates the histogram comparing OCP and control data. There is a difference between the two profiles as there is the appearance of what appear to be "OCP specific" glycoforms (O, P and Q in gold dashed box), which are observed to be more acidic than the most acidic control glycoform, CBG N. The appearance of CBG Q in the OCP sample group (which is very low in relative abundance) was only observed in one subject and was therefore ascribed to individual variation.

The OCP results demonstrate disappearance of the most basic glycoform, CBG A (in red dashed box), similar to the changes observed for pregnancy compared with control. Hence, the OCP appears to induce a similar effect on the CBG glycoform profile as pregnancy. The effect is equivalent to the change in CBG observed at mid gestation.

The OCP plasma samples demonstrated a comparable increase in CBG glycoform acidity to the CSP samples, however, when treated with con A, there was no evidence of any con A non-binding CBG or CAN-CBG like glycoforms (Discussed in more detail in chapter 4 and refer to Figure 4.1).

3.5 DISCUSSION

3.5.1 2D-E DEMONSTRATES A DIFFERENCE IN CBG GLYCOFORMS BETWEEN PREGNANCY AND CONTROL PLASMA

The serial and early pregnancy data demonstrate that pregnancy induces a change in CBG glycosylation, as observed in Figure 3.13, where there is disappearance of the most basic glycoform (CBG A), and appearance of two highly acidic glycoforms CBG O and P. These acidic glycoforms, when viewed on the Western blot images are of slightly higher MW indicating a possible shift towards increased tri-antennary glycosylation. Furthermore, CBG O and P were not observed in control plasma indicating that this was probably a pregnancy-induced alteration. The OCP induced a change in CBG glycoforms similar to pregnancy, however there appears to only be an increase in siallylation and nor glycosylation and sialylation as is the case in pregnancy.

The 2D CBG profiles in Figure 3.2, demonstrated only 8-10 elongated ellipsoid spots compared with up to 16 spots in Figures 3.8.1-3.10.5. This increase in the relative number of spots may have been due to an improvement in isoelectric focussing conditions with the new, lower 3-6 pH range, IPG strips. The shape of the CBG spots may be attributable to variable glycosylation, and Figure 3.16.a demonstrates the typical shape of the CBG glycoform spots. Increased retardation of the mainly tri-antennary glycoforms compared with the mixed (bi and tri-antennary) glycoforms in the 10% SDS-PAGE gel may possibly explain the dumb-bell shape of some of the CBG spots; thus indicating that the new 3-6 pH range strips provided much improved resolution conditions for CBG analysis.

The spots had a characteristic "lean" (Figure 3.16.b) suggesting that within each spot population there were some CBG glycoforms slightly more acidic than others and this may be attributable to the addition of more sialic acid residues, up to 18 being the estimated maximum (3 residues per site on 6 glycosylation sites, refer to Figure 1.2). There were some glycoforms, which appeared to have more than 18 sialic acid residues, and other acidifying PTMs may have been responsible for these.

The SAC becomes more acidic with increasing gestational age, in early pregnancy (3-15 weeks) the SAC is CBG H and by mid to late pregnancy (represented by CSP) the SAC has shifted one glycoform in the acidic direction, CBG I, compared to the control SAC which was CBG G. These observations are in keeping with previous observations (Avvakumov & Hammond, 1994) where it was found that the CBG present in plasma during pregnancy was highly tri-antennary glycosylated which would allow for the addition of increased sialic acid residues, and therefore decreased pI compared with control which had a mixture of bi- and tri-antennary glycosylation.

3.5.2 2D-E DEMONSTRATES A DIFFERENCE IN CBG GLYCOFORMS BETWEEN NORMAL WOMEN AND WOMEN ON THE OCP

The OCP CBG glycoform profile alterations were similar to those induced by gestation. In addition, the OCP is known to increase the plasma oestrogen concentration (Wiegratz *et al.*, 2003), and this is likely to underlie the increase in CBG levels with possible associated glycosylation alterations.. There may have been a shift to tri-antennary glycosylation, which was undetectable by the means used in the current experiments (see chapter 4 for details)

3.5.3 2D-E AS A MEANS OF STUDYING PLASMA PROTEINS

In order to visualise the most basic and most acidic spots (that is the outlying spots at either end of the profile) it was necessary to obtain an exposure that was not always optimal for analysis. There is a tendency for Western blot images produced by chemiluminescence to become over exposed as film exposure to the membranes is manual, leading to signal saturation and there is an upper limit beyond which it is not possible to measure intensity for these saturated images. Consequently, there is a "blunting" of the Gaussian analytical curves (see Figure 3.17). Fortunately, this did not affect the analysis of the CBG 2D-E Western blots as there was no change in the SAC (p value 0.7159). Therefore, although not ideal, image saturation did not introduce any systematic error or bias into the 2D-E Western blot image analysis. Other techniques that are effectively used for 2D-E analysis are staining of gels prior to image analysis and radioimmuno assays.

Despite the generally high resolution of the CBG glycoforms demonstrated in Figures 3.2, 3.8.1-3.10.5 and 3.14.1-10, there was reduced resolution at the low pH end of the images (representing the low pH end of the IPG strips, this occurred for both pH 4-7 and 3-6 strips). The poor resolution results in poor differentiation and spot quantitation of the CBG glycoforms with very low pI. The latter is characteristic of acidic proteins and has been demonstrated in CBG using 1D-E (Hammond, 1990), as well as in other glycosylated proteins. For instance, prion proteins from brain tissue samples used for investigation of the rare degenerative brain diseases, Creutzfeldt-Jacob disease and sporadic fatal insomnia (Pan *et al.*, 2001). 2D-E (with some form of purification) is generally a good method for serum protein analysis as it gives a range of the pI and MW of each protein form

3.6 CONCLUSIONS

The experiments presented in sections 3.2 and 3.5 have demonstrated using 2D-E and Western blotting that in plasma pregnancy there is a decrease in relative pI of CBG glycoforms with increasing gestational age. Furthermore this increase in glycoform acidity may be associated with increased glycosylation, that is, a shift towards increased tri-antennary glycosylation. In mid-to late pregnancy there were some very acidic glycoforms, which may have possessed more than 18 sialic acid residues, however, the information available in the literature along with the data did not adequately explain the existence of these glycoforms. Consequently, there may have been other acidifying modifications such as deamidation resulting in these very acidic glycoforms, and these glycoforms were present in the OCP plasma suggesting that oestrogen may play a role in the synthesis of these glycoforms. Pregnancy produces very dynamic and complex physiological changes; hence, the changes induced in CBG may be associated with these physiological changes in association with oestrogen.

