
Sex Hormone-Binding Globulin: Regulation and Role as a Marker of Chronic Disease Risk

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of ADELAIDE

*

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DECLARATION

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

Signature: _____

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Date: 17/07/2019

ADELAIDE, AUSTRALIA

ABSTRACT

Sex hormone-binding globulin (SHBG) is dimeric glycoprotein synthesised in hepatocytes and secreted into the circulation. SHBG binds sex-steroids and protects them from rapid degradation. SHBG is involved in the regulation of *de novo* lipogenesis in the liver. The synthesis and secretion of SHBG from the liver are regulated by hormonal, metabolic, nutritional, and genetic factors. There is increasing interest in SHBG because it is a determinant of total testosterone (TT) concentration, and maybe a marker of cardiometabolic disease risk. There are little data available from population-based studies on the determinants of serum SHBG. Furthermore, there is evidence for and against SHBG as a marker of risk for incident type 2 diabetes (T2D) and cardiovascular disease (CVD).

SHBG is also expressed in, but not secreted from the prostate. However, as in the liver, it modulates and may also be associated with lipid metabolism. Prostate cancer (PCa) is both androgen-dependent and dependent on fatty acids as an energy supply. I, therefore, hypothesised that 1) SHBG of hepatic origin may be a marked of dysregulated lipid metabolism and associated with cardiometabolic disease risk, and 2) the expression of SHBG in the prostate will be a marker of aggressive PCa and androgen ablation.

The studies that comprise this thesis aimed:

1. To determine, in a longitudinal cohort of community-dwelling men,
 - 1.1. the cross-sectional and longitudinal determinants of circulating SHBG levels;
 - 1.2. the associations of serum SHBG & sex steroids with incident T2D;
 - 1.3. the associations of serum SHBG & sex steroids with incident CVD and CVD-related mortality.

2. To test the hypothesis that SHBG expression in the prostate increased in PCa by determining the nature of the *SHBG* transcripts expressed and mRNA expression, together with SHBG protein, in normal, benign, and malignant prostate.

These studies have shown that:

1. We found that,
 - 1.1. Serum SHBG levels have a positive relationship with aging, thyroxine, and TT, and an inverse relationship with abdominal total fat mass, triglycerides, and oestradiol. My work has suggested that variation in serum SHBG reflects *de novo* lipogenesis within the liver that occurs in the context of insulin resistance.
 - 1.2. Low TT, not SHBG and other sex steroids, is independently associated with an increased risk of T2D development over a 5-year follow-up period.
 - 1.3. High SHBG and low TT, are independently associated with an increased risk of incident CVD, but not with increased risk of CVD related mortality.
2. *SHBG* mRNA and protein are increased in PCa at all stages compared with normal and with benign prostatic hyperplasia (BPH). Multiple *SHBG* transcripts of variable length were identified in the prostate. The dominant transcript varied between PCa cases and in response to androgen receptor antagonists.

Taken together, these data suggest that SHBG levels reflect the metabolic state in particular glucose, lipid and sex steroids metabolism and SHBG is a marker for incident CVD but not T2D. *SHBG* expression and transcript size may be markers for the aggressive behaviour of PCa and resistance to androgen ablation, deserves further attention in PCa studies.

DEDICATION

In loving memory of:

- *my life-coach, my late grandfather Lekh Nath Gyawali (1922-2005)*
- *my beloved sisters: Bijaya Gyawali (1981-1985) & Pratibha Gyawali (1986-2007)*

You are the determination on every page.

And

I dedicate this thesis to Professor Geoffrey L. Hammond, University of British Columbia who devoted much of his career understanding the pathobiology of SHBG.

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“Education is the leading of human souls to what is best, and making what is best of them “–John Ruskin

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LIST OF PUBLICATIONS ARISING FROM THIS THESIS

1. **Gyawali P**, Martin SA, Heilbronn LK, Vincent AD, Jenkins AJ, Januszewski AS, Taylor AW, Adams RJT, O'Loughlin PD, Wittert GA

Cross-sectional and longitudinal determinants of serum sex hormone-binding globulin (SHBG) in a cohort of community-dwelling men

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2. **Gyawali P**, Martin SA, Heilbronn LK, Vincent AD, Taylor AW, Adams RJT, O'Loughlin PD, Wittert GA

The role of sex hormone-binding globulin (SHBG), testosterone, and other sex steroids, on the development of type 2 diabetes in a cohort of community-dwelling middle-aged to elderly men

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RESEARCH PRESENTATIONS ARISING FROM THIS THESIS

1. **Gyawali P**, Butler LM, Selth LA, Vincent AD, Hanson AR, Irani S, Ryan NK, Martin SA, Heilbronn LK, Wittert GA

Sex hormone-binding globulin (SHBG) as a marker of aggressive prostate cancer

21st European Congress of Endocrinology, Lyon France

May 2019 - Poster presentation

(Recipient of the Hospital Research Foundation Travel Award)

2. Gyawali P, Martin SA, Heilbronn LK, Vincent AD, Jenkins AJ, Januszewski AS, Taylor AW, Adams RJT, O'Loughlin PD, Wittert GA

Higher serum sex hormone-binding globulin (SHBG) levels are associated with incident cardiovascular disease (CVD) but not CVD related mortality in a cohort of community-dwelling middle-aged to elderly men

92nd Annual Meeting of the Japan Endocrine Society, Sendai Japan

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3. Gyawali P, Martin SA, Heilbronn LK, Vincent AD, Jenkins AJ, Januszewski AS, Taylor AW, Adams RJT, O'Loughlin PD, **Wittert GA**

Associations of testosterone and sex hormone-binding globulin with the risk of cardiovascular disease and type 2 diabetes in Men

ENDO 2019 - Endocrine Society's Annual Meeting, New Orleans USA

March 2019 - Poster presentation

(Late-breaking abstract)

4. **Gyawali P**, Martin SA, Heilbronn LK, Vincent AD, Jenkins AJ, Januszewski AS, Taylor AW, Adams RJT, O'Loughlin PD, Wittert GA

Sex Hormone-Binding Globulin and sex steroids and the risk of type 2 diabetes and cardiovascular disease in men.

Asian Pacific Society of Cardiology Congress, Taipei Taiwan

May 2018 - Oral presentation

(In Competition for Young Investigator Award)

5. **Gyawali P**, Martin SA, Heilbronn LK, Vincent AD, Jenkins AJ, Januszewski AS, Taylor AW, Adams RJT, O'Loughlin PD, Wittert GA

Associations of sex hormone-binding globulin and testosterone with the risk of type 2 diabetes and cardiovascular disease in men.

2017 South Australian Cardiovascular Research Showcase, Adelaide Australia

October 2017 - Poster presentation

6. **Gyawali P**, Martin SA, Heilbronn LK, Wittert GA

Regulation of sex hormone-binding globulin (SHBG) and its role as a marker of fatty acid metabolism.

SAHMRI's Annual Scientific Meeting 2016, Adelaide Australia

October 2016 - Oral presentation

(In Competition for 3-minute Thesis)

7. **Gyawali P**, Martin SA, Heilbronn LK, Vincent AD, Jenkins AJ, Januszewski AS, Taylor AW, Adams RJT, O'Loughlin PD, Wittert GA

Cross-sectional and longitudinal determinants of serum sex hormone-binding globulin (SHBG) in a cohort of community-dwelling men.

20th World Meeting on Sexual Medicine, Beijing China

September 2016 - Oral presentation

(Recipient of International Society for Sexual Medicine Conference Travel Award)

8. **Gyawali P**, Martin SA, Heilbronn LK, Vincent AD, Jenkins AJ, Januszewski AS, Taylor AW, Adams RJT, O'Loughlin PD, Wittert GA

Cross-sectional and longitudinal determinants of serum sex hormone-binding globulin (SHBG) in a cohort of community-dwelling men.

Annual Meetings of the Endocrine Society of Australia and Society for Reproductive Biology and Australia and New Zealand Bone and Mineral Society 2016, Gold Coast Australia

August 2016 - Oral presentation

(Recipient of School of Medicine, the University of Adelaide, Conference Travel Award)

LIST OF ABBREVIATIONS

ATP, Adenosine triphosphate	BIA, Bioelectrical impedance analysis
ACL, ATP citrate lyase	BAT, Brown adipose tissue
ACC, Acetyl-CoA carboxylase	BMI, Body mass index
ACOX, Acyl-CoA oxidase	BP, Blood pressure
AMPK, Adenosine monophosphate-activated protein kinase	Ca ²⁺ , Calcium
AIC, Akaike's Information Criterion	CRP, Carbohydrate reactive protein
ALT, Alanine transaminase activity	cFT, Calculated free testosterone
AJCC, American Joint Committee on Cancer	cFE2, calculated free oestradiol
ATC, Anatomical Therapeutic Chemical	CVD, Cardiovascular disease
ADT, Androgen deprivation therapy	CPT I; Carnitine palmitoyltransferase I
AR, Androgen receptor	CRPC, Castration-resistant prostate cancer
A4, Androstenedione	CFM, Central fat mass
bp, base pairs	CES-D, Centre for Epidemiological Studies-Depression Scale
BDI, Beck Depression Inventory	COUP-TFI, Chicken ovalbumin upstream promoter transcription factor
BPH, Benign prostatic hyperplasia	CLIA, Chemiluminescent immunoassay
β, Standardized regression coefficients	COPD, Chronic obstructive pulmonary disease
bT, Bioavailable testosterone	CV, Coefficients of variation
BCR, Biochemical recurrence	

CATI, Computer-assisted telephone interviewing	E2, Oestradiol
CT, Computed tomography	E4, Oestetrol
CAR, Constitutive androstane receptor	ER, Oestrogen receptor
CHD, Coronary heart disease	ER α , Oestrogen receptor alpha
CBG, Corticosteroid-binding globulin	ER β , Oestrogen receptor beta
CS, Cross-sectional	EDTA, Ethylenediamine tetraacetic acid
cAMP, Cyclic adenosine monophosphate	ePLND, Extended pelvic lymph node dissection
CYP11A1, Cytochrome P-450 cholesterol side-chain cleavage enzyme	Erk-1/2, Extracellular-signal regulated kinase
DHEAS, Dehydroepiandrosterone sulphate	eSel, sE-selectin
DRE, Digital rectal examination	ECM, Extra-cellular matrix
DHT, 5 α -dihydrotestosterone	FAMAS, Florey Adelaide male aging study
DXA, Dual-energy x-ray absorptiometry scan	FA, Fatty acid
EMSA, Electrophoresis mobility shift assay	FABP-5, Fatty acid binding protein-5
eCLIA, Electrochemiluminescent immunoassay	FASN/FAS, Fatty acid synthase
EGF, Epidermal growth factor	FP, Footprint region
E1, Oestrone	FSH, Follicle stimulating hormone
E1S, Oestrone sulphate	FAI, Free androgen index
	FT, Free testosterone
	GGT, Gamma-glutamyltransferase
	GDM, Gestational diabetes mellitus

GS, Gleason score	HPG, Hypothalamic-pituitary-gonadal axis
GLP-1, Glucagon-like peptide -1	
GnRH, Gonadotrophin releasing hormone	HPA, Hypothalamus-pituitary-adrenal axis
GH, Growth hormone	IFA, Immunofluorometric assay
HRs, Hazard ratios	IFG, Impaired fasting glucose
HbA1c, Glycated hemoglobin	IR, Insulin resistance
Hep G2, Human hepatoma cells	IDF, International Diabetes Federation
HNF-4 α , Hepatocyte nuclear factor-4 alpha	IL1 β , Interleukin-1 beta
HNF- NF- κ B, Hepatocyte nuclear factor kappa B	ISUP; International Society of Urological Pathology
HFD, High fat diet	I, Interventional
HDL, High-density lipoprotein	IAD, Ischemic arterial disease
hS-CRP, High sensitivity C-reactive protein	IHD, Ischemic heart disease
HOMA, Homeostasis model assessment	IPW, Inverse probability weighting
HOMA- β , Homeostasis model assessment-beta	JNK, c-Jun N-terminal kinase
HSA, Human serum albumin	KLKs, Kallikrein-related peptidases
H-MR, Hydrogen 1 [^{1} H]) magnetic resonance (MR) spectroscopy	Ka, Association constants
HTN, Hypertension	kb, Kilobase
	kCal, Kilocalories
	IGF-1, Insulin-like growth factor-1
	LASSO, Least absolute shrinkage and selection operator

LC-MS/MS, Liquid chromatography-tandem mass spectrometry	MPO, Myeloperoxidase activity
L, Longitudinal	MI, Myocardial infarction
LDL, Low-density lipoprotein	MCF-7, Michigan cancer foundation-7
LEFM, Lower extremity fat mass	nM, Nanomolar
LH, Luteinizing hormone	NATA, National Association of testing authorities
LNCaP, Lymph node carcinoma of the prostate	NAFLD, Non-alcoholic fatty liver disease
LNI, Lymph node invasion	NAFL, Non-alcoholic fatty liver
MRI, Magnetic resonance imaging	NASH, Non-alcoholic steatohepatitis
MAILES, Men androgen inflammation lifestyle environment, and stress	NWAHS, North West Adelaide health study
mRNA, Messenger RNA	n.s., Not significant
MetS, Metabolic syndrome	NHR, Nuclear hormone receptor
MeOE2, Methoxyoestradiol	NMR, Nuclear magnetic resonance
μM, Micromolar	ORs, Odds ratios
mM, mill Mole	OGTT, Oral glucose tolerance test
MAPK, Mitogen-activated protein kinase kinase	PAD, Peripheral artery disease
MEK, MAPK kinase-1/2	PPAR _γ , Peroxisome proliferator-activated receptor gamma
MUFA, Monounsaturated fatty acid	PCOS, Polycystic ovarian syndrome
MFGRE, Multiecho fast gradient echo sequence	PUFA, Polyunsaturated fatty acid
	PCR, Polymerase chain reaction
	PCa, Prostate cancer

PSA, Prostate-specific antigen	TG, Triglyceride
PIN, Prostatic intraepithelial neoplasia	T, Testosterone
PAP, Prostatic acid phosphatase	T4, Thyroxine
PKA, Protein kinase A	TT, Total testosterone
PCT, Proximal convoluted tubule	TRUS, Transrectal ultrasonography
QUICKI, Quantitative insulin sensitivity check index	TIA, Transient ischemic attack
qRT-PCR, Real-Time Quantitative Reverse Transcription PCR	TF, Transcription factors
RIA, Radioimmunoassay	TU's, Transcription units
RSHBG, Sex hormone-binding globulin- receptor	T3, Triiodothyronine
RP, Radical prostatectomy	TG, Triglycerides
RT, Resistance training	TNF α , Tumour necrosis factor alpha
RR, Risk ratio	TNM, Tumour-node-metastasis system
RXR α , Retinoid X receptor alpha	T1D, Type 1 diabetes mellitus
SHBG, Sex hormone-binding globulin	T2D, Type 2 diabetes mellitus
SNP, Single nucleotide polymorphism	USG, Ultrasonography
StAR, Steroidogenic acute regulatory protein	USF, Upstream stimulatory factor
SAT, Subcutaneous adipose tissue	VAT, Visceral adipose tissue
TC, Total cholesterol	WC, Waist circumference
	WHR, Waist-to-hip ratio
	WHO, World Health Organization
	Zn ²⁺ , Zinc

CHAPTER 1

INTRODUCTION

1. Overview of the hypothalamic-pituitary-gonadal (HPG) axis in men

Androgens are a class of steroid hormones, synthesised from cholesterol, and are responsible for the development and maintenance of male secondary sexual characteristics and reproductive function [1, 2]. The predominant androgen of physiological importance produced by the testis and present in the circulation is testosterone (T) [1, 3]. T is biosynthesised by the action of 5 α -reductase, in a tissue-specific manner to the more potent androgen 5 α -dihydrotestosterone (DHT).

Neurosecretory cells of the hypothalamus secrete pulses of gonadotropin-releasing hormone (GnRH) which act on gonadotrophic cells in the anterior pituitary, leading to pulsatile secretions of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the bloodstream. LH binds to receptors on the surface of Leydig cells, activating adenylate cyclase and thereby causes increased production of intracellular cyclic adenosine monophosphate (cAMP). This rapid increase in cAMP results in the transport of cholesterol from the cell surface to the inner mitochondrial membrane with the involvement of steroidogenic acute regulatory protein (StAR) [1, 2]. Subsequently, the cytochrome P-450 cholesterol side-chain cleavage enzyme (CYP11A1) complex located on the inner mitochondrial membrane catalyses the conversion of cholesterol to pregnenolone and is the rate-limiting step in androgen biosynthesis. All subsequent enzymatic steps and conversion of pregnenolone into T occur in the Leydig smooth endoplasmic reticulum, and major pathways are illustrated in Figure 1.

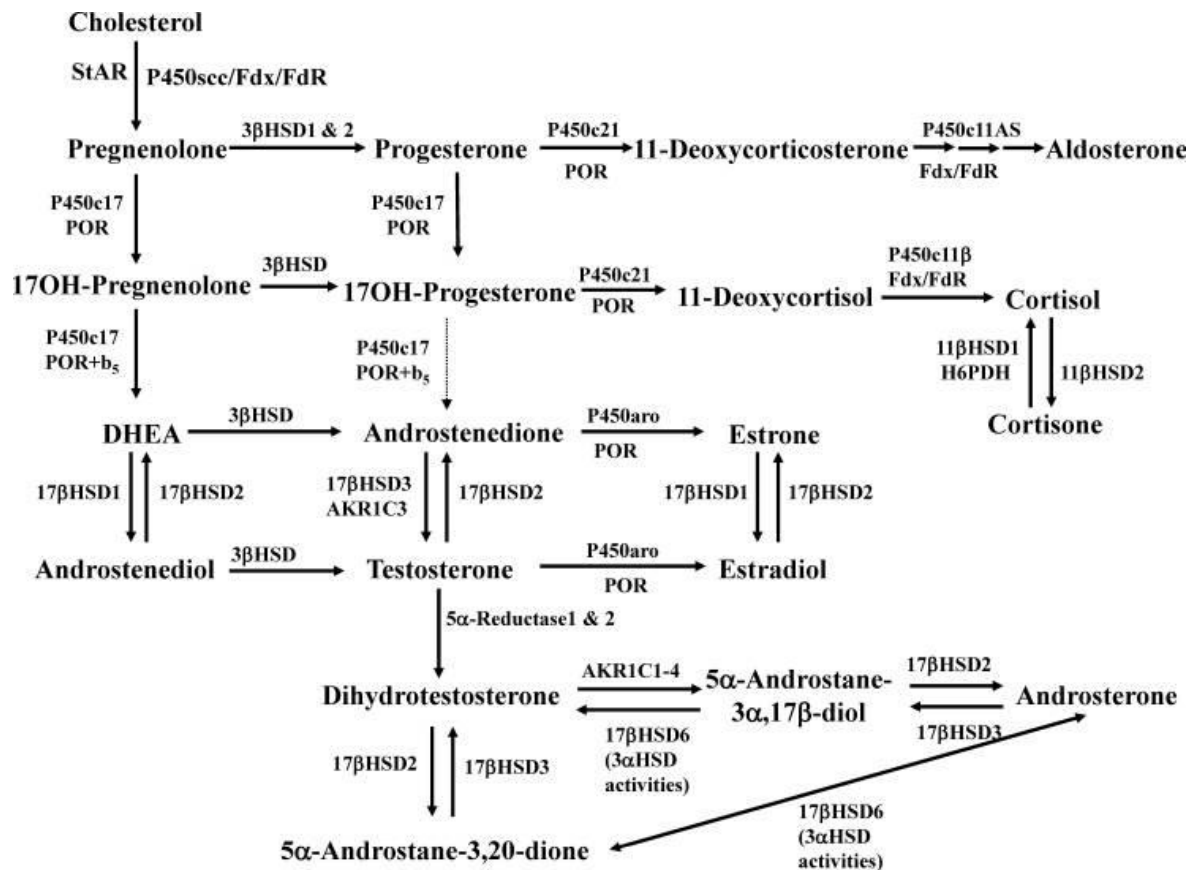


Figure 1. Major pathways of androgen biosynthesis and metabolism.

Reproduced, with permission, from Miller WL and Auchus RJ (2011) [2].

Key enzymes and cofactor proteins are shown near arrows indicating chemical reactions.

Not all intermediate steroids, pathways, and enzymes are shown.

Abbreviations: StAR, Steroidogenic acute regulatory protein; P450scc, P450 11A1, cytochrome P450, cholesterol side-chain cleavage; 3βHSD2, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase type II; POR, P450 oxidoreductase; 3β-HSD type 1 and 2, 3β-Hydroxysteroid dehydrogenase/Δ5-Δ4-isomerases; AKR1C3, Aldo-keto reductase family 1, member C3; P450arom, cytochrome P450 aromatase; 3α diol, androsterone or 3α, 17βdiol

In males of most mammalian species, 95% of circulating T is derived from testicular secretion [1, 2, 4]. The secretion of T is regulated by feed-forward and feedback mechanisms that operate within the HPG axis [1, 2] and is shown in Figure 2.

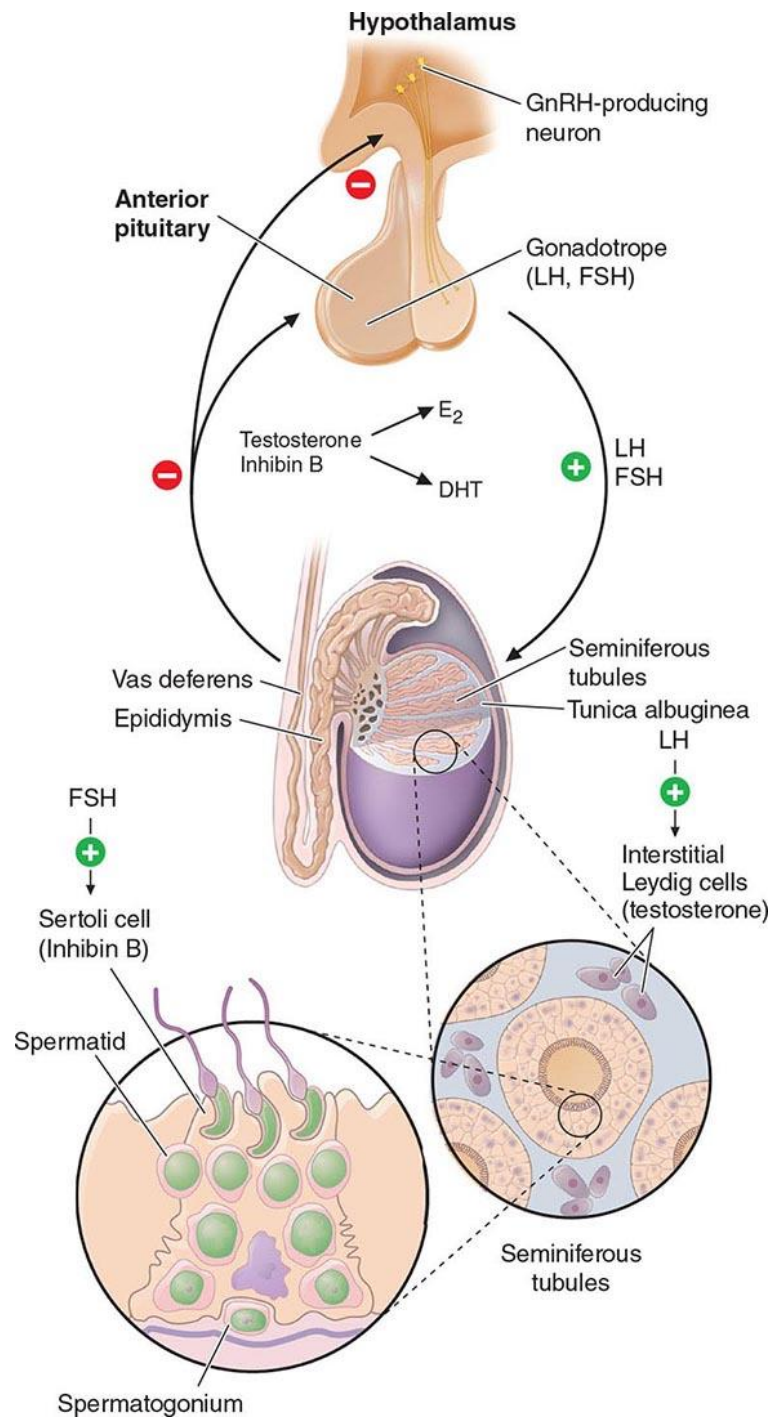


Figure 2. The HPG axis and hormonal feedback in male.

Adopted from Harrison's principles of internal medicine 19e; McGraw-Hill Companies, Inc.: www.accessmedicine.com

Abbreviations: E₂, Oestradiol; DHT, 5 α -dihydrotestosterone; FSH, Follicle-stimulating hormone; GnRH, Gonadotropin-releasing hormone; LH, Luteinizing hormone

1.1.Androgen biosynthesis and metabolism

While the adrenal glands only produce low levels of the active androgen T, they produce significant levels of the inactive androgen precursors dehydroepiandrosterone (DHEA) and its sulphated ester DHEAS and androstenedione (A4), which are released into the circulation [5]. After uptake into a peripheral tissue with the required enzyme machinery, these androgen precursors are converted into active androgens and is illustrated in Figure 1, which elicit a physiological response [6]. In human males, 3 to 10 mg of T is secreted daily by the testis; direct secretion of T by the adrenal and the peripheral conversion of DHEA, DHEAS and A4 secreted by the adrenal collectively account for another 500 µg of testosterone daily [1, 6].

Peripheral conversion

T is converted to most potent androgen, DHT by the action of 5 α -reductase. This enzymatic conversion is predominant in the epididymis and prostate, where it transduces most of its biological action [6, 7]. T can also be converted to oestradiol (E2) via the aromatase enzyme. Aromatase also converts A4 to oestrone (E4). The peripheral cells that express the key enzyme aromatase include the Sertoli cells, mature spermatocytes, osteoblasts, chondrocytes, and adipocytes [4, 8]. The regulation of these conversions is tissue specific [1, 9].

1.2.Transportation of sex steroids in blood

The hydrophobic steroids hormone are secreted from steroidogenic tissues by diffusion down a concentration gradient across cell membranes mainly into the bloodstream via capillaries, with smaller amounts appearing in the lymphatics and tubule fluid [1]. Testicular T constitute >95% of circulating T in post-pubertal male while the peripheral conversion of weak androgens constitutes the remainder of the circulating testosterone [1]. These weak androgens- DHEA, A4, and DHEA-S also comprise a large circulating reservoir of precursors for conversion to bioactive sex steroids in extragonadal tissues

including the liver, kidney, muscle, and adipose tissue [1]. T and its androgenic metabolite, DHT, exert biological effects directly through binding to the androgen receptor (AR) and indirectly through aromatisation of T to E2, which allows action via binding to the estrogen receptor (ER). The ARs and ERs are members of the steroid nuclear receptor superfamily with highly homologous structure differing mostly in the C-terminal ligand-binding domain [1].

Upon their release from steroidogenic tissues, hydrophobic steroids are transported to the target tissues through the circulatory system by binding to circulating plasma proteins. The most important is sex hormone-binding globulin (SHBG), a high-affinity but low-capacity binding protein, and other low-affinity but higher-capacity binding proteins include human serum albumin (HSA), corticosteroid-binding globulin (CBG) and Orosomucoid or α_1 acid glycoprotein as illustrated in Figure 3. SHBG possess 3 to 4 orders of magnitude greater affinity for androgens and estrogens than albumin, another carrier protein [10]. These binding proteins play an important role in regulating the transport, tissue delivery, bioactivity, and metabolism of sex-steroids [3, 11-14].

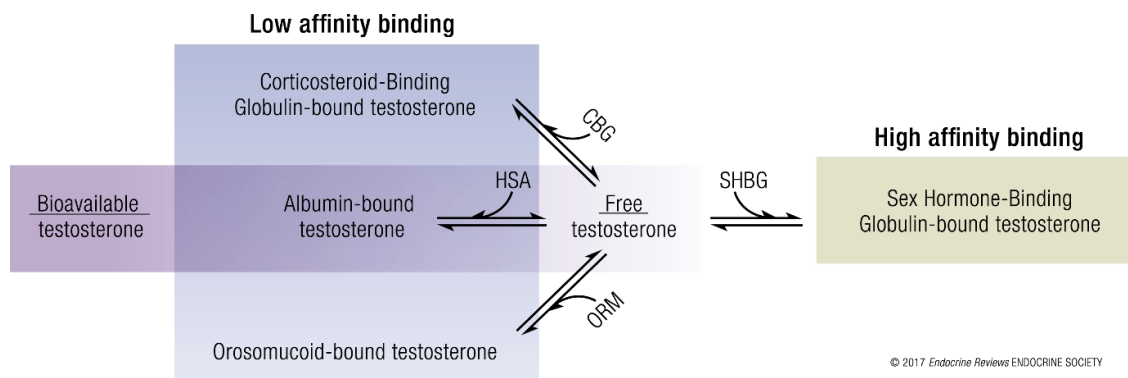


Figure 3. Partitioning of testosterone in the systemic circulation.

Reproduced, with permission from Goldman AL et al. (2017) [3].

Abbreviations: CBG; Corticosteroid-binding globulin; HSA; Human serum albumin, ORM, Orosomucoid, SHBG, Sex hormone-binding globulin.

SHBG is synthesised in the liver and secreted into the circulation, where it passively binds, transport sex steroids and plays a dynamic role in controlling steroid access to target tissues and cells. They bind steroids with high ($\sim$ nM) affinity and specificity. By contrast, albumin binds steroids with limited specificity and low ($\sim$ μ M) affinity, but its high concentration in blood buffers major fluctuations in steroid concentrations and their free fractions. Over the past few years, the biologic role of the specific and high-affinity globulin “the SHBG” has been remarkably expanded and are described in more detail below.

CHAPTER 2

INTRODUCTION: SEX HORMONE-BINDING GLOBULIN

2. Sex hormone - binding globulin (SHBG)

2.1. Historical overview

Sex hormone-binding globulin (SHBG) is a serum β_1 -globulin with a molecular mass of approximately 90 kDa, which was initially postulated in 1958 [15]. SHBG was first isolated and identified in 1965 by Mercier-Bodard and colleagues [16] in France, who separated a testosterone-binding β -globulin by electrophoresis. The same year an oestradiol-binding protein was independently isolated by Rosenbaum and colleagues in the US [17]. However, later these two proteins were shown to be identical by competitive steroid binding studies [18] and subsequently became known as testosterone-estradiol Binding Globulin (TeBG or TEBG) but has since been also shown to bind and act as a transport protein for many other sex steroids and drugs [3, 14, 19]. Now, it is more commonly known as Sex Hormone-Binding Globulin (SHBG) but has also been called Sex-Steroid Binding Protein (SBP), Androgen-Binding Protein (ABP), SSBG (Sex Steroid Binding Globulin), Testosterone-Estrogen-Binding globulin (TEBG) and Testis-specific Androgen-Binding Protein [3, 14, 19].

2.2. Structure of human SHBG

2.2.1. Human *SHBG* gene structure

The human SHBG is encoded by the 4.3 kb *SHBG* gene, located on the short arm (p12–p13) of chromosome 17 as revealed by *in situ* hybridisation to metaphase chromosome spreads [20]. The *SHBG* gene consists of eight exons and seven intervening introns [21–23] and is illustrated in Figure 4.

Exon 1 contains the sequence encoding a secretion signal polypeptide (29 amino acid) that are expressed primarily in the liver, as well as the first few amino-terminal residues of the mature secreted protein, and have also been found in the kidneys of transgenic mice expressing human SHBG transgenes [22–25] whereas exons 2–8 encode two contiguous laminins G-like (LG) domains. The amino-terminal LG domain contains the sites for the sex steroid binding and cation binding domains in addition to the dimer

interface while the functional significance of the C-terminal G domain is less well established, but it contains two sites for *N*-glycosylation [21-23, 25-27].

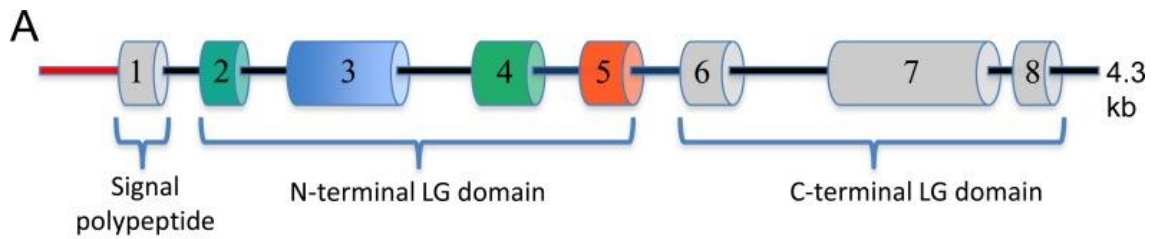


Figure 4. The organisation of the human SHBG gene, which in hepatocytes under the control of an approximately 800-bp promoter sequence (red).

Reproduced, with permission, from Hammond et al. (2011) [28].

Abbreviations: kb, kilo based; bp, base pairs

2.2.2. Human SHBG proximal promoter

The region of the human *SHBG* proximal promoter has been characterized in some detail in terms of transcription factor binding sites and functional studies, and DNase I footprinting, the gel electrophoresis mobility shift assay (EMSA) promoter analysis and reporter gene assays lead to the identification of key transcriptional elements in the promoter [28-31]. The molecular mechanisms of the transcriptional regulation of *SHBG* gene in the liver have been studied in the last decade and have been demonstrated that the expression of SHBG is regulated by the proximal promoter, consisting of a region located at about 850 base pairs of exon 1, which lacks the traditional TATA box [32].

Transcriptional regulation of the SHBG gene

The deoxyribonuclease (DNase) I footprinting (FP) analysis of the human SHBG proximal promoter with liver nuclear protein extracts have defined and identified several FB regions (FP1-FP6) - "cis-acting elements. Several studies have identified various transcription factors (TF) that interact with these regions to regulate SHBG expression [33, 34]. This TF are basically the members of the nuclear hormone receptor (NHR) family, including hepatic nuclear factor-4 α (HNF-4 α), chicken ovalbumin upstream promoter-TF (COUP-TF), upstream stimulatory factor (USF), and peroxisome-proliferator receptor γ (PPAR γ) and are known to regulate SHBG expression [28, 33-35]. The human SHBG promoter lacks a typical TATA box, and exist within four distinct footprints (FP) regions (FP1–FP4) that have been shown to have the highest activity in HepG2. The FP1 region is rich in TA that contains the TTAAAC sequence, appears to facilitate assembly of the transcriptional machinery at the *SHBG* promoter and seems to replace the "TATA-box" box (Figure 5).

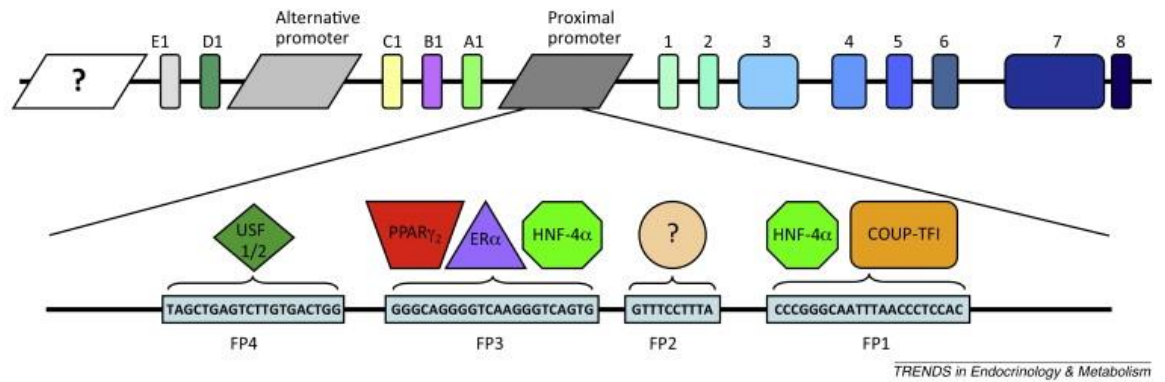


Figure 5. Scheme of human SHBG gene structure and regulation.

Reproduced, with permission from Simó R et al. (2015) [29].

Abbreviations: COUP-TF, Chicken ovalbumin upstream promoter-transcription factor; FP, footprint; ER α , Oestrogen receptor alpha; HNF-4 α , Hepatic nuclear factor-4alpha; PPAR γ , Peroxisome-proliferator receptor; USF, Upstream stimulatory factor

FP1 is located about 20 bp upstream of the transcription start site, and interacts with the nuclear receptors, HNF-4a and COUP-TFI, HNF-4a activating and COUP-TFI repressing *SHBG* gene expression [34]. Thus, FP1 appears to act as a critical on/off switch for *SHBG* transcription in hepatocytes.

FP3 is located about 50 bp further upstream *SHBG* transcription start site where HNF-4a and other members of the nuclear hormone receptor superfamily, ER α , PPAR γ and retinoid X receptor- α (RXR α) compete for the same binding site of FP3, with the former two activating and the latter two repressing *SHBG* gene expression [28, 33].

USF-1/2 has been shown to interact with FP4, although the interaction of USF with FP4 is not essential for the hepatic expression of *SHBG*, because the transgenic mice produce a high level of *SHBG* from their liver even though they lack USF in the *SHBG* promotor [28, 29, 35]

The FP2, FP5, and FP6 regions have been identified as potential binding sites for other factors of transcription, but have not yet been characterized.

Among the several TF, the HNF-4 α and PPAR γ have been identified as the most important TF that regulates *SHBG* expression. Moreover, both HNF4- α and PPAR- γ regulate hepatic *de novo* lipogenesis [29]. In this regard, in the last few years, several studies have described that an increase in *de novo* lipogenesis was able to reduce hepatic HNF-4 α and increase PPAR- γ protein levels, which in turn decreased *SHBG* production [29, 33, 36] (Figure 6).

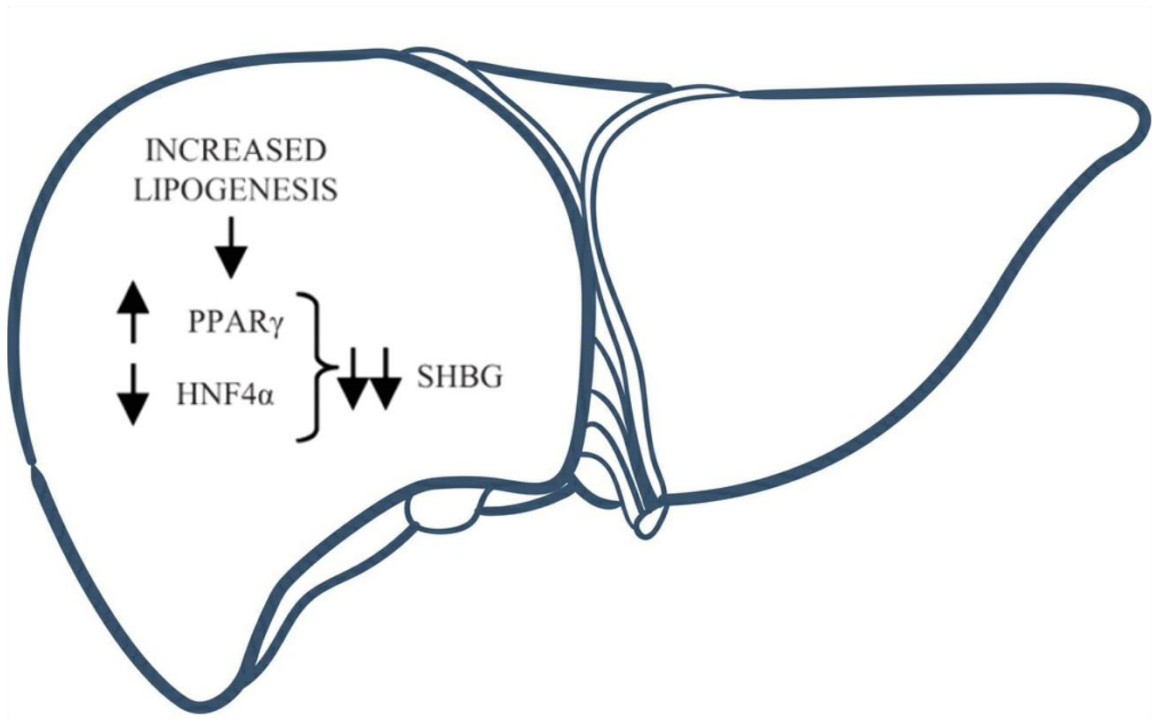


Figure 6. Hepatic de novo lipogenesis mediated crosstalk between SHBG and transcriptional factors.