CHAPTER 4

PREGNANCY INDUCED CBG GLYCOSYLATION CHANGES: A CLOSER LOOK

4.1 AFFINITY CHROMATOGRAPHY

4.1.1 INTRODUCTION CONCANAVALIN A

Glycoproteins may be separated from complex protein mixtures by affinity chromatography using Concanavalin A (Con A) coupled to sepharose beads. Con A is a Lectin tetrameric metalloprotein isolated from *Canavalia ensiformis* (jack bean), which binds molecules containing D-mannopyranosyl, D-glucopyranosyl and sterically related residues. Con A binding requires the presence of C3, C4 and C5 hydroxyl groups

The previously described characteristic, lack of Con A binding, of a subset of CBG appearing during pregnancy (Strel'chyonok OA *et al.*, 1984) was exploited for the subfractional purification of CBG during pregnancy. The subset was described as "pregnancy specific CBG" and later pregnancy associated CBG, however will be referred to here as Con A non-binding CBG or CAN-CBG. Tri-antennary glycosylated glycoproteins will not bind to Con A and this was the basis for selection of the affinity chromatography method for separating CAN-CBG from the normally glycosylated Con A binding CBG or CAB-CBG. In the afore mentioned study CAN-CBG was found to be in the "unbound" fraction (10% of total CBG in sample) during affinity chromatography, while the Con A bound fraction, eluted with α-D-methylmannopyranoside, represents CAB-CBG (90% of the total CBG).

Despite previous analysis of the molecular character of CBG glycoforms during pregnancy using a combination of Con A affinity chromatography, and Western blotting (Avvakumov & Strel'chyonok, 1987; Benassayag *et al.*, 2001b; Cerven E *et al.*, 1981; Mickelson *et al.*, 1982; Strel'chyonok *et al.*, 1984), there is a lack of high-resolution analysis of CBG glycoforms.

The main aim of this set of experiments therefore was to demonstrate separated plasma CBG affinity chromatography fractions with greater resolution than has been previously documented. It was hypothesised that Con A affinity chromatography would confirm the presence of pregnancy specific CBG glycoforms in 36 week gestation plasma and that similar glycoforms may be present in OCP plasma.

4.2 RESULTS PART C AFFINITY CHROMATOGRAPHY

4.2.1 AFFINITY CHROMATOGRAPHIC ANALYSIS OF SELECTED SERIAL SAMPLES

The experiments involved treatment of individual pregnancy, control, and OCP plasma samples with Con A sepharose in preparation for affinity chromatography. The chromatographic fractions (unbound and bound) were then analysed using one dimensional electrophoresis (1D-E) and Western blotting. The 1D-E experiments were necessary to establish the ratio of CAN-CBG to CAB-CBG in pregnancy plasma, with further analysis using 2D-E to compare the glycosylation profile of each fraction to the untreated CBG profile.

The random samples selected were 32 week gestation samples from 1, 6 and 8. In addition, a male and a non-pregnant OCP taking female sample were selected as controls.

4.3 ONE DIMENSIONAL ELECTROPHORESIS (1D-E)

4.3.1 EXPERIMENTAL RATIONALE

The 1D-E analyses were designed to assess the ratio of CAN-CBG to CAB-CBG in individual plasma samples rather than pooled pregnancy plasma as has been the case previously (Strel'chyonok *et al*, 1984). Con A sepharose binding capacity is usually quoted as approximately 20-30mg porcine thyroglobulin per ml of swelled resin on the product label (Sigma-Aldrich). Therefore, it was necessary to determine the amount of Con A required to sufficiently bind human glycoproteins, namely CBG; plasma glycoproteins generally account for only 10% of the total plasma protein volume.

4.3.2 PRELIMINARY TESTING

Initially a test volume of 50µl of Con A sepharose (the estimated binding capacity volume of which was 1µg of glycoproteins per µl of swelled resin) was incubated with 20µl of 32 week gestation plasma (the estimated total CBG content was 1.6µg). This was a working ratio of 2.5 to 1, Con A to pregnancy plasma, (respectively) for CAB-CBG binding.

The protocol for the partial separation of CAN from CAB-CBG is detailed in chapter 2 section 2.3.2. In summary, the 50µl prepared Con A resin in 400µl of binding buffer and 20µl (1600ng CBG) of the pregnancy plasma were mixed and incubated for 30 minutes, the mixture was centrifuged gently in a microfuge and the supernatant retained.

The mixture was then incubated in elution buffer (containing α-D-methylmannopyranoside, α-D-MMP), initially for 15 minutes then later for 30 minutes, and the supernatant collected. 50µl of each fraction (bound and unbound) was TCA precipitated with TritonX 100 and dissolved in a load buffer containing 6M urea and SDS for SDS-PAGE (20µl for unbound fraction and 180µl for bound fraction). The different load buffer volumes were to account for the proportions of CBG variants, unbound (10%) and bound (90%), in pregnancy plasma a 1 to 9 CAN-CBG to CAB-CBG ratio.

Hence, in the original 400µl unbound fraction there was an estimated 1600ng of total CBG, 10% of which was CAN-CBG (160 ng) and the other 90% was CAB-CBG (1440ng); a 50µl equivalent TCA precipitated concentrated sample of the unbound fraction would then contain 20ng of CBG. While a 50µl sample of the bound fraction (prepared as per the unbound fraction) contained the equivalent of 180ng of CBG – 9 times as much as the unbound based on the ratio. The volumes - 20µl for 20ng equivalent CBG in the unbound and 180µl for 180ng equivalent CBG in the bound fraction- were used to ensure an approximate concentration of 1ng/µl of CBG for each SDS-PAGE prepared sample and 8µl per well was loading onto the gels.

The calculations were based on pregnancy plasma and the assumption was that the control sample would contain half the amount of CBG (Avvakumov & Strel'chyonok, 1987). Thus, 1µl of pregnancy plasma contained 80ng of CBG and the equivalent volume of control plasma contained 40ng of CBG.

4.3.3 THE RATIO OF CAN-CBG TO CAB-CBG IN HUMAN PLASMA

The 2.5 to 1 ratio was found to be insufficient to bind CAB-CBG from human pregnancy plasma and further testing revealed that a 7.5 to 1 Con A to plasma ratio adapted from (Cerven E *et al*, 1981) was ideal for binding of CAB-CBG from human pregnancy plasma. In Figure 4.1 there is greater evidence of CAN-CBG in track 4 (the 32 week pregnancy sample) than track 8 (the non-pregnant OCP female control), indicating there was a higher concentration of the CAN-CBG in late pregnancy than in OCP taking non-pregnant female plasma. However, in track 7 there is less evidence of the CAB-CBG variant in OCP taking non-pregnant female plasma than in 23 week pregnancy plasma which showed almost double the amount of CAB-CBG. Thus indicating that the pill may have an effect on CBG glycosylation similar to pregnancy and since it was demonstrated in chapter 3 that the OCP did have an effect on the *acidity* of CBG, possibly due to other acidifying modifications not associated with glycosylation such as deamination, or branched sialylation.

Image Quant (IQ) analysis was used to quantify the 1D-E gels and the results revealed a 15 to 85%, unbound to bound (respectively) fraction ratio in the pregnancy plasma sample (Figure 4.2), which was in agreement with the previously documented 10 to 90% (bound to unbound CBG) ratio (Strel'chyonok *et al*, 1984).

4.4 TWO DIMENSIONAL ELECTROPHORESIS (2D-E)

4.4.1 EXPERIMENTAL RATIONALE

The lack of previous CBG glycoform resolution led to the investigation of the possibility of whether there were more CBG glycoforms during pregnancy with IEF and SDS-PAGE, known together as two-dimensional electrophoresis (2D-E). This technique would enable more enhanced CBG glycoform resolution than either technique alone, as well as visualisation of any changes in CBG profile or relative glycoform abundance for conditions such as pregnancy.