Abbreviations: $HNF-4\alpha$, hepatic nuclear factor-4 α ; $PPAR\gamma$, peroxisome-proliferator receptor γ ; SHBG, Sex hormone-binding globulin

Depending on the nutritional, hormonal, and metabolic status of the hepatocytes, the competition between this TF to interact with the FP regions of the SHBG promoter regulates their hepatic expression. The influence of a few physiological states and the corresponding transcriptional activity of the SHBG promoter is illustrated in Figure 7. Under conditions when HNF-4 α and USFs are bound to the FPs, activation of the transcript of SHBG, which results in high levels of the secreted protein (Figure 7A). Under conditions when FPs are occupied by PPAR γ or COUP-TFs repression of SHBG expression occurs, with consequent low levels of protein secretion (Figure 7B). Under conditions where HNF4A levels are reduced, the DR1 site closest to the transcription start site is occupied by COUP-TF, and this results in a marked reduction of SHBG transcription (Figure 7C).

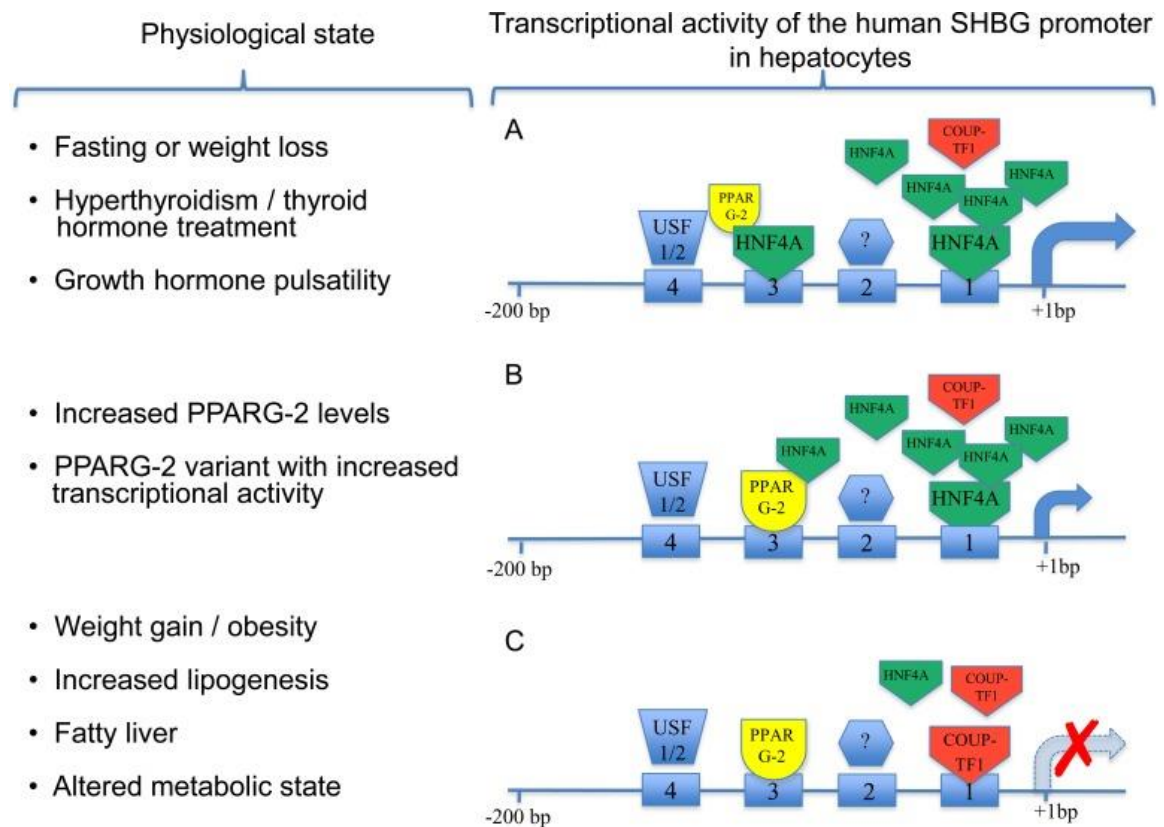


Figure 7. Human SHBG proximal promoter sequence, showing occupancy of cis-elements by transcription factors in response to different physiological states and the corresponding transcriptional activity of the promoter.

Reproduced, with permission, from Hammond et al. (2011) [28].

Abbreviations: bp, Base pairs; COUP-TF, Chicken ovalbumin upstream promoter-transcription factor; HNF-4A, Hepatic nuclear factor-4alpha; PPARG, Peroxisome-proliferator receptor gamma; USF, Upstream stimulatory factor

Recently, human constitutive androstane receptor (CAR) mediated increases in SHBG production by HepG2 cells has been suggested as an alternative mechanism. The proposed mechanism acts independently of alterations in HNF-4 α and PPAR γ levels in HepG2 cells and is thought to bind to a DR-1 element in the human SHBG proximal promoter. In mammals, CAR plays a crucial role in regulating drug metabolism and is a key drug/xenobiotic-sensitive transcriptional regulator and several CYP enzymes. Resveratrol, polyphenols present in red wine act through this mechanism [37] and is illustrated in Figure 8. Circulating resveratrol enters the liver and is degraded by enzymes, such as CYP's, SULT's and UGT's (Figure 8), it binds to different TFs regulating the expression of drug/detoxification genes (Figure 8), and through the binding, to CAR it increases SHBG production (Figure 8).

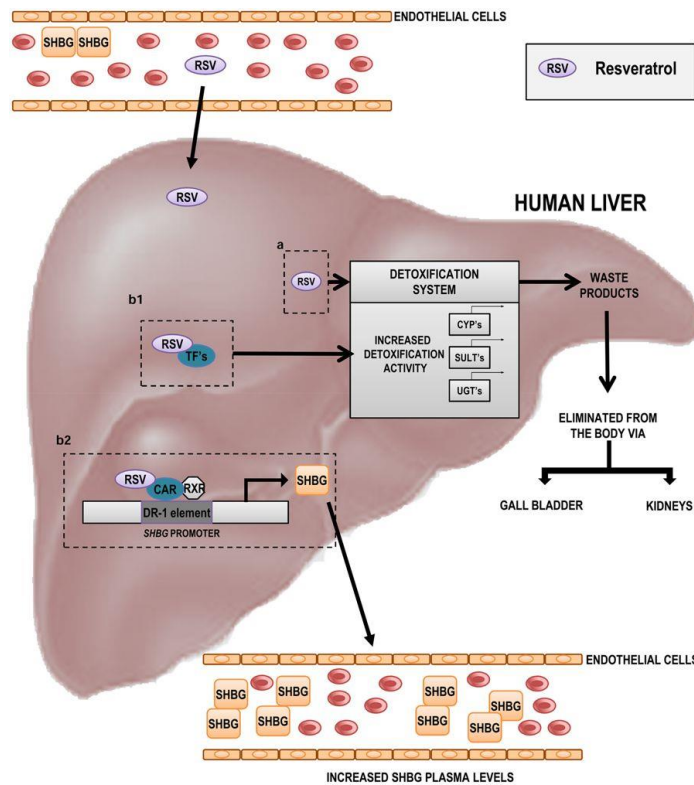


Figure 8. Resveratrol increases hepatic SHBG production through CAR.

Reproduced, with permission, from Saez-Lopez C et al. (2017) [37].

Abbreviations: CAR, Constitutive androstane receptor; RSV, Resveratrol; RXR, Retinoid X receptor; SHBG, Sex hormone-binding globulin; TF, Transcription factor

Plasma SHBG production by the liver varies during development and different physiological or pathophysiological conditions, and abnormalities in the plasma levels of SHBG or its ability to bind steroids are associated with a variety of pathologies [28, 29]. The potential mechanism involved in the regulation of SHBG production and secretion has been markedly expanded in recent years. However, several questions remain unanswered; *“How can we transfer the new concepts in SHBG regulation to clinical practice? And “What are the main mechanisms involved in SHBG production?”*

2.2.3. SHBG protein structure

Human *SHBG* mRNA encodes a native polypeptide consisting of 402 amino acids with lysine-rich, 29 amino acids long signal peptide at amino-terminal (N-terminal) [23, 25]. This signal peptide is cleaved at N-terminal that results in a polypeptide chain of 373 amino acids that undergoes posttranslational modifications and secreted by the hepatocytes [25, 38]. Thus, the mature, secreted form of SHBG circulates as a glycosylated homodimeric protein, approximately 90 kDa [13, 26, 39].

2.2.3.1. *Biochemical properties*

SHBG circulates as a glycoprotein and is a homodimer consisting of two identical, non-covalently bound 40.5 kDa monomers [38, 40]. Each monomer comprises 373 amino acids residues and exhibits two intramolecular disulphide bonds [13]. A highly conserved disulphide bridge is formed between C164 (strand 12) and C188 (strand 14) at the very C-terminus of the domain [39].

2.2.3.2. *Crystal structure of SHBG*

The quaternary structure of the SHBG homodimer has never been resolved, but there is evidence that it adopts a torpedo-like head to head conformation [41]. Crystallographic studies of SHBG have allowed determining their domains, steroid binding sites, and metals, as well as the conformational changes produced by dimerisation [27, 40, 42].

The tertiary structure of a monomer consists of a tandem repeat of laminin G-like (LG) domains at the N-terminal end of the SHBG, which is joined to the carboxyl-terminal domain by a short linker region [39, 40, 42, 43]. The N-terminal G domain (residues 1-194) in SHBG is comprised of two seven-stranded antiparallel β -sheets on top of each other [39, 42]. SHBG monomers possess high affinity for each other, thus spontaneously dimerises during synthesis and secretion and are solely found as a dimer in circulation [39]. The dimerisation occurs between each monomer of N-terminal LG domains in a head-to-head manner. Each monomer forms the interface of the two β -sheets, linked by

eight hydrogen bonds, leading to the formation of the two continuous 14-stranded β -sheets and ultimately an elongated, cylindrical shape of the mature homodimer [39, 42, 44, 45] (Figure 9). These dimerisation process of LG domains form pockets that enable the binding of sex steroids [46] and is promoted by high-affinity SHBG steroidal ligands as well as by divalent cations like Calcium (Ca^{2+}) or Zinc (Zn^{2+}). The SHBG homodimer has two distinct, and equally active, steroid binding sites capable of binding DHT, T, or E2 [39, 44-46]. The serine residue within this binding pocket (hydrophobic core) is important in ligands intercalate (androgen and estrogen binding) and forms hydrogen bonds between the protein and the ligands functional groups at C3 and C17 [39, 47, 48]. As a consequence C19 steroids, such as A ring of DHT and T [47], and C18 steroids, such as the C17 hydroxyl group in the D ring E2, occupy the steroid binding pocket in inverse orientations [48, 49]. Thus, the binding of androgens and estrogens imparts different conformations to the SHBG molecule and lead to slight differences in the positioning of distinct surface residues [39, 47, 50-52].

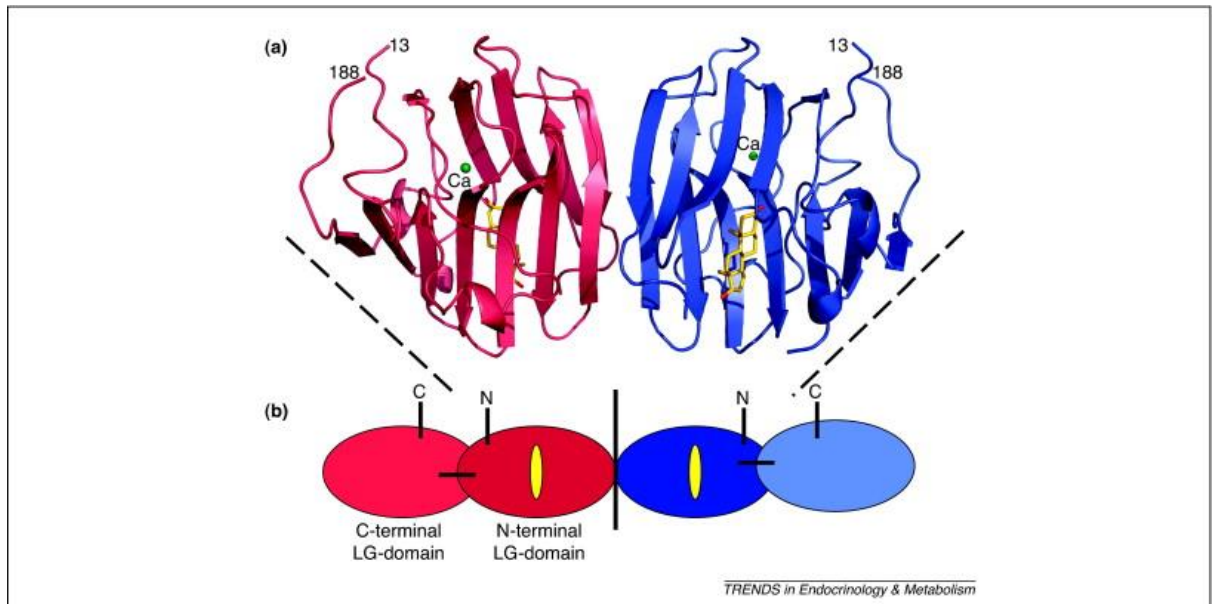


Figure 9. Crystal structure of SHBG protein.

(a) Ribbon representation demonstrating the interaction of the N-terminal domains (residues 13–188) of each SHBG subunit to form the homodimer. The two SHBG N-terminal laminin G-like (LG) domains, each containing a steroid-binding site, are shown in red and blue. (b) Representation of the domain composition of dimeric full-length SHBG. The steroids bound to the N-terminal LG domains are depicted as yellow ellipses. The twofold symmetry axis that relates the two monomers in dimeric SHBG is indicated as a vertical line at the centre of the sketch.

Reproduced, with permission, from Le TN et al. (2012) [53].

Each monomer consists of two laminin G-like domains: the N-terminal domain contains the steroid-binding pocket and binding sites for Ca^{2+} or Zn^{2+} , whereas the C-terminal domain contains residues for glycosylation (oligosaccharides) [39, 54]. Furthermore, each monomer consists of three oligosaccharides: two oligosaccharides are attached at two N-glycosylation sites on asparagine (Asn351 and Asn357) and one at an O-glycosylation site on threonine (Thr7) [38, 55]. These oligosaccharide sidechains are not essential for the steroid-binding activity of SHBG [39]. However, glycosylation of SHBG may be necessary for the interaction of the complete SHBG-steroid complex with plasma membrane receptors in target tissues [56]. Glycosylation prolongs serum SHBG half-life, thereby providing another putative regulatory mechanism for the duration of biological activity or interaction with other macromolecules [10, 53].

2.2.3.3. *Subunits and dimerisation (dimer interface)*

SHBG monomers possess high affinity for each other and spontaneously dimerise during synthesis and secretion [39, 46]. The dimerisation is required for the integrity of the steroid binding site of the dimer [46]. Each monomer forms the interface of the two β -sheets, linked by eight hydrogen bonds, leading to the formation of the two continuous 14-stranded β -sheets and ultimately an elongated, cylindrical shape of the mature homodimer [39, 42, 44, 45] (Figure 9). These dimerisation process of LG domains form pockets that enable the binding of sex steroids [46] and is promoted by high-affinity SHBG steroidal ligands as well as by divalent cations like Ca^{2+} and Zn^{2+} . Both Ca^{2+} and Zn^{2+} binding are required for holding the dimer together and their stability [57-59]. SHBG circulates as a homodimer; thus, chelating agents, such as ethylenediaminetetraacetic acid (EDTA), can dissociate the SHBG dimer. This explains why the steroid-binding activity of SHBG in EDTA plasma is disrupted by freeze-thaw cycles [57].

There is also evidence that dimer formation is promoted in the presence of steroid ligand [44]. The SHBG homodimer has two distinct, and equally active, steroid binding sites capable of binding DHT, T, or E2 [39, 44-46].

2.2.3.4. *Glycosylation*

Glycosylation is a most common post-translational modification observed in several extracellular proteins and is required for many biological processes including protein folding, stability, receptor activation and efficient secretion of many extracellular proteins [60, 61]. As mentioned above, less is known about the structural and functional importance of the C-terminal LG domain in SHBG, but it contains two sites for glycosylation, enabling functionally important post-translational modification undergoing glycosylation [39, 55, 62]. The mature human SHBG consists of three oligosaccharides; an O-glycosylation site in Thr7 and two N-glycosylation sites at positions Asn351 and Asn367 of the C-terminal LG domain. There is variety in the chains of carbohydrates that come together in the N-glycosylation sites whereas the site of O-glycosylation binds the disaccharide Gal-GalNAc, which can bind a sialyl group in one or both monosaccharides. O-linked glycosylation occurred during the final stages of glycoprotein modification within the endoplasmic reticulum and was suggested that the increased sialylation of mucin-type O-glycans prolongs the half-life of SHBG [10, 38, 55, 60].

Glycosylation is a hallmark of the secreted form of SHBG. The glycosylation prolongs serum SHBG half-life and protects against proteases, a more significant impact on their biological activity and the plasma clearance rate, without influencing its protein structure and steroid-binding properties [10, 39, 63]. Furthermore, based on other previous studies, the loss of an oligosaccharide at this position is not expected to influence the plasma half-life of SHBG appreciably. In contrast, use of the 2 N-glycosylation sites in the C-terminal LG domain will have a more significant impact on the half-life of the protein [62, 64]. The integrity of oligosaccharide sidechains linked to

SHBG may be necessary for the interaction of the complete SHBG-steroid complex with plasma membrane receptors in target tissues and possible interactions with other macromolecules [56]. Further, intracellular accumulation of incompletely glycosylated human SHBG after overexpression has been demonstrated in proximal convoluted tubule (PCT) epithelial cells [65], acrosome during spermatogenesis [66] and cell lines including PCT [65], Lymph Node Carcinoma of the prostate (LNCaP) [65] and Michigan Cancer Foundation-7 (MCF-7) cells [67].

2.2.3.5. Steroid binding site

The most important biologic property of human SHBG is its ability to bind sex steroids with equally high affinity as well as specificity [14]. The natural steroid ligands affinities for SHBG decrease in the following order: DHT, 2-methoxy-estradiol, T, A4, androstenol, E2, and E4 [3, 48, 50]. All of the listed molecules interact with high affinity (association constants K_a $1.5-55 \times 10^8 \text{ M}^{-1}$), whereas other steroid ligands bind with much lower affinity [54].

The current model of testosterone's binding to SHBG assumes that both monomers within the complex are capable of binding sex steroids i.e. each SHBG protein contains two steroid binding sites- a fact that was in debate for a long time, and that each of the two binding sites on SHBG dimer has similar binding affinity irrespective of the occupancy of the adjacent binding site (no allosteric) [3, 39, 44, 48] and is illustrated in Figure 10. It is possible that at any one time, zero, one or both binding sites may be occupied and also those different-sex steroids may be bound to the same SHBG protein simultaneously [3, 44, 54].

A linear model of testosterone (T) binding to SHBG as conceptualised by Vermeulen *et al.* [68], Södergard *et al.* [69], and Mazer [70] (Figure 10a). The new model (ZBJ, schematic adaptation) proposed by Zakharov *et al.* (34) incorporating the dynamics of allosteric regulation in testosterone binding to SHBG (Figure 10 b). The different

shapes represent conformationally distinct states of SHBG in the dynamic repartitioning of free testosterone into bound forms. Recent evidence derived from new biophysical techniques indicates that the binding of testosterone to SHBG is a dynamic, multistep process. The binding of one molecule of testosterone to the first binding site on an SHBG dimer leads to conformational rearrangement and allosteric between the two binding sites, such that the second testosterone molecule binds to the second binding site with a different binding affinity; there is a readjustment of equilibria between these interconverting microstates.