The aim of the affinity chromatography/2D-E experiments was to analyse the bound and unbound fractions from the Con A separation to confirm and extend the 1D-E experiments as well as correlate these findings with the chapter 3 results. In addition, the 2D-E analyses enabled the pI of the glycoforms in each of the fractions to be estimated, based on the CBG profile position relative to the internal standard, OVA.

4.4.2 CON A SAMPLE PREPARATION FOR 2D-E

Three 32 week gestation samples (from subjects 1, 6 and 8) were selected randomly from the serial pregnancy group of samples described in chapter 3 section 3.4. Con A sepharose was prepared as per section 4.3.3, using the 7.5:1 Con A to plasma ratio.

4.4.3 CON A 2D-E QUALITATIVE ANALYSIS

There are two forms of CBG occurring during pregnancy, which were demonstrated in section 4.3, and now for the first time with 2D-E and western blotting (Figures 4.3.1-3). The CBG glycoform profiles were similar to the profiles illustrated in chapter 3. The unbound fractions generally represented the more acidic portion of the untreated sample, that is, CBG glycoforms corresponding to CBG glycoforms A to H of the untreated profile. It is possible that this was due to mainly tri-antennary glycosylation with associated increased sialylation. In contrast, the bound fraction was more similar to the more basic portion of the untreated profile, as there would be a mixture of bi and tri-antennary glycosylation.

Although, the profiles in Figures 4.3.1-3 may appear similar to the other 2D-E profiles for CBG demonstrated in chapter 3, there was a difference between the bound and unbound fractions. The images (for example 4.3.1a-4b), demonstrate that the bound fraction, based on relative alignment with the IS, is more basic than the untreated sample and the unbound fraction is more acidic, which although expected based on what is known about the nature of each fraction it was not typical. A possible explanation may be experimental variation, which may have resulted in these differences, as well as possible enhanced glycoform profile separation of each fraction.

This enhancement of glycoform separation resulting in more separation of these acidic glycoforms in the unbound fraction compared with the untreated (control) fraction where the more acidic glycoforms were focussed in a mixture of basic glycoforms and appeared to blur into each other.

4.4.4 CON A 2D-E QUANTITATIVE ANALYSIS

The Gaussian curves (Figure 4.4) for the untreated 32 week gestation samples from subjects 1, 6 and 8 illustrate that there is a significant difference in DecSAC between the unbound (CAN-CBG) and bound fractions (CAB-CBG), $p<0.0001$.

There is significant difference between the CMG for the untreated compared with bound fraction indicating increased acidity in this fraction. While the unbound fraction CMG indicates that it is more acidic than the untreated sample, which is in keeping with previous findings and what is known about the CBG glycosylation and sialylation changes that occur in during pregnancy. These results are confirmed further by consideration of the $\triangle$SAC for the bound and unbound fractions, which are statistically significant (Table 4.1).

4.5 CONCLUSIONS

The bound and unbound fraction CBG 2D-E profiles are distinctive and demonstrate that there is indeed heterogeneity in both as the spots are not discrete dots but ellipsoids. This may indicate that especially for the unbound fraction, the tri-antennary glycosylation is heterogeneous and not homogeneous as mentioned in a previous study (Strel'chyonok *et al*, 1984). The timing of the appearance of the CAN-CBG in plasma appears to be mid to late pregnancy, despite the fact that there is evidence of a shift towards increased glycoform acidity in early pregnancy (slight shift in SAC to more acidic, Figure 3.11.2).

The observations made about pregnancy plasma are in keeping with (Strel'chyonok & Avvakumov, 1990) who found that the CAN-CBG appeared in the first trimester in some women and in all women by the third trimester. The alterations in CBG glycosylation resulting in increased CAN-CBG may be associated with the reported increased CBG syncytiotrophoblast membrane binding and prolonged half-life during pregnancy (Benassayag *et al.*, 2001a)

The presence of a CAN-CBG fraction of reduced volume in OCP plasma (referring to Figure 4.1, 1D-E analysis) may indicate that there is either a reduction in or a complete lack of a shift towards tri-antennary glycosylation with use of the OCP. Furthermore, increased sialylation induced by the OCP may underlie the increase in serum CBG concentration due to impaired interaction with the liver asialoglycoprotein receptors that are responsible for CBG degradation.

However, this reduction of the CAN-CBG fraction volume in OCP plasma suggests that the OCP does not induce an alteration in glycosylation in the same way as pregnancy has been shown to induce a shift to tri-antennary glycosylation (Avvakumov & Strel'chyonok, 1987)

There was limited time available for the completion of these experiments, thus the results were not as optimal as they could have been. The results of the experiments conducted for this section would have been strengthened by the inclusion of Con A chromatography controls in order to demonstrate the optimal workings of the method. Furthermore, the inclusion of results of experiments comparing different Con A to plasma ratios (2.5:1 and 7.5:1), and more 2D-E gels demonstrating the difference between the CAN-CBG and CAB-CBG fractions at different stages of pregnancy and non-OCP controls.

High-resolution separation of affinity chromatographic fractions from Con A treated pregnancy plasma has not previously been documented and this is a novel finding, demonstrating that the chromatographic fractions are indeed heterogeneous in terms of MW and perhaps pI (spot sloping referred to in chapter 3), further indicating the complexity of pregnancy induced CBG changes. The hypothesis that Con A affinity chromatography would confirm the presence of pregnancy specific CBG glycoforms in 36 week gestation plasma is accepted as the pregnancy glycoforms were visualised using 2D-E and Western blotting. Furthermore, the presence of a CAN-CBG fraction of reduced volume of in the OCP plasma sample indicated that oestrogen alone is not the only contributing physiological factor to the appearance of this variant of CBG during pregnancy as this is a very dynamic and complex physiological event.

CHAPTER 5

DISCUSSION

5.1 DISCUSSION: SIGNIFICANCE OF STUDY FINDINGS

5.1.1 PREGNANCY AND THE OCP ALTER THE CBG 2D-E PROFILE

5.1.1a CBG glycoforms specific to pregnancy vary with gestational age

This thesis reports for the first time, the application of 2D-E with western blotting for the analysis of CBG glycoforms in pregnancy and control plasma samples. Compared with previous results, for instance, where 8 individual bands were reported after 1D IEF of plasma from a normal individual (Figure 5.1A, lanes 1 and 2) (Hammond, 1990); the 2D-E experiments I conducted produced high resolution CBG profiles containing 9-11 elongated ellipsoid spots in a diagonal profile (example profile Figure 5.1B) for control samples and up to 16 spots for the pregnancy samples.

Figure 5.1B demonstrates a range in both pI and MW and Figures 3.8.1-3.10.10 demonstrate a range of pregnancy sample analyses from early (3 weeks) through to late (36 weeks) pregnancy. Hence, the hypothesis that CBG glycoforms change in relation to gestational age is accepted, and the data demonstrate that the difference in CBG glycosylation between mid to late pregnancy and control was significant.