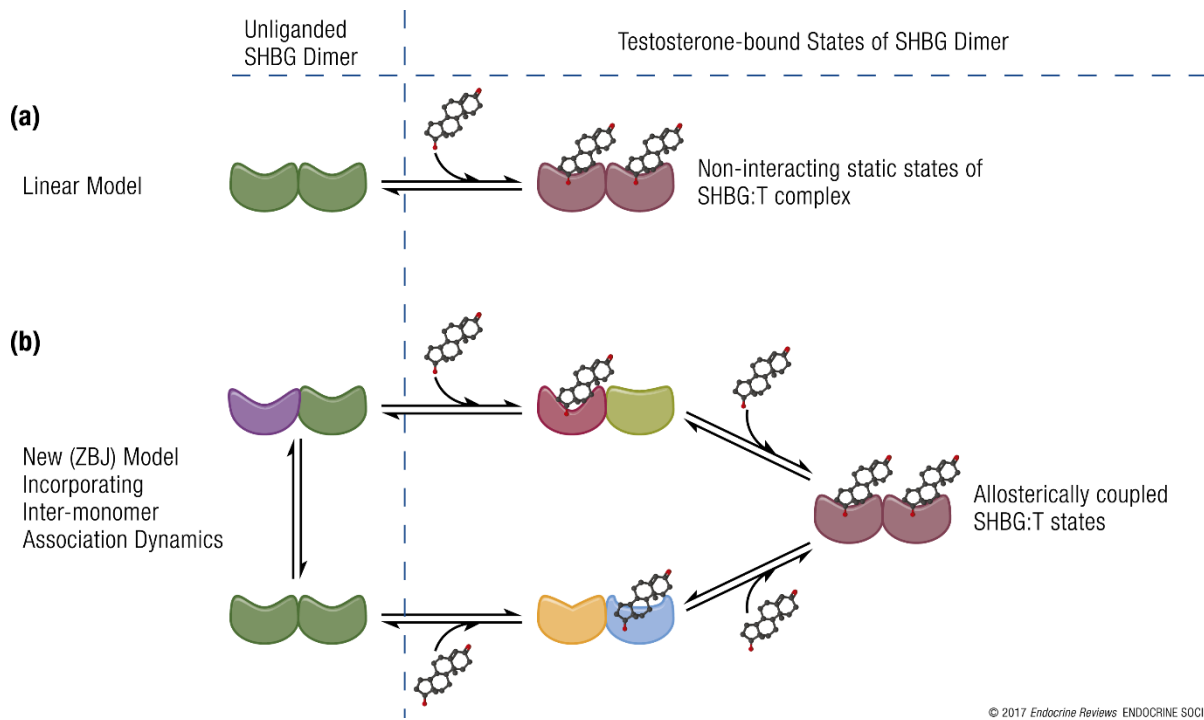


Figure 10. Schematic representation of experimental models of *T* binding to SHBG and showed that each monomer had a *T* binding pocket so that the homodimer binds two, not one, *T* molecules.

Abbreviations: SHBG, Sex hormone-binding globulin; *T*, Testosterone

Reproduced, with permission, from Goldman AL et al. (2017) [71].

Steroid binding ability is well located in the *N*-terminal LG domains. Steroids are deeply intercalated into its multiple hydrophobic cores and form a limited number of important, sequence-specific polar interactions with the protein and the steroids functional groups at C3 and C17 [3, 40, 48]. As a consequence C19 steroids, such as DHT and T, and C18 steroids, such as E2, occupy the steroid binding pocket in inverse orientations [47, 48, 50] and lead to slight differences in the positioning of distinct surface residues [47, 54] and is illustrated in Figure 11 and Figure 12. In case of preferred ligand DHT, the oxygen atom attached at C3 points into the interior of the protein and forms a hydrogen bond with the side-chain hydroxyl of Ser42 [39].

The steroids bound to the *N*-terminal LG-domains are depicted as yellow ellipses. The twofold symmetry axis that relates the two monomers in dimeric SHBG is indicated as a vertical line at the centre of the sketch and marked with a black ellipse (Figure 11 B). While DHT forms hydrogen bonds with SHBG via its oxygen atoms attached to C3 and C17, all additional interactions between the steroid and SHBG are restricted to hydrophobic interactions (Figure 11 C).

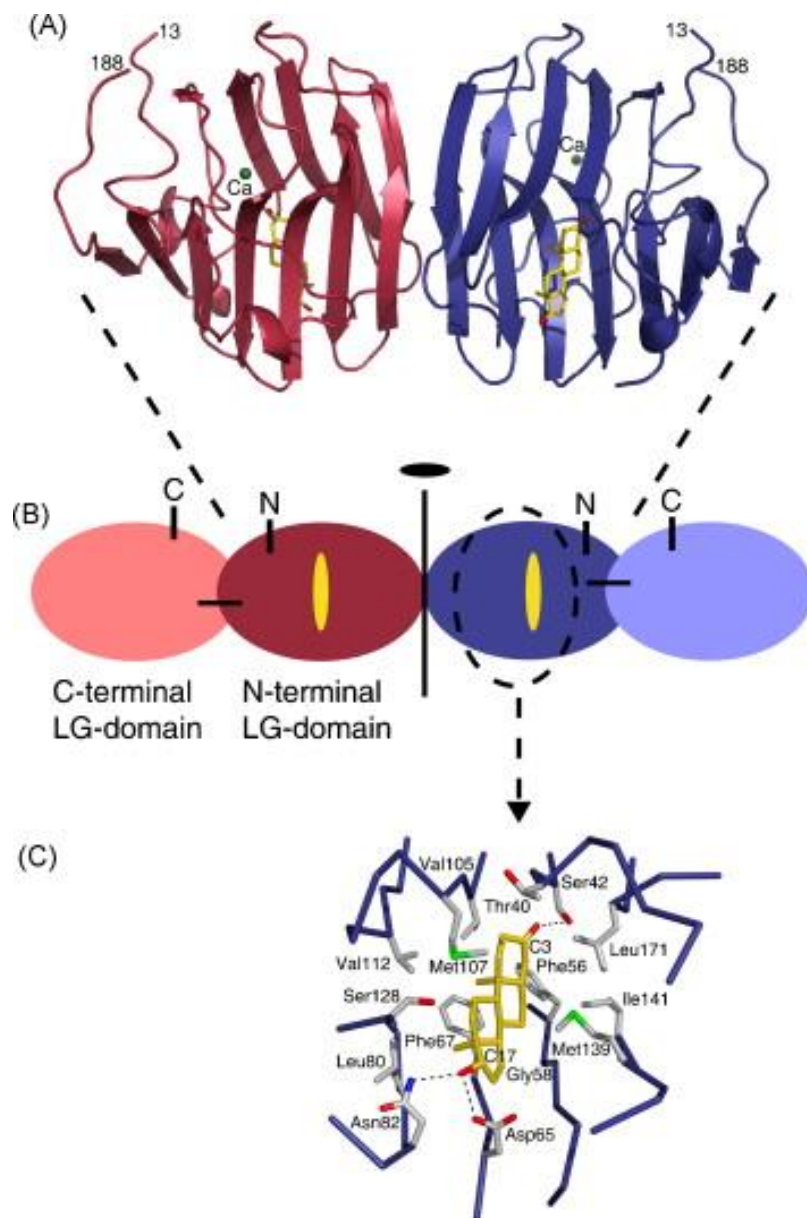


Figure 11. Crystal structure of SHBG homodimer, its domain composition and steroid binding site.

Reproduced, with permission, from Avvakumov et al. (2010) [39].

Two additional H-bonds are formed at the entrance of the binding pocket, namely between the hydroxyl group at atom C17 and the side-chains of Asp65 and Asn82. Besides these hydrophilic anchor points, all other residues lining the steroid-binding site are hydrophobic. The main contributors to the hydrophobic ligand interactions are Phe67, Met107 and Met139 (contact surface greater than 20Å²). Only 2% of the ligand surface is accessible to the solvent: rings A and B are completely buried and only atoms C12 and C17 from rings C and D, respectively, are partially solvent-accessible in the original DHT complex crystal structure [39, 40]. Next, to the ligand pocket, two additional binding sites are present in SHBG, namely a calcium-binding site and a second metal-binding site whose nature only became apparent after soaking SHBG-crystals with ZnCl₂ [39]. Site II is located at the entrance to the steroid-binding site, and Zn²⁺ is coordinated by the side-chains of His83 and His136 and the carboxylate group of Asp65, and its occupancy by zinc was found to alter the specificity of steroid binding by SHBG. The affinity of SHBG for oestradiol is reduced in the presence of 0.1–1mM Zn²⁺, whereas its affinity for DHT remains unaffected. This altered binding specificity was not observed when Zn²⁺ coordination at site II was modified by substituting glutamine for His136 [39]. The comparison of SHBG binding conformation with various steroids is illustrated in Figure 12.

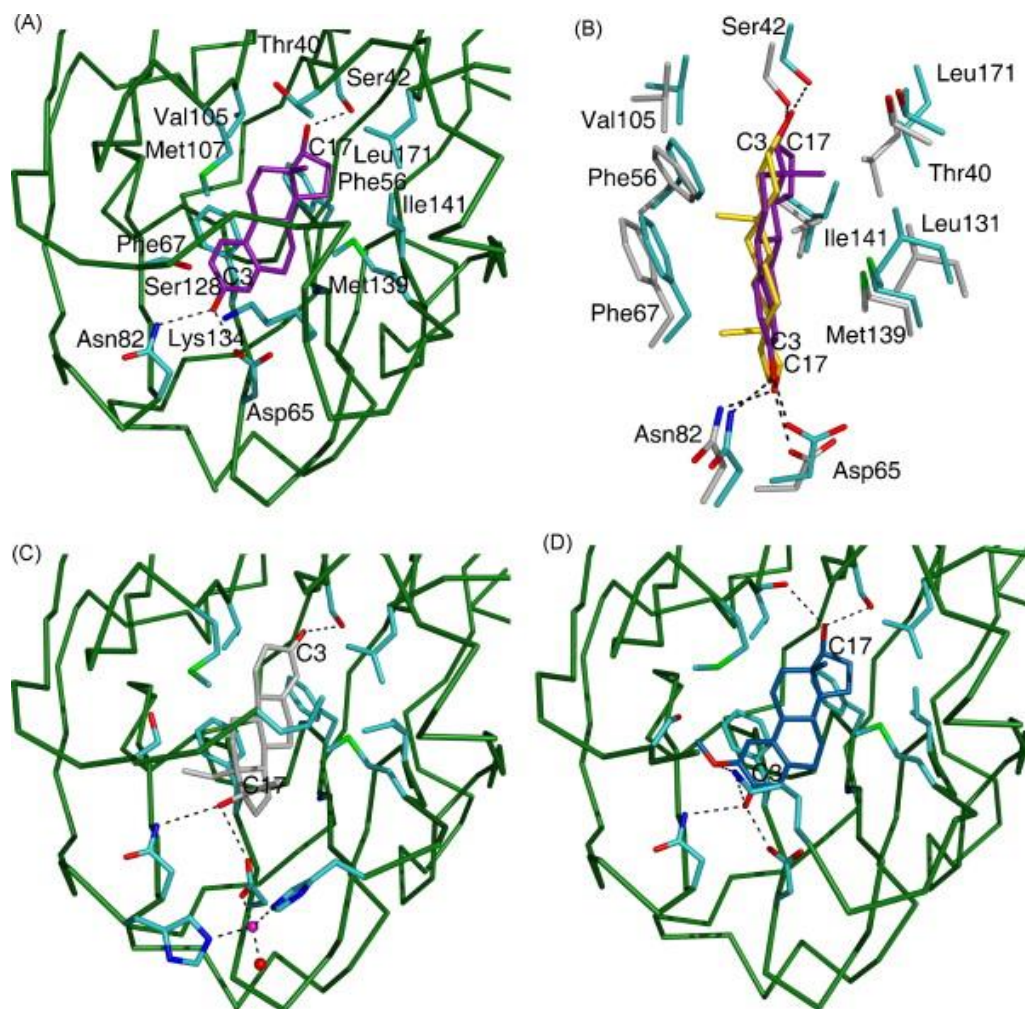


Figure 12. Crystal structures of the N-terminal LG-domain of SHBG in complex with various steroids.

(A) SHBG in complex with E2 (E2 shown in magenta), (B) the comparison of the E2 and DHT (E2 is shown in magenta and DHT is shown in yellow), (C) Crystal structure of SHBG in complex with norgestrel (norgestrel in grey), and (D) SHBG in complex with 2- MeOE2 (2- MeOE2 in dark blue).

Reproduced, with permission, from Avvakumov et al. (2010) [39].

Abbreviations: DHT, 5 α -dihydrotestosterone; E2, Oestradiol; MeOE2, Methoxyoestradiol; SHBG, Sex hormone-binding globulin; T, Testosterone

2.3. Synthesis and tissue specific expression of SHBG

The majority of plasma SHBG is of hepatic origin [25, 72], although several other tissues including the testis [73], prostate [74, 75], hypothalamus [76], cardiac myocytes [77], placenta [78], ovary [79], endometrium [80], breast [74, 81, 82] and many other tissues as shown in Figure 13, have also been shown to express *SHBG* mRNA in humans where their protein products function differently than in the plasma [11, 28]. The appropriate expression of SHBG by the placenta and the foetal liver during development may be critical in maintaining normal foetal exposures to androgens originating from the mother or produced by the foetus itself. As a consequence, variation in the levels of SHBG may influence sex steroid bioavailability and cellular effects with resultant clinical implications [21]. Due to the presence of signal polypeptide, almost all the SHBG produced in hepatocytes is actively secreted, very little intracellular SHBG is retained [65].

Compared to normal liver tissue, quantitative PCR analysis revealed that normal prostate expresses only 1/1000th the abundance of total SHBG promotor in liver (PL)-derived transcripts [83].

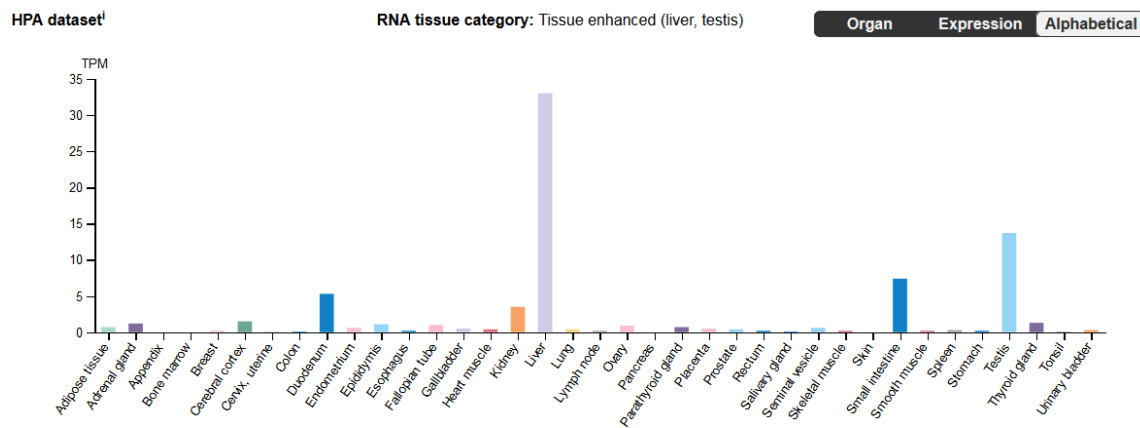


Figure 13. Tissue expression of SHBG in human tissue.

Source: <https://www.proteinatlas.org/ENSG00000129214-SHBG/tissue>

Abbreviations: HPA, Human protein atlas; RNA, Ribonucleic acid

2.3.1. Synthesis of SHBG

The human SHBG gene comprises, three distinct promoters - P_L , P_T , and P_N , and can initiate transcription from three separate sites in exon 1, resulting in three different transcription units-1L, 1T, and 1N [3, 22, 23, 53, 84]. P_L -, P_T -, and P_N - derived transcripts of SHBG are most abundantly expressed in the liver, testis, and prostate, respectively [53, 84]. The first transcription unit is responsible for the production of plasma SHBG by the hepatocytes and begins with the exon one sequence, which encodes for a leucine-rich signal secretion peptide. This transcription unit is under the control of promoter P_L and the other seven exons [3, 34, 84]. The second transcription unit (1TU) begins with the alternative exon one sequence, which replaces the exon 1 (1L) and lacks exon 7. These transcripts (1T) are produced under the control of alternative promoter P_T whose activity is confined in the testis to germ cells and encode an isoform of SHBG that accumulates in the acrosome of sperm [3, 22, 66, 84]. A variant and third transcription unit (1N) contains three novel alternative SHBG first exons (exons 1B, 1C and 1E) in prostate and is under the control of promoter P_N [53, 85, 86].

Differential activation of these three promoters in a tissue-specific manner allows for alternative splicing of SHBG exons, resulting in the expression of as many as 20 unique SHBG transcripts; the biologic roles of these variant *SHBG* transcripts are unknown [53, 84].

2.3.2. *SHBG* transcripts

Transcript variants play a critical role in diversifying gene expression. Alternative splicing is a major mechanism for generating transcript variants. The human *SHBG* gene encodes a large number of transcripts, which all contain alternative exon one sequences spliced to a common exon two sequence followed by up to six other exon sequences, but only two of them have been shown to encode a functional SHBG protein (Figure 14) [22, 57]. Three distinct promoters - P_L , P_T , and P_N , can initiate transcription from three separate

sites in exon 1, resulting in three major transcription units described in the Liver (L), Testes (T) and Prostate (N) [3, 22, 23, 32, 53, 84] and their aberrant expression of alternative transcripts may have pathophysiological significance.

A structure/function diagram for the eight exons long *SHBG* transcript is shown in Figure 14. The nucleotide positions of each exon are at the top (the exon one start codon defined as beginning with nucleotide +1); below that, the number of amino acids encoded by each exon. Conserved exons are numbered in white, alternatively spliced exons in yellow. Exon boundaries are denoted by horizontal white rectangles. Shown in red is the region of the *SHBG* transcript encoding the 29 amino acid long signal peptide, in light blue, the 383 amino acid long *SHBG* monomer? Steroid binding determinants in the mature *SHBG* protein are denoted by black rectangles, those forming the dimerisation domain are blue, and that encoding the decapeptide sequence is purple. Below, in black, are linear depictions of the two previously described human *SHBG* gene transcripts originating from P_L ; the four novel transcripts described in their study are depicted in grey. Transcripts are identified on the left by missing exons. Three of five alternatively spliced P_L transcripts retain the *SHBG* reading frame, whereas those lacking exon seven alone and exons 4-7 undergo identical frameshifts within exon eight that may affect their inherent stabilities [22].

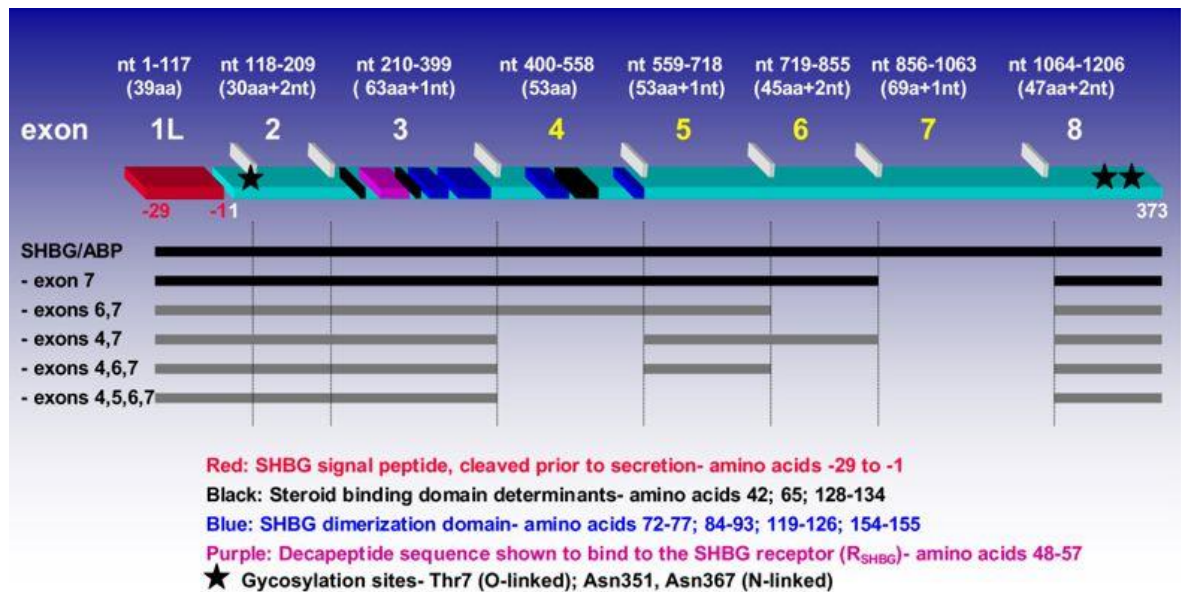


Figure 14. Schematic diagram of the contiguous human SHBG transcript and alternatively spliced transcripts arising from the downstream promoter, PL.

Reproduced, with permission, from Nakhla, AM et al. (2009) [22].

Abbreviations: PL, Promotor in liver

2.3.2.1. *SHBGL*

The first major and predominant transcript encodes a precursor for the secreted (plasma) form of SHBG and was originally described in the liver (SHBGL) [84], although it is also found in human prostate, breast, and brain [53]. SHBGL is regulated by promoter PL and is encoded by eight exons (exon 1L-8), ranging in size from 90 to 208 bp. Except a 733 bp intron separating exons 6 and 7 (which perhaps contains alternative splicing regulatory elements), the remaining introns are relatively small (133–331 bp). SHBGL is under the transcriptional control of a TATA-less promoter which possesses multiple protein-binding sites, including those for hepatocyte nuclear factor-4 and SP-1 [30, 31]. SHBGL is responsible for the production of plasma SHBG by the hepatocytes, although it is also found in human prostate, breast, and brain and begins with the exon one sequence, which encodes for a leucine-rich signal secretion peptide. [3, 34, 84].

Transcripts containing the exon one sequence encoding the signal for secretion have also been found in the kidneys of transgenic mice expressing human SHBG transgenes [24, 31].

2.3.2.2. *SHBGT*

The second transcript encoded a protein of unknown function and was originally described in the testis (SHBGT) [23, 84]. SHBGT is regulated by PT, located 1.9 kb upstream of PL and contains a noncoding alternative exon one sequence. SHBGL and SHBGT differ in their 5' sequences and the absence of exon 7 in the latter one. An AUG translation initiation codon within exon 2 produces an *N*-terminally truncated SHBG isoform starting with Met30 in the mature SHBG sequence. This SHBG isoform is produced in male germ cells and is not secreted, but it appears to bind steroids in the same way as plasma SHBG and accumulates in the acrosome of sperm [57, 84].

2.3.2.3. *SHBG*

A recently described third transcript encodes a protein of unknown function has been demonstrated in human prostate tissue and prostate cancer cell lines and is under control of PN (one other alternative promoter upstream of the proximal promoter) [22, 30, 86]. Nakhla *et al.* identified 19 different *SHBG* gene transcripts [22] while Pinós *et al.* describe additional transcripts arising from an alternative promoter upstream of the proximal promoter [86]. PN is found within intron 1 of the adjacent *FXR2* gene [22], also contains a unique first exon and contains at least five different transcription units (TU) (TU- 1A, -1B, -1C, -1D and -1E) [22, 85, 86]. However, apart from the singular transcript encoding *SHBG* itself, it is unclear whether any other *SHBG* gene transcript encodes a functional protein in humans, or whether they might act to regulate expression of the *SHBG* transcript. There are no reported data demonstrating their translation in human prostate tissue and prostate cancer cell lines, except TU-1A and -1B [85]. Moreover, although TU-1A and TU-1B are translated from the ATG situated in exon 2, their corresponding 5'UTR exons downregulate *SHBG* translation [85]. This *SHBG* isoform produced in the prostate is not secreted, and prostate *SHBG* being synthesized for local, or intracellular use [22, 57].

Moreover, Nakhla *et al.* reported that only the eight exons long *SHBG* transcript is generated in normal prostate tissue. However, normal prostate revealed a low abundance of transcripts derived from the two upstream promoters [22]. In striking comparison, the LNCaP prostate cancer cell line exhibited a dramatic relative increase in both the number of alternatively spliced transcripts and transcripts from upstream promoters [22, 86]. This raises the possibility that the alternative *SHBG* transcripts identified in human prostate cancer cells encode *SHBG* isoforms that function within the cells rather than having to be secreted to act, but this will only be resolved when these intracellular *SHBG* isoforms are isolated and characterised from human tissues. Taken

together, the *SHBG* transcripts may be a valuable provider of diagnostic, prognostic, and predictive biomarkers for individuals with prostate cancer.

2.3.3. Transgenic Mice

Unlike other mammals, the rodent liver does not express the *SHBG* gene postnatally. Thus, rodents do not have *SHBG* in their blood circulation [12, 34, 87]. The gene encoding *SHBG* in the rat is expressed transiently in the liver during foetal life, but not postnatally. Instead, they express the *SHBG* in the Sertoli cells of the testis and contribute to a small amount of circulating *SHBG* [31, 87, 88]. Only new world monkeys and chimpanzees have the same gene structure in terms of promoters and footprint regions of human [72, 87]. A USF-1/2 binding site present in the human *SHBG* promoter is lacking in the *SHBG* promoters of sub-primate species and has recently been shown to account for phylogenetic differences in testicular *SHBG* expression between rodents and humans [24, 35]. This site has little effect on hepatic *SHBG* expression, as transgenic mice expressing a human *SHBG* transgene lacking this USF binding site, maintain a high level of *SHBG* expression in the liver [24, 35].

Interestingly, whereas the wild type 4.3 kb *SHBG* transgene is not expressed in the testis, the deletion of this USF1/2 binding site permitted the expression of *SHBG* in the testes of transgenic mice harbouring the modified *SHBG* transgene [24, 35]. Therefore, rodent models cannot be used to study the *SHBG* expression and regulation. However, the creation of transgenic mice carrying the human *SHBG* isoform (h*SHBG* transgenic mice) under the control of its native promoter has facilitated research [31, 87, 89]. These h*SHBG* transgenic mice or “humanised mice” express human *SHBG* in their liver and extrahepatic tissues, which results in the presence of *SHBG* in blood and urine [31, 90]. The extrahepatic expression of *SHBG* occurs in some cell types, such as the androgen-sensitive epithelial cells of the PCT of the kidney, and testicular germ cells, much of the h*SHBG* produced by these cells remain within them [29]. For instance, the human *SHBG*

produced by PCT of the kidney is most likely secreted into the renal tubule because it can be detected in the urine, but substantial amounts of immunoreactive human SHBG are retained within these cells. This is remarkable because the human SHBG transcripts in PCT cells encode the SHBG precursor polypeptide that includes the signal polypeptide necessary for secretion, and they are identical in sequence to the SHBG mRNA in hepatocytes that very actively secrete almost all the SHBG they produce and retain very little intracellular SHBG [31, 90]. The blood levels of SHBG in this model are almost 10-fold higher than in humans [31, 87]. These SHBG transgenic mouse has been used extensively to study human SHBG expression and molecular mechanisms for regulation of SHBG by various factors e.g. TNF- α [91, 92], IL1 β [36], adiponectin [93], insulin and monosaccharides [94] and several other factors [33, 35-37, 91, 93-96]. The molecular mechanisms and transcription factors causing the SHBG down-regulation during obesity development [87] and non-alcoholic fatty liver disease (NAFLD) [89] development, were elucidated by using SHBG transgenic mouse.

2.4. The physiological role of SHBG

Transfer of hydrophobic sex steroids into tissues is presumed to occur passively according to physicochemical partitioning between the hydrophobic protein-binding sites on circulating binding proteins, the hydrophilic aqueous extracellular fluid, and the lipophilic cellular plasma membranes [1]. SHBG possess 3 to 4 orders of magnitude greater affinity for androgens and estrogens than albumin, another carrier protein [10]. These binding proteins play an important role in regulating the transport, tissue delivery, bioactivity, and metabolism of sex-steroids [3, 11-14]. In the classic paradigm, the main function of SHBG was to passively bind and transport sex-steroids within the bloodstream to extravascular target tissues [14, 97]. Over the past few years, the biologic role of SHBG has been more thoroughly investigated from simple role to regulate the biological activity to complex interactions with high-affinity receptors on cell membranes [19, 28, 84].

2.4.1. Binding of sex steroids in plasma

SHBG binds androgens and oestrogens with nanomolar affinity: DHT is the preferred ligand, followed by T and E2. The sex steroids not bound to SHBG (on average 55% of T and E2 in healthy adult men and women, respectively) are considered bioavailable because they circulate either freely (~1–3%) or loosely bound to other proteins such as albumin [98, 99].

HSA binds all classes of steroids with low (μM) affinity, but its very high plasma concentrations and ligand-binding capacity allow it to buffer fluctuations in steroid levels and their distribution between other steroid-binding proteins and the free fraction in plasma. SHBG binds major sex steroids with high (nM) affinity and specificity. Although SHBG is present in much lower concentrations in plasma than albumin, their high affinity and specificity for steroids enable them to play much more dynamic roles in determining the plasma concentrations of their main ligands [11, 100]. The new binding model depicts a multi-step dynamic process, encompassing at least two inter-converting

microstates in unliganded SHBG, readjustment of equilibria between unliganded states upon binding of the first ligand molecule, and allosteric interaction between two binding sites of SHBG dimer. All dissociation constants are depleted in nM [47] and are illustrated in Figure 15.

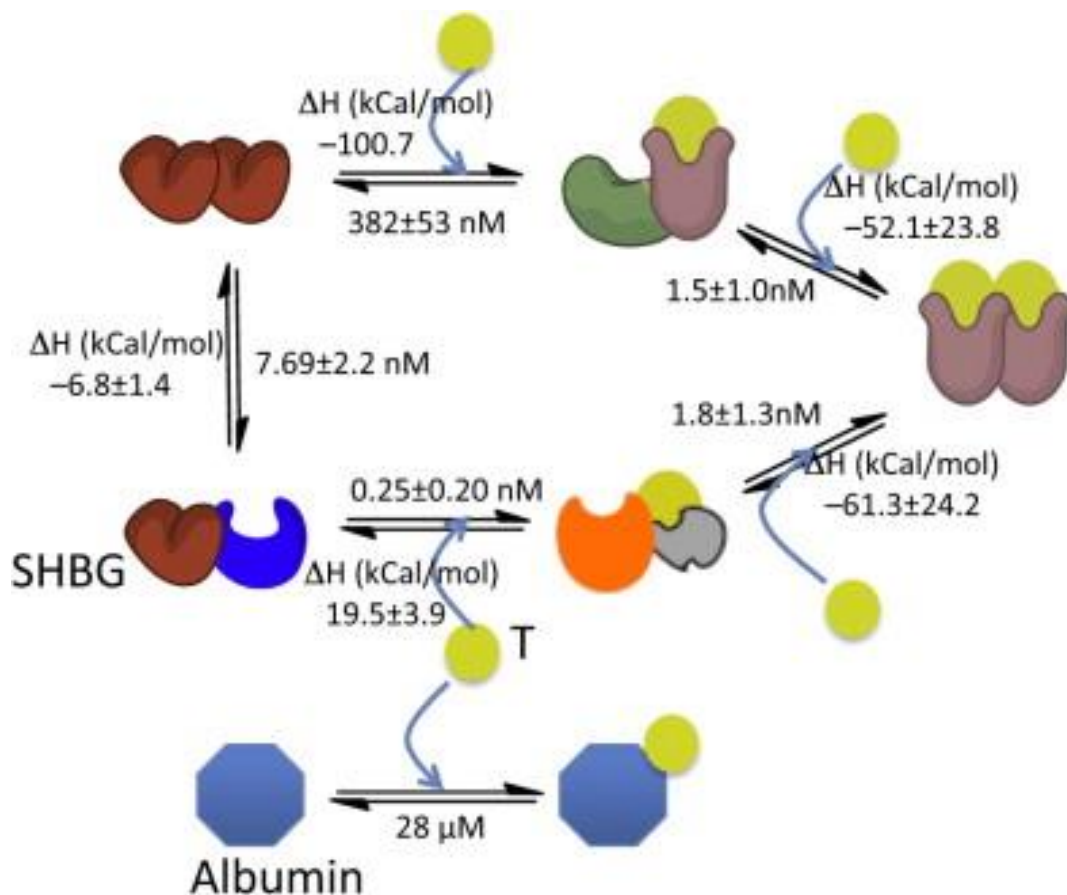


Figure 15. Multi-step, dynamic allosteric model of testosterone's binding to HSA and SHBG.

Reproduced, with permission, from Zakharov MN et al. (2014) [47].

Abbreviations: ΔH , Enthalpy; HSA, Human serum albumin; kCal, Kilocalories; mol, molar; nM, nanomolar; SHBG, Sex hormone-binding globulin; T, Testosterone

2.4.1.2. Sex steroids carrier in plasma

Steroid hormones are highly lipophilic, and therefore share a common requirement for serum carrier proteins to ensure effective delivery to target cells (Figure 16).

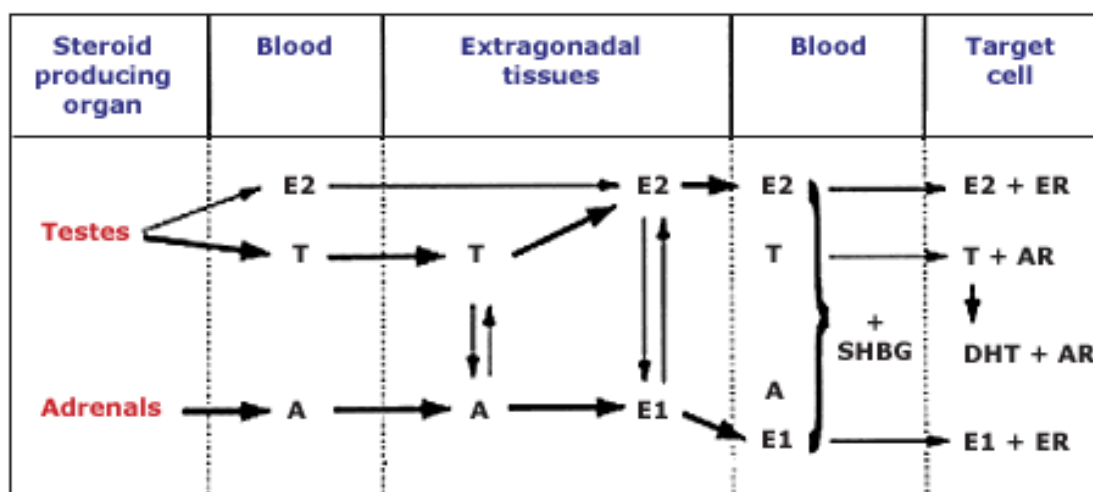


Figure 16. Sex steroid production in endocrine glands, circulation in bloodstream, and conversion in extra-glandular tissues.

Adapted from Braunstein, GD. Pubertal gynecomastia. In: Paediatric Endocrinology, Lifshitz, F (Ed), Marcel Dekker, New York, 1996, p. 197.

Abbreviations: AR, Androgen receptor; DHT, Dihydrotestosterone; E1, Oestrone; E2, Oestradiol; ER, Oestrogen receptor; A, Androstenedione, SHBG, Sex hormone-binding globulin; T, Testosterone

Under physiologic conditions, 30-45% of circulating testosterone is SHBG bound, with the remainder (50-68%) bound to lower affinity, high-capacity binding sites (albumin, α_1 acid glycoprotein, corticosteroid-binding protein), with 0.5-3.0% remaining non-protein bound [1, 101, 102] and is illustrated in Figure 17. Although oversimplified, the currently favoured view called the free hormone hypothesis postulates that it is the free and albumin-bound forms of sex steroid hormones that are available for diffusion out of capillaries and into cells, where they initiate an appropriate response [47, 101-104]. Moreover, as the affinity of SHBG for T being higher than that for E2, differences in SHBG concentrations might differentially modulate relative tissue exposure to androgens and oestrogens [105].

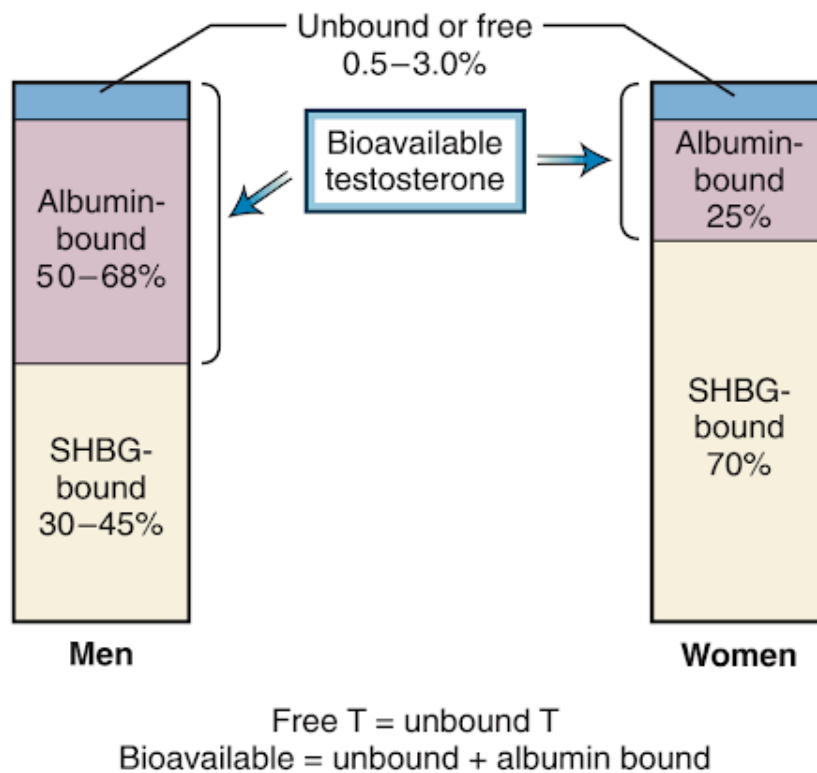


Figure 17. Binding of circulating T to plasma proteins in men and women.

Adopted from Williams Textbook of Endocrinology, 13e Elsevier/Saunders:

www.accessmedicine.com

Abbreviations: SHBG, Sex hormone-binding globulin; T, Testosterone

2.4.1.3. *The affinity of SHBG for sex steroids*

The most prominent feature of human SHBG is its ability to bind sex steroids with nanomolar (nM) affinities [14, 100]. SHBG binds sex steroids: dihydrotestosterone (DHT) → 2-methoxy-estradiol → testosterone (T) → androstenediol → androstanediol → oestradiol (E2) → Oestrone (E1) in order of respective affinity [50, 54, 106]. All these sex steroids molecules interact with high affinity (association constants K_a $1.5-55 \times 10^8$ M⁻¹), whereas other steroid ligands bind with much lower affinity. SHBG binds the most physiologically important androgen, DHT, with about five times greater affinity than T and about 20 times higher affinity than E2 [50, 106]. Each SHBG protein contains two steroid binding sites and is capable of binding two sex steroids [3, 39, 44]. E4 does not bind to human SHBG [107].

2.4.1.4. *Occupancy of SHBG in plasma*

SHBG levels are sexually dimorphic, and the degree of occupancy of the SHBG steroid-binding sites in men and women differs considerably, such that women have more SHBG than men. SHBG circulates in women with only a small fraction of sites occupied by sex steroids, whereas SHBG in men is largely occupied by testosterone [100]. In plasma of healthy humans, 20% of the steroid binding sites of SHBG in males and 50% of the steroid binding sites of SHBG in females is unoccupied [28, 89]. Unoccupied sites could be joining other ligands, such as xenobiotics [21].

2.4.2. Metabolic clearance of steroids

SHBG is the major plasma transport protein for biologically active sex steroids, and changes in its plasma concentrations influence the plasma half-lives of its steroid ligands [49]. SHBG also controls the rate of metabolic clearance of sex steroids in plasma by regulating their uptake by target cells. SHBG is believed to keep sex steroids inactive and to control the number of free hormones that enter cells by passive diffusion [75, 94,

108, 109]. Binding of T to SHBG decreases its metabolic clearance rate and its conversion rate to androstenedione [110].

2.4.3. Access to sex steroids to target tissues

The SHBG plays a key role in regulating the bound hormone from diffusing out of the bloodstream and their access and possibly action in target tissues [49, 84, 99, 111]. SHBG also has profound effects on the balance between bioavailable androgens and E2, thereby preventing hormone binding to the intracellular androgen or oestrogen receptors in responsive tissues [110]. Therefore, any fluctuations in SHBG levels affect the distribution and access of these molecules to target tissues [112]. Several studies have postulated that HSA-bound T, being loosely bound, could dissociate in the tissue capillaries, enter the cell, and thus become bioavailable. This led to the concept that unbound testosterone plus HSA-bound testosterone fractions in circulation were biologically relevant to tissue action, and these two fractions together came to be known as bioavailable T [3, 54, 113] and are illustrated in Figure 18.