Pregnancy-induced CBG glycosylation changes have previously been defined as a shift to solely tri-antennary glycosylation with associated sialylation increases (Avvakumov & Strel'chyonok, 1987). In this thesis any change in glycosylation was defined as a shift in the CBG profile towards greater acidity (represented by the CMG, as discussed in chapter 3) and appearance of new acidic glycoforms not present in the control samples.

The CBG glycosylation heterogeneity previously reported in the literature (Avvakumov & Strel'chyonok, 1987; Strel'chyonok *et al*, 1984) is evident as both a range of pI and apparent MW in the representative CBG 2D-E profiles in chapter 3 and 4. There was a difference in CBG glycoform retardation in 10% SDS-PAGE gels as the higher pI glycoforms travelled further than the most acidic and this may have been due to variable terminal branching along with variable sialylation, which may have contributed to the pI differences. The glycosylation changes observed in pregnancy plasma appeared (in some subjects) in early pregnancy peaking in mid to late pregnancy, in agreement with previous studies (Avvakumov & Strel'chyonok, 1987; Strel'chyonok *et al*, 1984) where it was found that the pregnancy associated variant (referred to here as con A non-binding or CAN-CBG) in the plasma of some women as early as the first month of pregnancy.

The glycosylation and acidity changes observed in the CBG 2D-E glycoform profiles during pregnancy were in keeping with previous findings (Avvakumov & Strel'chyonok, 1987; Strel'chyonok *et al*, 1984). Furthermore, the experiments detailed in chapter 4 confirm the existence of the previously documented "pregnancy-associated CBG variant" (Strel'chyonok *et al*, 1984), CAN-CBG variant, and the combination of 2D-E and affinity chromatography has extended the description of CBG during pregnancy.

The CAN-CBG variant, is readily separable from pregnancy plasma based on its unique inability to bind con A due to a higher degree of tri-antennary glycosylation (possibly 3 tri-antennary glycosylations, compared with 2; see Figure 1.2) and comprises 15% of total CBG during pregnant plasma. The other subsets of CBG glycoforms which bind con A (con A binding CBG or CAB-CBG) comprise the remaining 85% of CBG present in pregnant plasma. CAB-CBG has a slightly lower MW than CAN-CBG, indicating this variant possesses a similar glycosylation profile to normal non-physiologically altered CBG (NNA-CBG) with a 3 to 2 bi- to tri-antennary.

A novel observation was made during 1D-E testing of pregnancy CBG that the CAN-CBG variant appeared to run as a doublet on SDS-PAGE gels. This finding was in direct contrast to a previous report (Avvakumov & Strel'chyonok, 1987) where it was found that CBG from non-pregnancy plasma demonstrated a doublet band pattern in SDS-PAGE due to mixed bi- and tri-antennary glycosylation. However, the doublet observed here for the CAN-CBG may indicate the presence of a variant with a slightly different glycosylation profile, or in fact it was not completely all CAN-CBG. It is possible too that all six N-linked glycosylation sites have tri-antennary glycosylation in a sub-fraction of CAN-CBG variant or some positions are *tetra*-antennary glycosylation (Avvakumov & Strel'chyonok, 1985b).

The other novel finding of this study was the appearance of two highly acidic CBG glycoforms in mid to late pregnancy plasma which were not evident in control plasma, possibly demonstrating the validity of the high resolution nature of the current experiments facilitating a more detailed description of the CBG glycosylation profile during pregnancy than was possible previously (see Figures 5.1a and b above for comparison). In addition the change in CBG 2D-E profile of CAN-CBG substantially overlaps with that of CAB-CBG, in fact they are quite similar except for the highly acidic glycoforms only seen in CAN-CBG.

5.1.1b Pregnancy associated physiology related to CBG glycosylation changes

It is postulated that the increased oestrogen levels observed during pregnancy (Braunstein, 1980) may not be the only physiological factor influencing the alterations in CBG glycosylation observed here and previously (Strel'chyonok *et al*, 1984). The complex nature of pregnancy and the associated hormonal and physiological alterations in the maternal circulation may all play a part in CBG glycosylation alteration. Furthermore, it appears that alterations in CBG glycosylation may be associated with increases in CBG biosynthesis and this has been demonstrated to be the case in foetal sheep (Berdusco, 1993). However, it may be possible too that the CBG synthesised during pregnancy persists in the circulation longer due to reduced degradation by the associated liver enzymes.

Investigations of plasma samples in Dr Bagley's lab by 2D-E and Western blotting from septic shock patients were unable to demonstrate discernable alterations in CBG glycosylation. Conditions of physiological stress (including septic shock) are known to decrease the concentration of CBG in the circulation by up to 50% (Bernier *et al.*, 1998; Garrel *et al.*, 1993; Garrel, 1996; Hammond *et al*, 1990), and the CBG that is produced may be normally glycosylated. Thus the latter indicated that the reduction in circulating CBG may be due to increased degradation and not increased biosynthesis, as biosynthesis would involve glycosylation changes. Indeed this may further support the proposition of increased CBG biosynthesis, especially in the presence of increased oestrogen, being associated with altered glycosylation.

Cortisol levels have been documented to increase during pregnancy (Carr *et al*, 1981). Furthermore, Beitins *et al*, (1973) demonstrated a lower cortisol metabolic clearance rate (MCR) for active or free cortisol during pregnancy, and it is suggested that CBG may play a role as a protector of cortisol from metabolism as well as a plasma transporter (Benassayag *et al*, 2001). The reduced cortisol MCR may be related to the decreased rate of CBG catabolism especially the CAN-CBG variant in pregnancy plasma leading to an increased plasma half-life. Furthermore, the altered glycosylation of CBG during pregnancy may significantly extend the half-life of the glycoprotein up to day 40 after parturition and it may even persist for up to 8 weeks (Potter *et al*, 1987; Strel'chyonok & Avvakumov, 1991). The increased half-life of CAN-CBG may be related to the reduced rate of CBG degradation post partum and not the site of synthesis, and the mechanism(s) behind the prolonged elevation of CBG levels post partum are yet to be determined.

5.1.1c The OCP causes CBG glycosylation changes that only partially mimic pregnancy-induced changes

The profile changes observed for the OCP subjects are novel as these are the first documented demonstrations of high resolution CBG 2D-E profiles in plasma from women taking the OCP. There have been several studies investigating the levels of CBG associated with oral oestrogen treatment (Coenen *et al.*, 1996; Hammerstein *et al.*, 1993; Humpel *et al.*, 1990; van, V *et al.*, 1989; Wiegratz *et al.*, 1995; Wiegratz *et al.*, 2003e), however, there has been no documentation of the effects of OCP use on the CBG glycosylation profile.

The current study demonstrated a shift towards greater acidity compared with control as well as the appearance of new highly acidic glycoforms compared with control, however, there is no CAN-CBG like variant in OCP plasma (see Figure 4.2). The presence of low pI CBG glycoforms in OCP plasma may have been due other acidifying events including deamidation, other PTMs not associated with alterations or increases in CBG glycosylation. This result further increases the possibility that oestrogen alone may not be the main cause of pregnancy induced CBG glycosylation changes, as it may only increase siallylation without affecting the branching significantly.