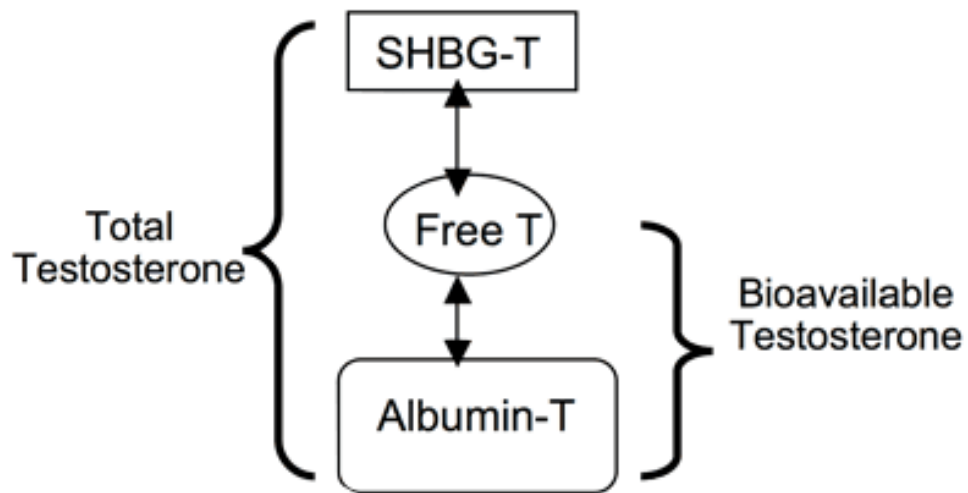


Figure 18. *Fractions of circulating testosterone in men.*

Adopted from McAninch JW, Lue TF: *Smith & Tanagho's General Urology*, 18e:

www.accessmedicine.com

Abbreviations: SHBG, Sex hormone-binding globulin; T, Testosterone

2.4.3.2. *SHBG and free hormone hypothesis*

The free hormone hypothesis postulates that only the “free” steroid hormones are available for diffusion out of capillaries and then can traverse the plasma membrane of target cells, where they exert an appropriate biologic response [3, 114-117].

Experimental evidence for the tenuous theoretical basis of free hormone hypothesis suggesting that only the “free hormones” can enter the cells, that first came from studies in which the entry of testosterone into the cell was blocked by the addition of SHBG [104, 116]. Later, sex steroids bound to albumin (bioavailable) has also been proposed to be accessible to target tissues, but steroids still have to dissociate from albumin before they diffuse into target cells and exert their response while the SHBG-bound fraction is generally considered inaccessible [11, 47, 68, 114, 115]. The small free fractions are the most biologically active fraction of circulating testosterone for its greater accessibility to tissues. As a result, the measurement of this free testosterone (FT) has been of increasing interest. At present, the proposition that only free steroids diffuse into cells therefore still best explains the clinical manifestations of either steroid hormone excess or deficiency and knowledge of free steroid concentrations in plasma is critical to understanding their biological activities.

2.4.3.3. *The fatal flaw of the free hormone hypothesis*

Even though the “free hormone hypothesis” is widely accepted in endocrinology, the physiological facts are oversimplified, only have a theoretical basis and is not adequately supported robust experimental evidence [71, 104, 118].

2.4.3.3.1. *Accessibility to tissues*

The access of ‘free’ sex steroids (e.g. free T) to target cells varies considerably between tissues. Moreover, the diffusion of free steroids and their accessibility to specific target cells vary considerably in terms of their tissue vascularity, blood flow and transit time, the composition of the extravascular fluids and extracellular matrix, cell lipid content in

particular [11, 71, 104]. Precisely, how sex steroids enter cells? Has never been defined completely. Moreover, this theoretical approach cannot be resolved and requires robust and empirical evaluation along with efficient methods for measurement of free hormones [119].

2.4.3.3.2. *Difficulty in free T measurements*

Equilibrium dialysis and centrifugal ultrafiltration are the gold standard methods used for free T measurements. However, both manual methods are too cumbersome and laborious, and not widely available in current high-throughput commercial pathology laboratories [3, 71, 120, 121]. This has led to the creation of empirical formulae to calculate FT (cFT), all based on measuring testosterone, albumin and SHBG in the same samples and is widely used in clinical practice [68-71, 119, 120, 122-124]. These empirical formulae come in two forms [71]:

- Model-based formulae based on equilibrium binding equations with an obligatory requirement for plug-in estimates for testosterone's binding stoichiometry and affinity for SHBG;
- Model and an assumption-free, purely empirical formulae derived from large databases;

These widely used empirical formulae to calculate free in current clinical practice are mathematical models based on the law of mass action [68-70]. However, all these methods attribute the inaccuracies, lack validation and have major sources of potential systematic error: [121-123, 125, 126].

2.4.3.3.3. *Erroneous stoichiometry and the binding affinity*

Although SHBG is a homodimer, the stoichiometry assumption to derive above mentioned empirical formulas speculated that homodimer binds only a single T molecule. However, the SHBG crystallisation studies have shown that each monomer had a binding pocket so that the homodimer binds two, not one, testosterone molecules,

therefore, the erroneous stoichiometry should be corrected to these equations [3, 39]. By using mutated recombinant SHBG proteins, Wu and Hammond found that three naturally occurring mutants had 2.5-5.0 fold reduced binding affinity for DHT[127]. Similarly, several non-synonymous single nucleotide polymorphisms (SNPs) in SHBG were identified, which may influence androgen binding affinity and thus free T concentrations [10, 57].

2.4.3.4. *Why is SHBG important?*

The access of reproductive steroids to their target cells varies considerably between tissues and is influenced to a great extent by their interactions with plasma steroid-binding proteins, and with SHBG in particular [128]. Thus, SHBG is regarded as an important regulator of circulating sex steroid bioactivity. SHBG measurements are used to estimate the amounts of 'free' or bioavailable testosterone in the blood. Because T production is regulated by gonadotropin feedback in men, changes in SHBG, which alter FT concentrations, will be compensated by autoregulation of T production but not so in women [129]. On the other hand, changes in SHBG are associated with parallel changes in TT and occur only in men with an intact hypothalamic-pituitary-testicular (HPT) axis. However, hypogonadal men with HPT axis pathology are incapable of increasing T production in response to increasing SHBG concentrations, limiting the diagnostic utility of TT [130]. Thus, reliance on TT may lead to an over diagnosis of hypogonadism, particularly in situations where SHBG concentrations are altered [130] and is illustrated in Figure 19.

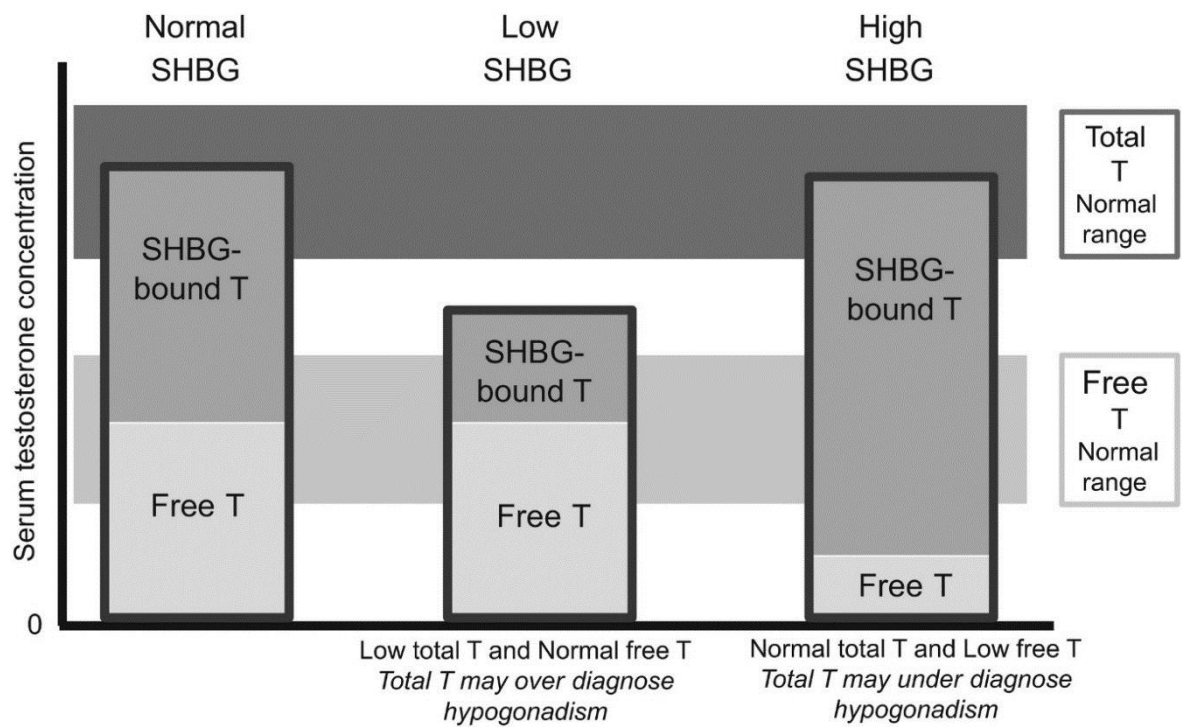


Figure 19. Relationship between TT and SHBG levels, in situations of altered SHBG levels.

Reproduced, with permission, from Grossman M (2018) [130].

Abbreviations: SHBG, Sex hormone-binding globulin; T, Testosterone

2.4.4. Interaction with cell membranes

Emerging evidence suggests that the function of SHBG extends beyond those of a plasma transport protein and has been presented that SHBG is also able to interact with other proteins, especially binding with plasma membrane based receptors to trigger cellular actions [3, 84, 128, 131, 132]. These include internalisation of steroids bound to SHBG to their target cells by a receptor-mediated endocytic mechanism [133-135] or a cAMP based signal transduction system with various biological effects according to the cell type [128, 131, 136, 137]. It was anticipated that the oligosaccharide side-chains possess by SHBG may be necessary for the SHBG-steroid complex to interact with plasma membrane receptors in target tissues [138]. Furthermore, the specific binding of SHBG to specific SHBG membrane receptor located in cell membranes of various cells has been widely observed for e.g. LNCaP cells [84, 128, 139], hepatocytes (Chang liver cells) [128, 140] thus making the interaction reasonably specific to tissues that are responsive to the steroids which bind to SHBG [128]. Several theoretical mechanisms for the interaction of SHBG with the cell membrane is shown in Figure 20. The disagreements about the precise function of SHBG persist and have been reinforced by early and current data demonstrating the interactions of SHBG to cell membranes. Thus, the physiological and wider clinical implications of these interactions remain to be established.

Several interactions between SHBG and extracellular proteins have been identified, which are suggested to aid efficient steroid transport and delivery. Multiple hypothetical mechanisms for the cellular uptake of testosterone and downstream signalling (Figure 20a). The model is depicting the “free” hormone hypothesis. In this model, T that is not bound to SHBG or HSA or other binding proteins diffuses across the plasma membrane and binds to the androgen receptor (AR). The liganded AR recruits co-regulators and chaperone proteins translocates to the nucleus and binds to androgen response elements (AREs) on androgen-responsive target genes, which activates the

transcription of target genes (Figure 20b). The megalin-dependent mode of T entry. According to this model, SHBG-bound testosterone is internalised into the cell through an endocytic process mediated by the membrane protein megalin. Once internalised, SHBG-bound T is released at the low pH within the lysosome (Figure 20c). The RSHBG - T system. The SHBG dimer has multiple binding sites—two sites (simplified as one in this model) bind T, and one site binds to a membrane receptor. It may be that only unbound SHBG can bind to the receptor, then the RSHBG - T complex is coupled to the activation of a G protein (GP), the accumulation of intracellular cyclic adenosine monophosphate (cAMP), and activation of protein kinase A (PKA). PKA may modulate AR function by activating AR through phosphorylation (Figure 20d). Steroid ligand-dependent interactions between SHBG and at least two matrix-associated proteins in the fibulin family (fibulin-1D and fibulin-2) contribute to the extravascular sequestration of SHBG in some tissues, such as the breast, prostate, and endometrial stroma. According to this model, ligand-dependent interactions between SHBG and fibulins modulate their binding to various signalling molecules, such as integrins, to modify signalling pathways that regulate cell adhesion, proliferation, and migration.

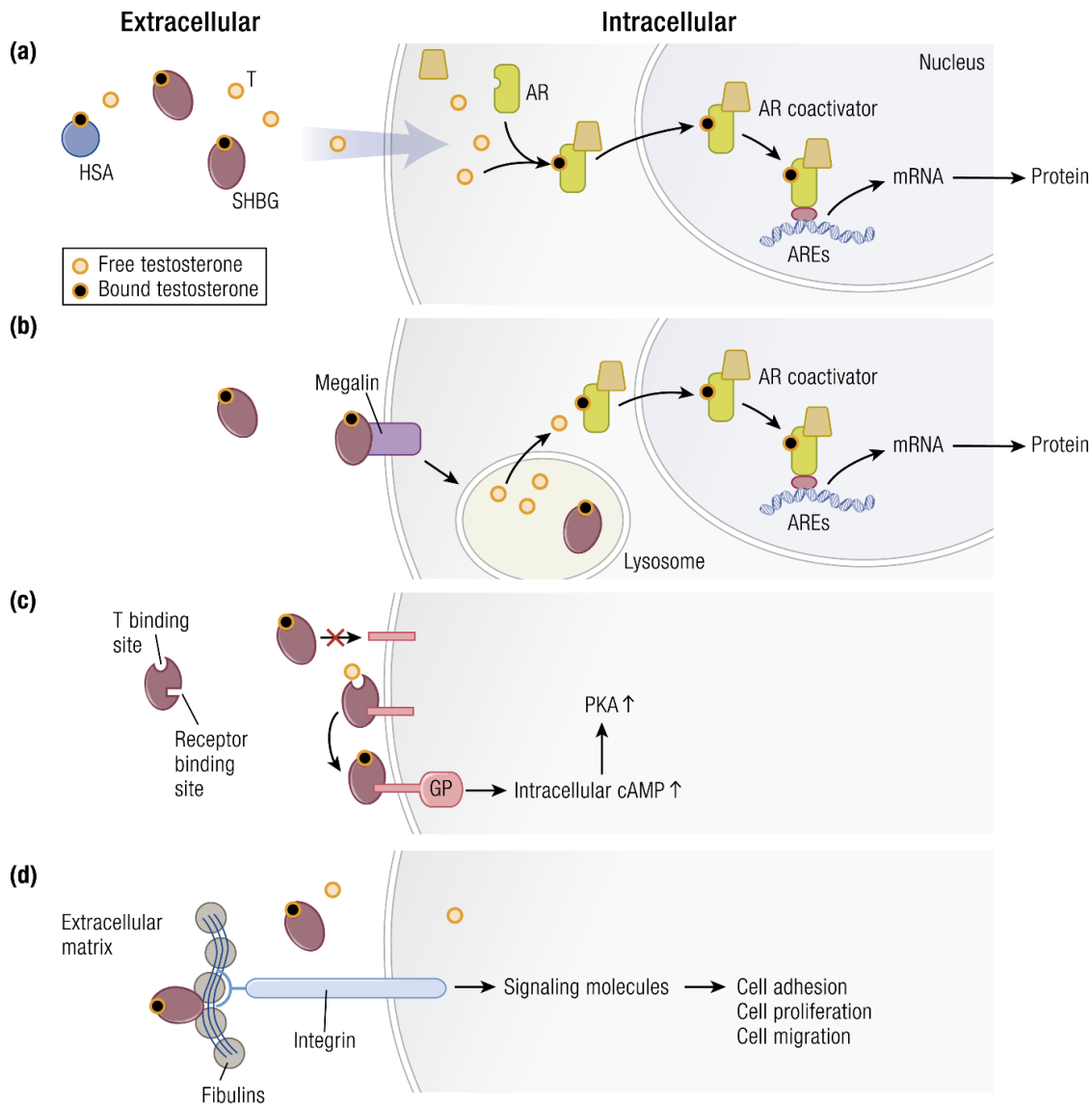


Figure 20. Model of different mechanisms for the cellular uptake of T and SHBG uptake and downstream signalling pathways.

a) Classical pathway depicting the “free” hormone hypothesis, **b)** Megalin-dependent endocytic pathway of testosterone entry, **c)** SHBG receptor-testosterone signalling pathway, and **d)** Steroid ligand-dependent interactions between SHBG and extracellular matrix.

Reproduced, with permission, from Goldman AL et al. (2017) [3].

Abbreviations: AR, Androgen receptor; AREs, Androgen response elements; cAMP, cyclic adenosine monophosphate; HSA, Human serum albumin; mRNA, messenger RNA; PKA, Protein kinase A; T, Testosterone

2.4.4.1. SHBG receptors (RSHBG) and cAMP-mediated signalling

SHBG has its own specific and high-affinity receptors (RSHBG) on the cell surface of various hormone-responsive tissues, which is thought to be a G-protein coupled receptor. It participated in a novel steroid-signalling system independently of the classical intracellular androgen receptors and was first demonstrated in 1984, and reasonably well characterised since then [3, 19, 74, 83, 84, 131, 136, 141].

The current evidence postulates that the specific RSHBG is capable of recognising ligands for SHBG and can bind unoccupied SHBG (i.e. steroid free SHBG) forming an SHBG-RSHBG complex. After exposure to an appropriate steroid, the anchored SHBG-RSHBG complex is activated and causes the downstream biological response via cAMP [84, 128, 131]. Activated SHBG-RSHBG complex leads to a rapid and sustained increase in cAMP, then activation of cAMP-dependent PKA [128, 131, 136, 137] and is shown in Figure 21. However, the final effect exerted might be agonist or antagonist and depends on target cell type and specific steroid that trigger activation of the complex [84, 131]. SHBG binds to these cells with high affinity, is not significantly internalised by them, and causes the intracellular accumulation of cAMP. The accumulation of cAMP is critically dependent upon the presence of a steroid with biological activity in the SHBG-binding site [139]. For instance, the complex with oestradiol has anti- oestrogenic response (inhibit cell proliferation) in human breast carcinoma cell line, MCF-7 [142, 143], whereas growth enhancing property has been shown by both oestradiol and DHT along with complex [81, 84, 136]. Although support for the presence of an RSHBG comes from observations that it induces cAMP-dependent, PKA in LNCaP prostate [83, 136, 142] and MCF-7 breast cancer cells [83, 142-144], has not been cloned - a major lack.

It has been reported that SHBG may have testosterone-independent actions to regulate the growth of prostate cancer (PCa) cells and to interact with the estrogen receptor [145].

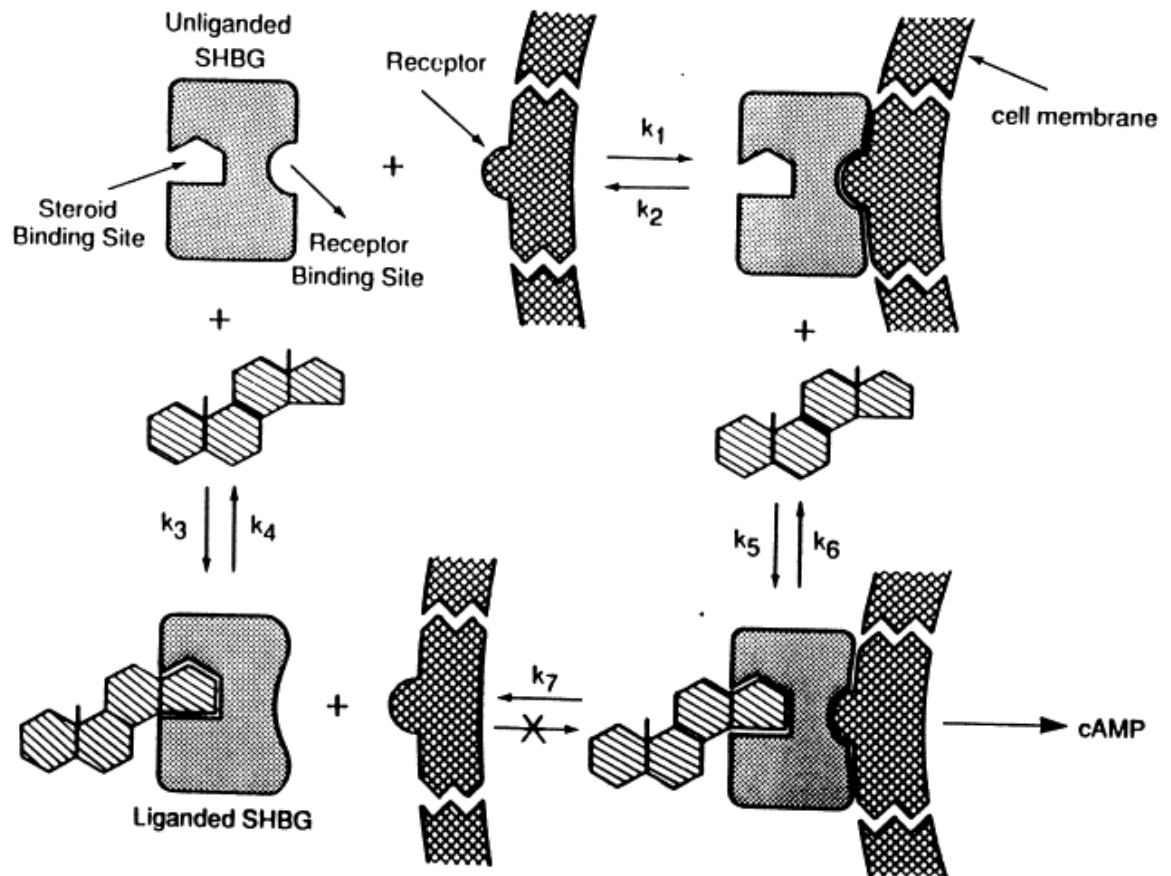


Figure 21. Schematic illustration of the SHBG-RSHBG signalling system presenting unliganded and liganded SHBG.

Reproduced, with permission, from Hryb DJ et al. (1990) [131].

Abbreviations: K , association constants; cAMP, cyclic adenosine monophosphate; SHBG, Sex hormone-binding globulin

2.4.4.2. *Biologic effect of RSHBG and cAMP-mediated signalling in prostate cells*

As will be described in later sections, SHBG has been demonstrated in tissue sections of human Prostate Cancer (PCa) as well as PCa cell lines PC-3, DU145 and human PCa cell line (LNCaP) by immunohistochemistry for protein examination and in situ hybridization for *SHBG* mRNA, suggesting that SHBG is locally regulated and produced [75, 146]. As briefly described earlier, SHBG participates directly in cellular signalling pathways in the prostate epithelial cell [147]. E2 also has been implicated in contributing to the growth and progression of human PCa through SHBG [148-150]. In this regard, E2 binding to SHBG has been shown to stimulate cAMP accumulation in an LNCaP [147, 150].

Details regarding the downstream effects of steroid signalling through SHBG exist but are not extensive. SHBG is most abundant in luminal epithelial cells of the prostate, whereas stromal cells from prostate explants possess the greatest SHBG binding ability, i.e., contain a specific receptor (RSHBG) on prostate cell membranes [83, 142, 147]. SHBG, in its steroid-free configuration, binds to a high affinity, but yet to be cloned membrane receptor (RSHBG), forming a bipartite complex (SHBG-RSHBG). SHBG-RSHBG complex causes a rapid and sustained increase in cAMP, then activation of cAMP-dependent PKA within the cancer PCa cells [142]. However, this primes, but does not activate downstream signalling, but depending on an appropriate androgen or oestrogen that binds to SHBG prior activation of SHBG-RSHBG complex determines the final effect [131, 136, 137, 139, 145, 151, 152]. The early steps of this pathway cascade rapidly and independently of the AR and ER. The testosterone exerts antagonist effects while DHT exerts either agonist or antagonist effects [19, 74, 83, 84, 139, 142]. Downstream effects reported in prostate cell lines include prostate specific antigen (PSA) induction, increased apoptosis, and regulation of cell growth; however, the overall biology of this pathway is not well understood [84, 142].

Prostate explants secrete PSA when treated with DHT; however, they do not know when treated with E2, which does not bind to the AR. When such explants were treated first with SHBG, and then with E2, they produced PSA at concentrations similar to those seen when they were exposed to DHT. Neither DHT or E2 alone nor SHBG alone duplicated these effects. Furthermore, anti-oestrogens did not block E2–SHBG–RSHBG-mediated PSA induction, whereas anti-androgens and a steroid (2-methoxyestradiol) did. These results indicate that E2–SHBG–RSHBG initiates ligandin dependent activation of PSA secretion [84, 148] as illustrated in Figure 20.

2.4.4.3. *Endocytic receptor megalin*

Experimental studies have shown that SHBG-bound androgens (either T or DHT) and E2 may be internalised by interacting with megalin, a member of the low-density lipoprotein (LDL) receptor-related protein (LRP) family [3, 135, 153]. The plasma membrane-anchored LRP serve as an endocytic receptor for a wide variety of extracellular ligands, facilitating their entry into target cells by endocytosis of the receptor-ligand complex [153]. Megalin facilitates the entry of SHBG-bound sex steroids by endocytosis into reproductive tissues through three step process: 1) SHBG-bound sex steroids are embedded in clathrin-coated pits in the plasma membrane of target cells, 2) formation of an endocytic vesicle and 3) finally lysosomal degradation of SHBG to release steroids for its genomic action [133, 135]. The presence and role of megalin are supported by the evidence that megalin knockout mice showed impairment in testicular function [135]. However, regardless of crucial mechanisms megalin mediated endocytosis of SHBG-sex steroids complex remains controversial [104, 154] and does not seem to be sufficiently supported by earlier evidence. However, Hammes and colleagues [135] proposed that megalin was essentially the RSHBG though no further data have appeared either to substantiate or refute their observations, and RSHBG has never been cloned. In recent years, RSHBG or megalin has attracted attention to the possibility of important

biological function, the specific down-stream effect of the SHBG-RSHBG complex merit further investigation, especially in hormone responsive tissues (Figure 20).

2.4.4.4. *Interaction of SHBG with extra-cellular matrix-associated proteins*

Steroid ligand-dependent interactions between SHBG and extra-cellular matrix (ECM) associated proteins contribute to the extravascular sequestration of SHBG in some tissues, such as prostate, the breast and endometrial stroma. According to this model, ligand-dependent interactions between SHBG and ECM modulate their binding to various signalling molecules, to modify signalling pathways that regulate cell adhesion, proliferation, and migration [3], as illustrated in Figure 20.

2.4.4.4.1. *Interaction with fibulins*

The biochemical characterisation of the RSHBG has never been recognised, but an alleged interaction of SHBG with ECM associated proteins (e.g. Fibulins) was suggested [39, 155]. The Fibulins are a family of secreted glycoproteins, comprising several different members (e.g. Fibulins-1D, Fibulins-4, Fibulins-7, etc.) that are localised in the basement membrane and ECM [156]. The amino-terminal LG-domain of SHBG in the stromal matrix of some tissues (e.g. endometrium, breast) interacts with these ECM-associated proteins, Fibulins 1D and Fibulin-2; allow SHBG to reach extracellular space from circulation. The final effect of SHBG, for example, in endometrium, was cell adhesion, migration, proliferation, and differentiation [155, 157]. Fibulin-1 are highly expressed in cancer cells, and interactions of SHBG with Fibulin-1D and two was promising in breast cancer [156, 157]. The biological significance of this remains to be determined, but it provides *in vivo* evidence that SHBG can act in extravascular compartments, extending its functions beyond that of a transport protein that regulates free sex steroids levels in the blood. Given, the prostate is hormone responsive tissue, the interaction of SHBG and Fibulins are good candidates to be studied specifically in PCa cells.

2.4.4.4.2. *Interaction with kallikrein (KLK) in prostate epithelium*

Kallikrein-related peptidases (KLKs) are serine proteases, which are synthesised in hepatocytes and secreted into the blood. PCa is accompanied by deregulated expression of KLKs, and prostatic KLKs was demonstrated to be useful biomarkers for PCa, most notably KLK3 or prostate-specific antigen (PSA), which is the 'gold standard' clinical biomarker for PCa detection. These KLKs comprises several different members KLK1-15 that are abundant in biological fluids, thus have a clinical potential to improve PCa diagnosis and prognosis [158]. SHBG has been shown to interact with KLK4 and KLK14 in glutathione S-transferase interaction analyses. It has been proposed that KLK4 and KLK14 cleave human SHBG at unique sites and in an androgen-dependent manner and demonstrate that a stable fragment derived from proteolysis of steroid-bound SHBG retains binding function. KLK4 separated androgen-free SHBG into its two laminin G-like (LG) domains that were subsequently proteolytically stable even after prolonged digestion, whereas a catalytically equivalent amount of KLK14 reduced SHBG to small peptide fragments over the same period. Thus, KLKs-SHBG interaction might have a role in the regulation of bioavailability of androgens in the prostate epithelium cells [159] and merits further investigation.

2.5.SHBG: measurement, reference values and half-life

The source of plasma SHBG is exclusively the liver, and the measurement of blood levels of SHBG, albumin and total testosterone are used together to determine 'free' or bioavailable testosterone [100], although the utility of free or bioavailable testosterone is debatable. Measurements of plasma SHBG have also been used as a biomarker of androgen sensitivity. Changes in SHBG are associated with parallel changes in TT, limiting its diagnostic utility. Therefore, the measurements of TT is affected by the concentration bound to SHBG. It essential to measure serum SHBG concurrent with TT in men to evaluate hypogonadism and has a central importance in the clinical evaluation of male reproductive function [57, 130]. The most recent Australian position statement on male hypogonadism [160] have proposed that 1) rather than relying on FT measured by suboptimal methodology, at high and low ends of the reference interval, serum TT concentrations should primarily be interpreted in relation to SHBG concentrations; and 2) at high and low ends of the reference interval, serum testosterone level must be interpreted in relation to serum SHBG concentrations [160].

Although the plasma concentrations of SHBG in a given individual is relatively constant and is unrelated to meals or time of day, there is a 10-fold variation among individuals that are influenced by developmental, genetic, hormonal, metabolic, and nutritional factors [161].

2.5.1. Measurement

Immunofluorometric assay (IFA) and chemiluminescent assay (CLIA) or by dihydrotestosterone binding assays are the recommended methods for estimation of serum SHBG, as proposed by National Institute for Biological Standards and Control (NIBSC) [162-164].

For all recommended methods, plasma samples with EDTA should not be used, because of EDTA chelates calcium ions that are critically important in stabilising both the structure and function of SHBG [54, 100]. SHBG is stable in heparinised plasma or serum for many years when stored at -20°C, and is not adversely affected by freezing and thawing [100, 165, 166].

2.5.2. Reference values

Adult Males: 10 to 57 nmol/L

Children (Males):

Tanners Stages*	Mean Age	Reference Range (nmol/L)
Stage I	7.1	31-167
Stage II	11.5	49-179
Stage III	13.6	5.8-182
Stage IV	15.1	14-98
Stage V	18.0	10-57

*Puberty onset (transition from Tanner stage I to Tanner stage II) occurs for boys at a median age of 11.5 (+/-2) years. For boys, there is no definite proven relationship between puberty onset and body weight or ethnic origin. Progression through Tanner stages is variable. Tanner stage V (young adult) should be reached by age 18 [167].

Plasma levels of SHBG are usually about twofold higher in women than in men, but far fewer SHBG steroid-binding sites are occupied in women than in men, in whom SHBG is largely occupied by testosterone [100].

2.5.3. Biological half-life

The plasma half-life of SHBG in healthy male subjects is about 38 hours [64]. The glycosylation prolongs serum SHBG half-life and protects against proteases, a more significant impact on their biological activity and the plasma clearance rate, without influencing its protein structure and steroid-binding properties [10, 39].

2.5.4. Metabolic clearance rate

The metabolic clearance of SHBG from the intravascular compartment is biphasic, with a rapid initial distribution from vascular compartment into extracellular space (half-life of a few hours), followed by a slower degradation phase (half-life of several days) [100, 138].

REGULATION OF PLASMA SHBG

3. Factors affecting plasma SHBG

Plasma SHBG varies considerably between and within individuals. Several factors have been suggested to influence the levels of SHBG, most of which are believed to affect the rate of SHBG synthesis and secretion from the liver rather than the rate of SHBG elimination from plasma [11, 24, 29, 97, 100].

Abnormal plasma SHBG levels have been linked to the risk for chronic diseases and their associated pathologies [3, 53, 54, 57, 87, 89, 100, 138, 161]. Higher circulating SHBG is favourably linked with lower cardiometabolic risk and wide range of cardiometabolic risk factors, including lipoprotein lipids and subclasses, fatty acids, amino acids and inflammation-linked glycoproteins [29, 57, 168]. Body composition has been thought to be a major determinant of circulating SHBG concentrations and an inverse correlation between several body compositions and anthropometric measures including percentage total and trunk fat mass, body mass index (BMI), waist circumference (WC), and waist-to-hip ratio (WHR) and plasma SHBG levels has been consistently reported [169-173]. However, in recent years, it has been demonstrated that liver fat content rather than total body or visceral fat is the major determinant of circulating SHBG [174-176]. Low serum SHBG concentrations in over-weight individuals are a biomarker for insulin resistance [161, 177-179], MetS [180-183] and are predictive of type 2 diabetes (T2D) [168, 177, 184-186], and cardiovascular disease (CVD) risk [187-190], as well as CVD events [184, 191, 192], that accompanies these conditions. Higher adiposity has been demonstrated to be causal for lower levels of circulating SHBG [171, 175]; however, this mediation is insufficient to explain the strong observational associations between SHBG and circulating metabolites independent of obesity and insulin resistance [57, 161, 168]. A greater understanding of the main mechanisms involved in SHBG production and clearance is required. *How can we transfer the new concepts in SHBG regulation to clinical practice? Is integral to understanding their regulation and role as a marker of chronic disease risk?*

Pioneering studies carried out in cellular and animal models, as well as epidemiological investigations, have identified that SHBG production is under multifactorial regulation with a broad range of nutritional, metabolic hormonal, and genetic factors [11, 24, 29, 57, 100, 193]. The proposed molecular mechanism involves the modulation of the nuclear hormone receptor (NHR) family, hepatic nuclear factor 4 α (HNF4- α) [29] and peroxisome proliferator-activated receptor γ (PPAR- γ) [33] indirectly through hepatic lipid metabolism. In this regard, adiponectin increases SHBG production by activating adenosine monophosphate-activated protein kinase (AMPK), which reduces hepatic lipid content and increases HNF4- α levels [93], whereas TNF- α regulates SHBG production by decreasing HNF4- α levels indirectly through the activation of nuclear factor kappa B (NF- κ B) [91, 92]. Moreover, monounsaturated fatty acid (MUFA) such as oleic acid upregulates SHBG production by HepG2 cells through the reduction of PPAR- γ levels [95]. The molecular mechanisms of regulation of hepatic SHBG by various factors via their receptors and activating various signal cascades by affecting HNF-4 α or PPAR γ are illustrated in Figure 22. Several factors have been suggested to influence the levels of SHBG by few cross-sectional and prospective cohort studies. However, various factors effects not assayed in these large population cohorts could potentially drive the observed metabolic signature of SHBG, the molecular underpinnings remain elusive. Moreover, SHBG is not extensively studied, especially in the context of its regulation and its epidemiological associations in large prospective cohort studies. Better knowledge of factors controlling the production of SHBG is important not least because of strong evidence for the relationship between SHBG and cardiometabolic disorders.

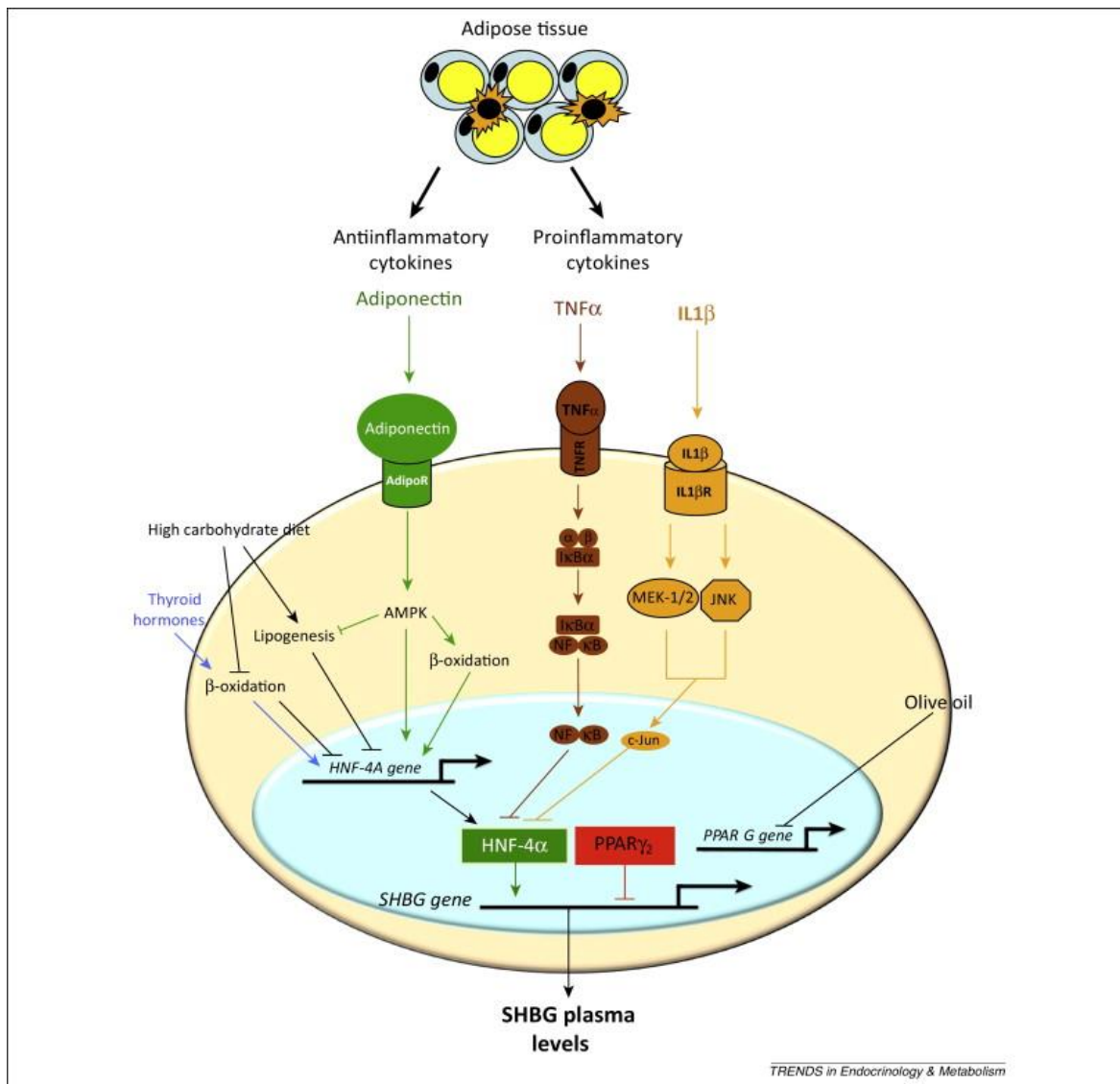


Figure 22. Molecular mechanisms of regulating hepatic SHBG expression by various factors.

Reproduced, with permission, from Simo R et al. (2015) [29].

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; HNF4- α , hepatic nuclear factor 4 α ; IL1 β , Interleukin 1 β ; JNK, c-Jun N-terminal kinase; MEK; MAPK kinase-1/2; PPAR- γ , peroxisome proliferator-activated receptor γ ; TNF, tumour necrosis factor- α

3.1.Ageing and SHBG

SHBG circulates at high concentrations in childhood, reach a plateau, decreases with puberty, and then increases again with aging [28, 194], perhaps optimising steroid bioavailability. During infancy and childhood, SHBG concentrations are elevated in both boys and girls and decrease to a greater extent in boys than girls as they approach puberty. As a result, SHBG concentrations are about two times lower in men than women [100, 195]. Circulating SHBG concentrations among middle-aged to elderly men are illustrated in Figure 23.

Epidemiological studies have consistently shown a gradual increase in SHBG levels in men, principally after middle age and are summarised in Table 1.