5.2 CONCLUSIONS AND FUTURE CONSIDERATIONS

Two-dimensional electrophoresis is a useful and powerful tool in proteomics, as the technology has allowed the separation of plasma CBG glycoforms during pregnancy and for the first time with the use of the OCP. The introduction of IPG strips for the first dimension has enabled easier and more improved sample preparation although it is only possible to load one sample per gel, the second dimension step is time consuming and cumbersome. Furthermore, post separation analysis is the most important part of 2D-E and there are three main methods, staining for total protein, gel excision of the protein of interest followed by mass spectrometric (MS) analysis or immunoblotting using compatible antibodies. Although highly specific, immunoblotting is a time consuming process and it is difficult to conduct further experiments on the samples once membranes have been probed.

2D-E gels are usually stained for quantitative analysis using computer image analysis programs (eg PDQuest). However, as separation of the CBG from plasma for the current experiments was not possible, Western blotting was the method of choice for visualising the protein; and the image analysis programs were not originally designed for blot analysis. Thus data analysis for the current experiments was difficult as CBG has a unique profile and the 2D image analysis programs available are mainly designed for the analysis of standard 2D-E profiles.

This study has highlighted the need for more investigation into the use of internal standards with 2D-E, particularly in relation to Western blotting and as their utility was demonstrated in glycoprotein glycoform identification. The analysis may have been enhanced if the IS was compatible with the antibodies used for probing for CBG as the digitised images would have contained the exact position of the OVA, thus not being subject to possible human error in the process of transference from the membrane to the film.

The nature of the 2D-E method has enabled more detailed analysis of the CBG glycoforms than 1D-E as it was possible to assess both MW and charge heterogeneity. Furthermore, 2D-E analysis of glycoproteins is becoming useful in following any PTM alterations associated with proteins in development, differentiation and disease. Glycosylation pattern differences may be used as markers for disease states (Packer *et al.*, 1997b) and now for investigation of the progression of gestation and other physiological effects of OCP use, not previously investigated.

Despite the novel findings documented in the current study there are some limitations associated with the methodology. One of the limitations affecting the analysis was the fact that the more acidic CBG glycoforms were not completely resolved especially near the very low pH end of the IPG strips, regardless of the use of narrow pH range strips. The result is that some of the very low pI glycoforms were not completely resolved leading to spot blurring and these spots being analysed as one. This is a common problem associated with IEF of highly acidic proteins and the resolution of glycoforms depends inversely upon the ability of neighbouring acidic residues to buffer each other (in this case sialic acid).

Furthermore, excision (from stained gels of) low pI glycoforms from CBG and similar profiles may be difficult due to this lack of resolution, although, longer IPG strips may have reduced this effect, as the glycoforms would spread more.

This thesis reports the utility of 2D-E as a high resolution method for the study of glycoproteins in the general. Furthermore the Western blotting technique has been demonstrated as a useful method for illustrating glycoproteins in a complex mixture such as plasma especially when purification methods are not readily accessible.

2D-E is a very effective method for the analysis of glycoproteins in order to investigate MW and charge heterogeneity especially where alterations in glycosylation are suspected or previously proven. Post separation methods such as MS may then be applied to further characterise these alterations.

REFERENCES

Ain, K. B. & Refetoff, S. (1988). Relationship of oligosaccharide modification to the cause of serum thyroxine-binding globulin excess. J Clin Endocrinol Metab 66, 1073-1043.

Ali, S. & Baseett, J.R. (1995). Studies on the role of glycosylation in the origin of the electrophoretic variants for rat corticosteroid binding globulin. Steroid, 60, 743-52.

Allen, H. J. & Kisailus, E. C. (1992). Glycoconjugates: A composition, structure and function. New York: Marcel Dekker.

Aron, D., Findling, J., & Tyrell, B. (2001). Glucocorticoids and Adrenal adreogens. edited by F. Greenspan & D. Gardener, pp. 334-376. New York: McGraw-Hill.

Avvakumov, G. V. (1991). Membrane recognition of steroid-glycoprotein complexes: a model for steroid delivery to the target cells, Lectins and Cancer, edited by H.-J. Gabius & S. Gabius, pp. 263-272. Heidelberg: Springer-Verlag.

Avvakumov, G. V. (1995). Structure and function of corticosteroid-binding globulin: role of carbohydrates. J Steroid Biochem Mol Biol 53, 515-522.

Avvakumov, G. V. & Hammond, G. L. (1994). Glycosylation of human corticosteroid-binding globulin. Differential processing and significance of carbohydrate chains at individual sites. Biochemistry 33, 5759-5765.

Avvakumov, G. V. & Strel'chyonok, O. A. (1987). Properties and serum levels of pregnancy-associated variant of human transcortin. Biochemistry 925, 11-16.

Avvakumov, G. V. & Strel'chyonok, O. A. (1985). Methylation analysis in glycoprotein chemistry: a comparative study of the carbohydrate structures of three hormone-binding glycoproteins from human serum. Carbohydr. Res. 138, 91-98.

Avvakumov, G. V. & Strel'chyonok, O. A. (1988). Evidence for the involvement of the transcortin carbohydrate moiety in the glycoprotein interaction with the plasma membrane of human placental syncytiotrophoblast. Biochim Biophys Acta 938, 1-6.

Avvakumov, G. V., Warmels-Rodeniser, S., & Hammond, G. L. (1993). Glycosylation of Human Corticosteroid-binding Globulin at Asparagine 238 Is necessary for Steroid Binding. J Biol Chem 268, 862-866.

Ballard, P. L. (1979). Delivery and transport of glucocorticoids to target cells, Glucocorticoid hormone action, edited by J. D. Baxter & G. Rousseau, New York: Springer-Verlag.

Beitins, I. Z., Bayard, F., Ances, I. G., Kowarslo, A., & Migeon, C. J. (1973). The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy and near term. Pediatr Res 7, 509-519.

Benassayag, C., Souski, I., Mignot, T. M., Robert, B., Hassid, J., Duc-Goiran, P., Mondon, F., Rebourcet, R., Dehennin, L., Nunez, E. A., & Ferre, F. (2001). Corticosteroid-binding globulin status at the fetomaternal interface during human term pregnancy. Biol Reprod. 64, 812-821.

Berdusco, E. T., Jacobs, R. A., Grolla, A., Akagi, K., Langlois, D., & Challis, J. R. (1993). Glucocorticoid-induced increase in plasma corticosteroid-binding globulin levels in fetal sheep is associated with increased biosynthesis and alterations in glycosylation. Endocrinology 132, 2001-2008.

Bergstrom, M., Nilsson, M., Isaksson, R., Ryden, I., Pahlsson, P., & Ohlson, S. (2004). Lectin affinity capillary electrophoresis in glycoform analysis applying the partial filling technique. J Chromatogr. B Analyt. Technol. Biomed. Life Sci 809, 323-329.

Bernier, J., Jobin, N., Emptoz-Bonneton, A., Pugeat, M., & Garrel, D. (1998). Decreased corticosteroid binding globulin in burn patients: Relationship with interleukin-6 and fat in nutritional support. Crit Care Med 26, 456-460.

Bernutz, C., Hansle, W. O., Horn, K., Pickardt, C. R., Scriba, P. C., Fink, E., Kolb, H., & Tschesche, H. (1979). Isolation, characterization and radioimmunoassay of corticosteroid-binding globulin (CBG) in human serum -- clinical significance and comparison to thyroxine-binding globulin (TBG). Acta Endocrinol. (Copenh) 92, 370-384.

BIORAD, 2-D Electrophoresis for Proteomics A Methods and Product Manual, Bio-Rad Laboratories, Inc., Basel, Switzerland, ProteomeWorksSystem.com.

Blithe, D. L., Khan, M. S., & Rosner, W. (1992). Comparison of the carbohydrate composition of rat and human corticosteroid-binding globulin: species specific glycosylation. J Steroid Biochem Mol. Biol 42, 475-478.

Braunstein, G. D. (1980). Endocinre Changes in pregnancy, Endocrinology and the Life span, pp. 795-807.

Breuner, C. W. & Orchinik, M. (2002). Plasma binding protein as mediators of corticosteroid action in vertebrates. J Endocrinol 175, 99-112.

Brien, T. G. (1981). Human corticosteroid binding globulin. Clin. Endocrinol. (Oxf) 14, 193-212.

Carr, B. R., Parker, C., Madden, J., MacDonald, P., & Porter, J. (1981). Maternal plasma adrenocorticotropin and cortisol relationships throughout human pregnancy. Am J Obstet Gynecol 139, 416-422.

Cerven E, Ronquist G, Rimsten A, & Agren G (1981). The use of Con A sepharose as an affinity absorbent in a simple assay of serum sialyl and fucosyltransterase and its application in tumour diagnosis. Upsala J Med Si 86, 237-247.

Challis, J. R., Bassett, N., Berdusco, E. T., Han, V. K., Lu, F., Riley, S. C. , Yang, K., (1993). Foetal endocrine maturation. Equine Vet J Suppl. Apr;(14):35-40.

Challis, J. R. G. & Bennett, M. J. (1977). Cortisol binding in human amniotic fluid. Am J Obstet Gynecol, 129, 655-661.

Charlwood, J., Bryant, D., Skehel, J. M., & Camilleri, P. (2001). Analysis of N-linked oligosaccharides: progress towards the characterisation of glycoprotein-linked carbohydrates. Biomol. Eng 18, 229-240.

Clark, P. M., Hindmarsh, P., Shell, A., Law, C., Honour, JW., & Barker, D. J. P. (1996). Size at birth and adrenocortical function in children. Clin Endocrinol (Oxf) 45, 721-726.

Coenen, C. M., Thomas, C. M., Borm, G. F., Hollanders, J. M., & Rolland, R. (1996). Changes in androgens during treatment with four low-dose contraceptives. Contraception 53, 171-176.

Conrad, C. C., Malakowsky, C. A., Talent, J., Rong, D., Lakdawala, S., & Gracy, R. W. (2001). Chemiluminescent standards for quantitative comparison of two-dimensional electrophoresis western blots. Proteomics 1, 365-369.

Daughaday, W. H. (1958). Binding of corticosteroids by plasma proteins. III. The binding of corticosteroid and related hormones by human plasma and plasma protein fractions as measured by equilibrium dialysis. J Clin. Invest 37, 511-518.

de Fenci, M. & Tulchinsky, D. (1975). Total cortisol in amniotic fluid and fetal lung maturation. N. Engl. J Med 292, 133-136.

de Jong G & et al (1992). Adaptation of transferin protein and glycan synthesis. Clin Chim Acta 212, 27-45.

Dell, A. & Morris, H. R. (2001). Glycoprotein structure determination by mass spectroscopy. Science 291, 2351-2356.

Demey-Ponsart, E., Foidart, J., Solon, J., & Sodayez, J. (1982). Serum CBG free and total cortisol and circadian patterns of adrenal function in normal pregnancy. J. Steroid Biochem. 16, 169-165.

Dennis, J. W., Gramovsky, M., & Warren, C. E. (1999). Protein glycosylation in development and disease. BIoessays 21, 412-421.

Doe, R. P., FERNANDEZ, R., & Seal, U. (1964). Measurement of corticosteroid-binding globulin in man. J Clin. Endocrinol. Metab 24:1029-39., 1029-1039.

Economides, D. L., Nicolaides, K., Linton, E., Perry, L., & Chard, T. (1988). Plasma cortisol and adrenocorticotrophin in approximated and small for gestational age fetuses. Fetal Ther 3, 158-164.

Garrel, D. (1996). Corticosteroid-binding globulin during inflammation and burn injury; Nutritional modulation and clinical implications. Horm Res 45, 245-251.

Garrel, D., Zhang, L., Zhao, X., & et al (1993). Effect of burn injury on corticosteroid levels in plasma and wound fluid. Wound Repair Regen 102, 411-419.

Hammerstein, J., Daume, E., Simon, A., Winkler, U. H., Schindler, A. E., Back, D. J., Ward, S., & Neiss, A. (1993). Influence of gestodene and desogestrel as components of low-dose oral contraceptives on the pharmacokinetics of ethinyl estradiol (EE2), on serum CBG and on urinary cortisol and 6 beta-hydroxycortisol. Contraception 47, 263-281.

Hammond, G. L. (1990). Molecular properties of corticosteroid binding globulin and the sex steroid binding protein. Endocr Rev 11, 79.

Hammond, G. L., Smith, C. L., Goping, I., Unerhill, D., Harley, M., Reventon, J., Musto, N., Gunsalus, G., & Bardin, C. W. (1987). Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs, exhibits homology with serine protease inhibitors. Proc Nat Acad Sci USA, 84, 5153-5157.

Hammond, G. L., Smith, C. L., Paterson, N. A. M., & Sibbald, W. J. (1990). A role for the corticosteroid binding globulin in delivery of cortisol to activated neutrophils. J Clin Endocrinol Metab 71, 34-39.

Helenius, A. & Aebi, M. (2001). Intracellular functions of N-linked glycans. Science 291, 2364-2369.

Heyns, W. & Coolens, J. L. (1988). Physiology of corticosteroid-binding globulin in humans. Ann. N. Y. Acad Sci 538:122-9., 122-129.

Hryb, D., Khan, M., Romas, N., & Rosner W. (1986). Specific binding of human corticosteroid binding globulin to cell membranes. Proc Nat Acad Sci USA, 83, 3253-3256.

Humpel, M., Tuber, U., Kuhnz, W., Pfeffer, M., Brill, K., Heithecker, R., Louton, T., & Steinberg, B. (1990). Comparison of serum ethinyl estradiol, sex-hormone-binding globulin, corticoid-binding globulin and cortisol levels in women using two low-dose combined oral contraceptives. Horm. Res 33, 35-39.

Huntington, J. A. & Stein, P. E. (2001). Structure and properties of ovalbumin. J Chromatogr. B Biomed. Sci Appl. 756, 189-198.

Ishihara, H., Takahashi, N., Ito, J., Takeuchi, E., & Tejima, S. (1981). Either high-mannose-type or hybrid-type oligosaccharide is linked to the same asparagine residue in ovalbumin. Biochim Biophys Acta 669, 216-221.

Kato, E., Hsu, B., & Kuhn, RW. (1988). Comparative structural analysis of corticosteroid binding globulin. J Steroid Biochem 29, 213-220.

Kirk, L. F., Jr., Hash, R. B., Katner, H. P., & Jones, T. (2000). Cushing's disease: clinical manifestations and diagnostic evaluation. Am. Fam. Physician 62, 1119-4.

Kobata, A. (1992). Structures and functions of the sugar chains of glycoproteins. Eur j Biochem 209, 483-501.

Kornfeld, R. & Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem 54:631-64., 631-664.

Kuster, B., Krogh, T. N., Mortz, E., & Harvey, D. J. (2001). Glycosylation analysis of gel-separated proteins. Proteomics 1, 350-361.

Larsson-Cohn, U., Fahraeus, L., Wallentin, L., & Zador, G. (1982). Effects of the estrogenicity of levonorgestrel/ethinylestradiol combinations of the lipoprotein status. Acta Obstet. Gynecol. Scand. Suppl 105:37-40., 37-40.

Le Gaillard, F., Han, K. K., & Dautrevaux, M. (1975). Characterization and physico-chemical properties of human transcortin. Biochimie 57, 559-568.

Lis, H. & Sharon, N. (1993). Protein glycosylation: Structural and functional aspects. Eur j Biochem 218, 1-27.

Liu, T., Li, J. D., Zeng, R., Shao, X. X., Wang, K. Y., & Xia, Q. C. (2001). Capillary electrophoresis-electrospray mass spectrometry for the characterization of high-mannose-type N-glycosylation and differential oxidation in glycoproteins by charge reversal and protease/glycosidase digestion. Anal. Chem 73, 5875-5885.

Lopez-Soto-Yarritu, P., ez-Masa, J. C., Cifuentes, A., & de, F. M. (2002). Improved capillary isoelectric focusing method for recombinant erythropoietin analysis. J Chromatogr. A 968, 221-228.

MacCoss, M. J., McDonald, W. H., Saraf, A., Sadygov, R., Clark, J. M., Tasto, J. J., Gould, K. L., Wolters, D., Washburn, M., Weiss, A., Clark, J. I., & Yates, J. R., III (2002). Shotgun identification of protein modifications from protein complexes and lens tissue. Proc Nat Acad Sci USA 99, 7900-7905.

Magiakou, M. A., Mastorakos, G., Rabin, D., Margioris, A. N., Dubbert, B., Calogero, A. E., Tsigos, C., Munson, P. J., & Chrousos, G. P. (1996). The maternal hypothalamic-pituitary-adrenal axis in the third trimester of human pregnancy. Clin. Endocrinol. (Oxf) 44, 419-428.

Mickelson, K. E., Harding, G. B., Forsthoefel, M., & Westphal, U. (1982). Steroid-protein interactions. Human corticosteroid-binding globulin: characterization of dimer and electrophoretic variants. Biochemistry 21, 654-660.

Milstein, C. P. (1968). An application of diagonal electrophoresis to the selective purification of serine phosphate peptides. Serine phosphate peptides from ovalbumin. Biochem J 110, 127-134.

Misao, R., Iwagaki, S., Sun, W., Fujimoto, J., Saio, M., Takami, T., & Tamaya, T. (1999). Evidence for the synthesis of corticosteroid binding globulin in human placenta, 1999; 51: 162-7. Horm Res 51, 162-167.

Misao, R., Nakanishi, Y., Fujimoto, J., Ichigo, S., Hori, M., & Tamaya, T. (1995). Expression of corticosteroid binding globulin mRNA in human uterine endometrial cancers. Steroids 60, 720-724.

Mitchell, E., Torpy, D. J., & Bagley, C. J. (2004). Pregnancy-associated corticosteroid-binding globulin: high resolution separation of glycan isoforms. Horm. Metab Res. 36, 357-359

Moore, D. E., Kawagoe, S., Davajan, V., Nakamura, R. M., & Mishell, D. R. (1978). An in vivo system in man for quantitation of estrogenicity. II. Pharmacologic changes in binding capacity of serum corticosteroid-binding globulin induced by conjugated estrogens, mestranol, and ethinyl estradiol. Am. J Obstet. Gynecol. 130, 482-486.

Nakamura, R. M., Tanaka, K., & Murakawa, S. (1960). Specific protein of legumes which reacts with animal proteins. Nature 188, 144-145.

Nisbet, A. D., Saundry, R. H., Moir, A. J., Fothergill, L. A., & Fothergill, J. E. (1981). The complete amino-acid sequence of hen ovalbumin. Eur j Biochem 115, 335-345.

Nolten, W., Linheimer, M., Rueckert, P., Oparil, S., & Ehrlich, E. N. (1980). Diurnal patterns and regulation of cortisol secretion in pregnancy. J Clin Endocrinol Metab 51, 466-472.

O'Connell, M. & Welsh, G. (1969). Unbound plasma cortisol in pregnant and Enovid-E treated women as determined by ultrafiltration. J Clin Endocrinol Metab 29.

O'Gray, G., Rundle, S., Leavitt, W. W., (1987). Purification and partial characterization of a corticosteroid-binding globulin from hamster serum. Biochim Biophys Acta. Oct 8;926(1):40-53.

Packer, N. H., Pawlak, A., Kett, W. C., Gooley, A. A., Redmond, J. W., & Williams, K. L. (1997). Proteome analysis of glycoforms: a review of strategies for the microcharacterisation of glycoproteins separated by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis 18, 452-460.

Pan, T., Colucci, M., Wong, B. S., Li, R., Liu, T., Petersen, R. B., Chen, S., Gambetti, P., & Sy, M. S. (2001). Novel differences between two human prion strains revealed by two-dimensional gel electrophoresis. J Biol Chem 276, 37284-37288.

Pemberton, P., Stein, P., Pepys, M., Potter, J., & Carrell, R. W. (1988). Hormone binding globulins undergo serpin conformational change in inflammation. Nature 336, 257-258.

Pepe, G. J. & Albrecht, E. D. (1995). Actions of placental and fetal adrenal steroid hormones in primate pregnancy. Endocr. Rev. 16, 608-648.

Perlman, G. E. (1952). Enzymatic dephosphorylation of ovalbumin and plakalbumin. J Gen. Physiol 35, 711-726.

Potter, J., Mueller, U., Hickman, P., & Micheal, C. (1987). Corticosteroid binding globulin in normotensive and hypertensive human pregnancy. Clin Sci 72, 725-735.

Rabin, M., Watson, M., Kidd, V., Woo, S. L., Breg, W. R., & Ruddle, F. H. (1986). Regional location of alpha 1-antichymotrypsin and alpha 1-antitrypsin genes on human chromosome 14. Somat. Cell Mol. Genet. 12, 209-214.

Raynes J (1982). Variation in the relative proportion of microheterigeneous forms of plasma proteins in pregnancy and disease. Biomed Pharmacol 39, 77-86.

Rosner, W. (1990). The function of corticosteroid binding globulin and sex-hormone binding globulin recent advances. Endocr Rev, 11, 80-91.

Sandberg, A. A., Woodruff, M., Rosenthal, H., Nienhous, S., & Slaunwhile, W. R. (1964). Transcortin: A corticosteroid-binding protein of plasma. VII. Half-life in normal and estrogen treated subjects. J Clin Invest 43, 461-466.

Schroeder, W. T., Miller, M. F., Woo, S. L., & Saunders, G. F. (1985). Chromosomal localization of the human alpha 1-antitrypsin gene (PI) to 14q31-32. Am. J Hum. Genet. 37, 868-872.

Schwartz, U., Volger, H., Schneller, E., Moltz, L., & Hammerstein, J. (1983). Effects of various replacement oestrogens on hepatic transcortin synthesis in climacteric women. Acta Endocrinol. (Copenh) 102, 103-106.

Scrocchi, L., Orava, M., Smith, C. L., Han, V., & Hammond, G. L. (1993). Spatial and temporal distribution of corticosteroid binding globulin and its messenger ribonucleaic acid in embryonic and fetal mice. Endocrinology 132, 903-909.

Seal, U. & Doe, R. P. (1962). Corticosteroid-binding globulin. I. Isolation from plasma of diethylstilbestrol-treated men. J Biol Chem 237:3136-40., 3136-3140.

Seralini, G. E., Berude, D., Gagne, R., & Hammond, G. L. (1990). The human corticosteroid binding globulin gene is located on chromosome 14p31-q32.1 near two other serine protease inhibitor gene. Hum Genet, 86, 73-75.

Stolk, R. P., Lamberts, S. W., de Jong, F. H., Pols, H. A., & Grobbee, D. E. (1996). Gender differences in the associations between cortisol and insulin in healthy subjects. J Endocrinol 149, 313-318.

Strel'chyonok OA, Avvakumov, G. V., & Akhrem, A. A. (1984). Pregnancy associated molecular variants of human serum transcortin and thyroxine-binding globulin. Carbohydrate Research 134, 133-140.

Strel'chyonok, O. A. & Avvakumov, G. V. (1990). Specific steroid-binding glycoproteins of human blood plasma: novel data on their structure and function. J. Steroid Biochem. 35, 519-534.

Strel'chyonok, O. A. & Avvakumov, G. V. (1991). Interaction of human CBG with cell membranes. J Steroid Biochem Mol Biol 40, 795-803.

Strel'chyonok, O. A., Avvakumov, G. V., & Akhrem, A. A. (1984). Pregnancy-associated molecular variants of human serum transcortin and thyroxine-binding globulin. Carbohydr. Res. 134, 133-140.

Strel'chyonok, O. A., Avvakumov, G. V., Matveentseva, I. V., Akhrem, L. V., & Akhrem, A. A. (1982). Isolation and characterization of glycopeptides of human transcortin. Biochim Biophys Acta 705, 167-173.

Suzuki, T., Kitajima, K., Emori, Y., Inoue, Y., & Inoue, S. (1997). Site-specific de-N-glycosylation of diglycosylated ovalbumin in hen oviduct by endogenous peptide: N-glycanase as a quality control system for newly synthesized proteins. Proc Natl. Acad Sci U. S. A 94, 6244-6249.

Torpy, D. J., Bachmann, A. W., Grice, J., Fitzgerald, S., Phillips, P., Whitworth, J., & Jackson, R. (2001). Familial corticosteroid-binding globulin deficiency due to a novel null mutation: association with fatigue and relative hypotension. J Clin Endocrinol Metab 86, 3692-3700.

Torpy, D. J. & Chrousos, G. P. (1997). General adaptation syndrome: An overview. J Intens Care Med 12, 125-238.

Underhill, D. & Hammond, G. L. (1989). Organisation of the human corticosteroid binding globulin gene and analysis of its 5'-flanking region. Molec Endocrinol 3, 1448-1454.

Van Baelen, H. V., Brepoels, R., & Demor, P. (1982). Transcortin Leuven: A variant of human corticosteroid binding globulin with decreased cortisol binding affinity. Molec Chem 257, 3397-3400.

van, d., V, van der, B. H., Kloosterboer, H. J., & Haspels, A. A. (1989). Effects of seven low-dose combined contraceptives on vitamin B6 status. Contraception. 40, 377-384.

Wells, C., Bog-Hansen, T. C., Cooper, E. H., & Glass, M. R. (1981). The use of concanavalin A crossed immuno-affinoelectrophoresis to detect hormone-associated variations in alpha 1-acid glycoprotein. Clin. Chim. Acta 109, 59-67.

Westphal U. (1971). Steroid -protein interactions. Berlin: Springer.

Westphal, U. (1983b). Corticosteroid binding globulin. Molec Chem 55, 145-157.

Westphal, U. (1983a). Steroid-protein interaction from past to present. J. Steroid Biochem. 19, 1-15.

Westphal, U. (1986). Corticosteroid binding globulin (CBG) Steroid protein interactions II Monographs on Endocrinology. Berlin, Springer-Verlag.

Wiegratz, I., Jung-Hoffmann, C., & Kuhl, H. (1995). Effect of two oral contraceptives containing ethinylestradiol and gestodene or norgestimate upon androgen parameters and serum binding proteins. Contraception 51, 341-346.

Wiegratz, I., Kutschera, E., Lee, J. H., Moore, C., Mellinger, U., Winkler, U. H., & Kuhl, H. (2003). Effect of four oral contraceptives on thyroid hormones, adrenal and blood pressure parameters. Contraception 67, 361-366.

Wiseman, R. L., Fothergill, J. E., & Fothergill, L. A. (1972). Replacement of asparagine by aspartic acid in hen ovalbumin and a difference in immunochemical reactivity. Biochem J 127, 775-780.

Zeng, R., Xu, Q., Shao, X. X., Wang, K. Y., & Xia, Q. C. (1999). Characterization and analysis of a novel glycoprotein from snake venom using liquid chromatography-electrospray mass spectrometry and Edman degradation. Eur j Biochem 266, 352-358.

APPENDIX 1

Abbreviation	Complete text
1D-E	One Dimensional Electrophoresis
2D-E	Two Dimensional Electrophoresis
A1-PI	α1-antitripsin protease inhibitor
α-D-MMP	α-D-methylmannopyranoside
ACT	Anti-chymotrypsin
ACTH	Adrenocorticotrophic Hormone
Asn	Asparagine
Asp	Aspartic acid
CA	Carrier ampholytes
CAB-CBG	Con A binding CBG
CAN-CBG	Con A non-binding CBG
CBG	Corticosteroid binding globulin
Con A	Concanavalin A
CSP	Collated serial pregnancy data
DTT	Dithiothreitol

GlcNAc	N-acetyleglucosamine
HPA axis	Hypothalamic pituitary adrenal Axis
IEF	Isoelectric focussing
IPG	Immobilised pH Gradient
IQ	Image Quant
IS	Internal standard
MQ water	MilliQ water
NC	Nitrocelluose membrane
OCP	Oral contraceptive pill
OVA	Ovalbumin
PDQ	PD Quest
SAC	Sialic acid residues per CBG molecule

APPENDIX 2

Mitchell, E., Torpy, D. J., & Bagley, C. J. (2004). Pregnancy associated corticosteroid-binding globulin: high resolution separation of glycan isoforms. *Hormone and Metabolic Res*earch, *36*, 357-359