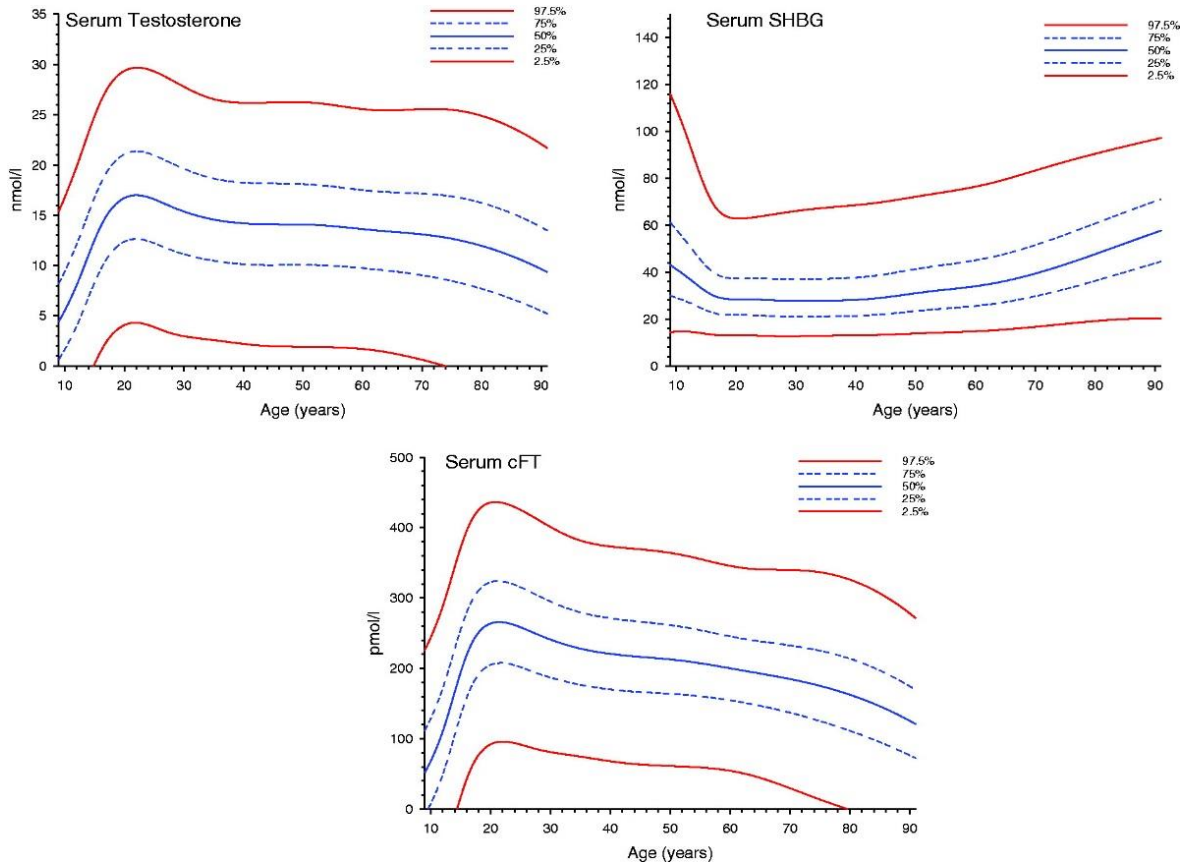


Figure 23. Serum SHBG, circulating TT and cFT concentrations across the lifespan in Men.

Smoothed centile plots of circulating testosterone (left panel), SHBG (middle panel) and cFT (right panel) in males plotted for each year of age at centiles 2.5%, 25%, 50% (median), 75% and 97.5%. The median plot is a solid [blue] line, 25% and 75% in broken [blue] lines, and 2.5% and 97.5% in solid [red] lines.

Reproduced, with permission, from Handelsman DJ et al. (2016) [195].

Abbreviations: cFT, calculated free testosterone; SHBG, Sex hormone-binding globulin;

TT, total testosterone

Table 1. Main outcomes from prospective cohort studies investigating SHBG and aging in men.

Author	Study design	Study population	Age of participants	Sex steroids included	Sex steroid assay	Primary endpoint*	Main findings
Fabbri E et al. (2016) [196]	L (BLSA)	444	30-96	TT, SHBG	LC-MS/MS	Aging	– In men, SHBG increases with age over the life span, and the rate of increase is steeper after the age of 70 years.
Hsu B et al. (2015) [197]	L (CHAMP)	1367 at two years 958 at five years	≥70	TT, DHT, E2, E1, SHBG	LC-MS/MS	Cognitive Decline	– SHBG concentrations increased with age.
Liu PY et al. (2007) [198]	L (the Busselton cohort)	480	≥18	TT, SHBG	CLIA	Aging	– Plasma SHBG increases while TT falls with ageing.
Andersson AM et al. (2007) [199]	L (MONICA & Inter99)	5350	≥30	TT, SHBG	Fluoro immunoassay	Aging	– SHBG showed a significant increasing trend with ageing.
Feldman HA et al. (2002) [200]	L (MMAS)	1156	≥40	TT, DHT, E1, SHBG	RIA	Aging	– SHBG rise by 1.3%/year longitudinally at, a rate similar to the cross-sectional rise.
Leifke E et al. (2000) [201]	CS	572	20-80	TT, E2, E1 SHBG	RIA	Aging	– SHBG concentrations increased with age.

		(None obese)					– Potential confounding effects of adiposity and health status was accounted (BMI≤ 30)

*the main endpoint to be investigated about aging

Abbreviations: CLIA, Chemiluminescent immunoassay; CS, Cross-sectional; DHT, Dihydrotestosterone; E2, Oestradiol, E1, Oestrone; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; L, Longitudinal; RIA, Radioimmunoassay; SHBG, Sex Hormone-Binding Globulin; TT, Total testosterone

BLSA, Baltimore Longitudinal Study of Aging; CHAMP, Concord Health and Ageing in Men Project; MMAS, Massachusetts Male Aging Study; MONICA I, II and III, three Danish Monitoring Cardiovascular risk factor surveys

3.2. Effect of dietary and nutritional factors on SHBG

Several reports in the literature have previously addressed the relationships between dietary composition and SHBG levels with contradictory results.

3.2.1. Effect of fatty acid on hepatic SHBG: *in vitro*

It has been demonstrated that monounsaturated fatty acid (MUFA) such as oleic acid upregulates SHBG production by HepG2 cells through the reduction of PPAR- γ levels (Figure 21). In this study they have demonstrated that oleic acid treatment did not influence HNF-4 α gene expression and HNF-4 α protein levels instead reduces PPAR- γ gene expression and PPAR- γ protein levels. They also shown that polyunsaturated fatty acid (PUFA) such as linoleic acid does not have any effect on SHBG production in HepG2 cells [95]. Another study from same research group have demonstrated that palmitate, a saturated fatty acid, downregulates SHBG production by decreasing PPAR- γ levels in HepG2 cells [94, 96].

3.2.2. Effect of monosaccharides and disaccharides on hepatic SHBG: *in vitro*

Elevated monosaccharides (glucose or fructose) and disaccharide (sucrose) play an essential role in the downregulation of SHBG production in the liver *in vivo* and *in vitro* [57, 94]. This occurred via downregulation of hepatocyte nuclear factor-4 α (HNF-4 α) and replacement of HNF-4 α by the chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) at a *cis*-element within the human *SHBG* promoter, coincident with the repression of its transcriptional activity (Figure 22). The dose-dependent reduction of HNF-4 α levels in HepG2 cells after treatment with glucose or fructose occurred in concert with parallel increases in cellular palmitate levels and could be mimicked by treatment with palmitoyl-CoA. Moreover, inhibition of *de novo* lipogenesis prevented Monosaccharide-induced downregulation of HNF-4 α and reduced SHBG expression in HepG2 cells. Thus, Monosaccharide-induced lipogenesis reduced hepatic HNF-4 α levels, which in turn, attenuated SHBG expression [24, 94].

3.2.3. Dietary and nutritional factors and SHBG

Regarding protein intake, it has been reported that a low-protein diet in elderly men increases SHBG levels [202]. While feeding a diet rich in carbohydrates does not affect SHBG levels [202]. Moreover, changing the diet to low fats (less than 20 g fat per day) from high fat for two weeks resulted in a significant increase in SHBG levels in men [203]. It has been reported that a high-fibre diet decreases SHBG levels [202]. Associations between few dietary and nutritional factors and SHBG are summarised in Table 2.

Table 2. Associations of dietary and nutritional factors and SHBG in men.

Reference (Year)	Study design	Sample size (Age, Years)	Dietary and Nutritional factors	Main findings
Frey et al. (2018) [204]	CS	1,410 (≥20)	Coffee and soft drink consumption	– Coffee consumption was positively associated with SHBG concentration ($P_{trend} = 0.045$) in multi-adjusted model (age, race/ethnicity, WC, BMI, physical activity, cotinine concentration, alcohol consumption. Further mutually adjusting TT and E2 attenuated the association ($P_{trend}=0.14$). – No association with SHBG was observed for soft drink consumption in any model.
Saez-Lopez el al. (2014) [95]	CS (a part of the experimental study)	315 (Mean±SD=46.8±14.4)	Dietary MUFA (Olive oil rich in oleic acid) Serum concentrations: MUFA, PUFA, Oleic to Stearic fatty acid ratio	– SHBG levels was positively associated with serum phospholipid MUFA ($r=p < 0.001$) and ratio of oleic to stearic fatty acid ($r = 0.34$; $p < 0.0001$) in age and BMI adjusted model. – SHBG levels correlated positively with serum phospholipid MUFA ($r = 0.35$; $p < 0.0001$) and negatively with phospholipid saturated fatty acids ($r=-0.[205]17$; $p = 0.003$). – No significant correlation between SHBG levels and either PUFA $n-6$ ($r = -0.10$; $p =0.07$) or PUFA $n-3$ ($r = -0.02$; $p = 0.73$) in serum phospholipids.
Longcope et al. (2000) [202]	CS	1563 (40-70)	Dietary Carbohydrate Protein Fat (animal, vegetable,	– SHBG was positively associated with fibre intake and negatively associated with protein and animal fat intake. – Total caloric intake, carbohydrate, alcohol, and vegetable fat intake showed no association with SHBG. – Multiple regression analysis:

			and total), Dietary fibres, Alcohol intake	• No association with vegetable and total fat; • None of the three fat variables (animal, vegetable, and total) was associated with SHBG.

Abbreviations: CS, Cross-sectional; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid

3.3. Metabolic and hormonal factors

Circulating SHBG is intrinsically linked with the complex myriad of metabolic perturbations accompanying insulin resistance and adiposity [161, 168]. Comprehensive metabolic profiling here revealed that higher circulating SHBG concentrations in young adults were related to numerous metabolic deviations characterising a less risk-prone cardiometabolic profile [53, 54, 57, 87, 89, 168].

3.3.1. SHBG with systemic lipid and metabolite measures

Recently a high-throughput serum nuclear magnetic resonance (NMR) metabolomics platform was used to quantify 69 metabolites that represent a broad molecular signature of systemic metabolism [168]. They reported that higher circulating SHBG is favourably linked with a wide range of cardiometabolic risk factors, including lipoprotein lipids and subclasses, fatty acids, amino acids and inflammation-linked glycoproteins and is illustrated in Figure 24. The novel, strong associations were observed for various fatty acids, branched-chain and aromatic amino acids, and ketone bodies. The magnitudes of the cross-sectional associations of SHBG with the metabolic measures remained broadly similar when further adjusting for BMI, insulin and testosterone [168].

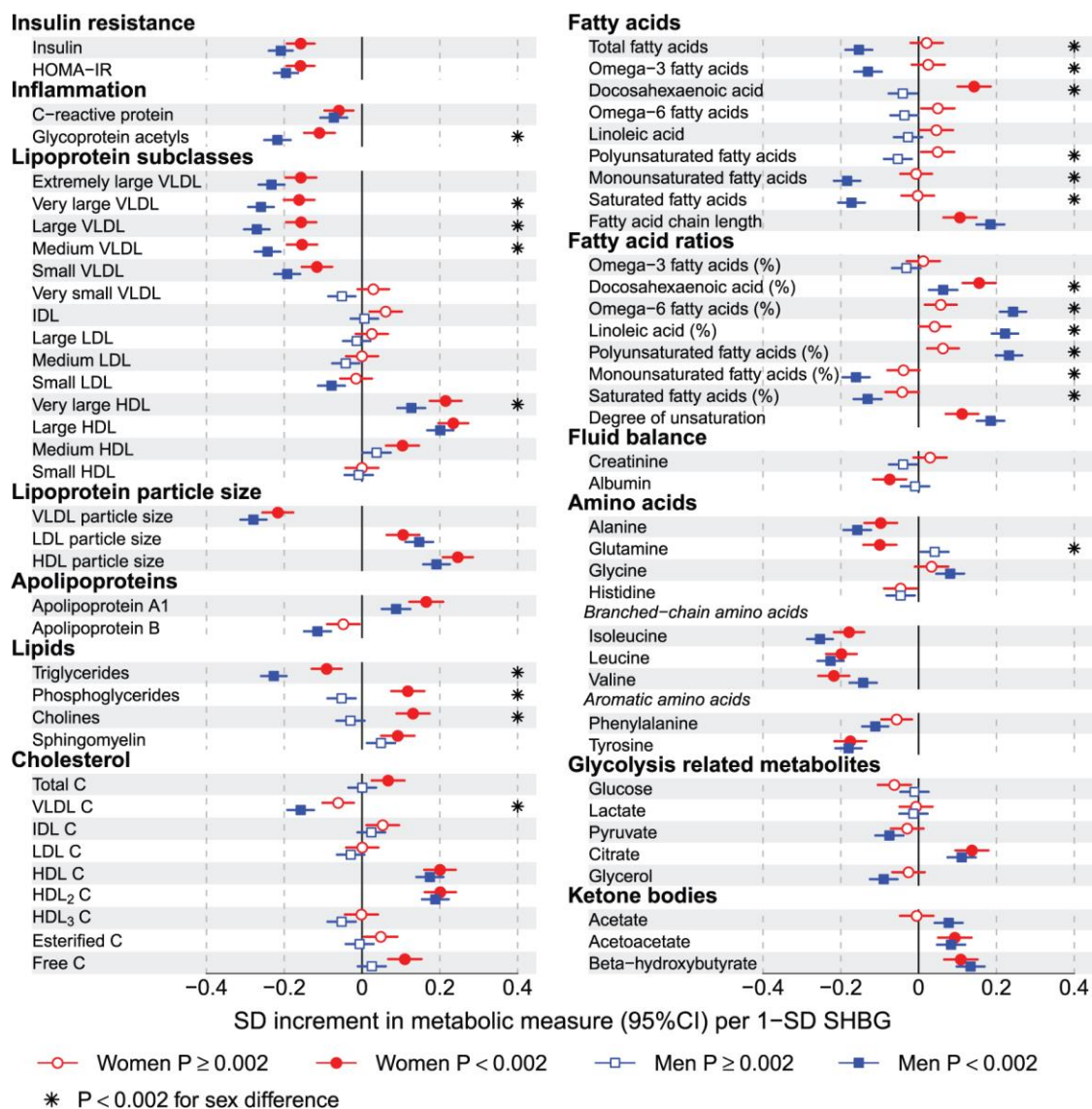


Figure 24. Cross-sectional associations of SHBG with the 69 metabolomics measures (NMR metabolomics data) and insulin, HOMA-IR, and CRP are illustrated for men and women.

Open and closed symbols indicate $P \geq 0.002$ and $P < 0.002$, respectively. Sex differences with $P < 0.002$ are marked by asterisks.

Reproduced, with permission, from Wang Q et al. (2015) [168].

Abbreviations: C, Cholesterol; CRP, C-reactive protein; HDL, High-density lipoprotein; HOMA-IR, Homeostasis model assessment-insulin resistance; IDL, Intermediate density lipoprotein; LDL, Low-density lipoprotein; NMR, Nuclear magnetic resonance; VLDL, Very low-density lipoprotein

3.3.2. Effect of fasting and refeeding on SHBG

There is evidence that circulating SHBG concentrations are influenced by fasting [206] and refeeding [207, 208]. SHBG represents a reliable value in assessment of refeeding in diseases such as anorexia nervosa and kwashiorkor, while not in marasmus [193, 209, 210]. In the latter case, SHBG can be used to differentiate it from the diseases above [209]. It was found that restoring body mass in anorexic individuals was followed by a decrease in SHBG serum levels [209, 211] while SHBG concentrations are inversely correlated with weight gain [212, 213].

3.3.3. Body compositions and SHBG

As will be described in later sections, liver fat accumulation as a consequence of dietary factors is the main reason accounting for the reduction of plasma SHBG levels during obesity development [87]. This decrease in plasma SHBG is caused by a reduction in HNF-4 α and the increase in PPAR γ levels [87]. People with normal or low body weight have levels of high levels of SHBG in plasma, while these levels are low in people who are overweight [205]. The body mass index (BMI) is considered a major determinant of SHBG plasma concentrations [29, 57, 87, 91]. The summary of the relationships between various measures of body composition and SHBG are shown in Table 3.

Table 3. Association between serum SHBG levels and body compositions in men.

Reference (Year)	Study design	Sample size (Age, Years)	Body composition measures	Main findings
Gagnon SS et al. (2018) [214]	CS	846 (18-48)	BIA as well as BMI & WC	– $\uparrow$ BMI = $\downarrow$SHBG ($\beta = -0.435$)* – WC, % body fat, fat free mass $\leftrightarrow$ SHBG.
He Z et al. (2018) [205]	CS	242 (17-65)	CT scan	– $\uparrow$ body composition & fat distribution = $\downarrow$SHBG – $\uparrow$ fat-free mass/H^2 = $\downarrow$SHBG – Fat free mass $\leftrightarrow$ SHBG.
Kim C et al. (2017) [169]	CS & L (DPP)	246 (25- $\geq$ 60)	CT scan as well as BMI	– SAT & VAT were not associated with SHBG at both cross-sectional examination changes in both VAT and SAT were inversely related to changes in SHBG
Lee K et al. (2015) [170]	CS	1083 (29-79)	DXA	– All body composition measures were inversely associated with the levels of testosterone and SHBG, with an exception for percentage total lean mass and skeletal muscle index.
Cooper LA et al. (2015) [215]	CS	3671 (20-98)	BMI	– Each unit $\uparrow$BMI = $\downarrow$ SHBG by 1.26
Gates MA et al. (2013) [171]	L (4.8 years) (BACH)	821 (30-79)	DXA	– $\uparrow$ Fat mass, BMI, WC & WHR = $\downarrow$SHBG at baseline * but no correlation on follow up.
Bonnet F et al. (2013) [175]	CS	233 (27-77)	MRI MFGRE	– BMI was not associated with SHBG concentrations ($\beta = 0.04$, $P = 0.53$)

Trabert B et al. (2012) [172]	CS (NHANES)	898 (20-90)	DXA as well as BMI & WC	– ↑ Fat mass, BMI, WC & WHR = ↓SHBG at baseline * but no correlation on follow-up.
Cao J et al. (2012) [216]	CS	314 (65-92)	BMI & WC	– BMI is inversely associated with SHBG (β: -0.163; p = 0.02) in multiple regression analysis. – WHR was also inversely associated with SHBG (β: -0.24; $p < 0.01$) but reported in the univariate model only.
Vanbillemont G et al. (2012) [217]	CS	677 (20-45)	DXA	– Increased percent body fat was associated with low SHBG (β = -1.11; $p < 0.01$) – 1 kg ↑ fat mass (g) = ↓SHBG (β = -0.59 $p < 0.01$) – 1 kg ↑ Total fat mass (g) = ↓SHBG (β = -0.29 $p < 0.01$)
Rohrmann S et al. (2011) [218]	CS (NHANES III)	1265 (20-90)	BMI, WC and % body fat (BIA)	– SHBG is more strongly affected by abdominal than overall obesity. – Each unit change in body fatness was associated with ↑ in SHBG by 0.7 unit by BMI, 1.6 unit by WC and 0.9 by % body fat.
Peter A et al. (2010) [173]	CS & L (9 months)	90 (19-69)	BMI, WC, MR tomography, Total body fat, Visceral fat, H-MR Spectroscopy, Liver fat	– ↑Body weight, BMI or whole body fat mass, whole body lean mass, truncal fat mass & appendicular fat mass = ↓SHBG * – Liver fat was the strongest determinant ($p < 0.001$) of circulating SHBG while the total body and visceral fat were not independent determinants.
Osuna JA et al. (2006) [219]	CS	77 -Nor wt 21, Over wt 31, Obese 25 (20-60)	BMI, WC and % body fat	– ↑ extent of each measure of body fatness BMI (r = -0.334; $p < 0.01$) & WC (r = -0.332; $p < 0.01$) = ↓SHBG concentration

Longcope (2000) [202]	C	CS (MMAS)	1552 (40-70)	BMI & WHR	– ↑ BMI & WC = ↓SHBG (r=- 0.334)*
Vermeulen A et al. (1996) [220]		CS	250 (25-100)	TT	– No Association with BMI & WHR
Haffner SM et al (1993) [221]		CS	178	BMI & WHR	– Each unit ↑BMI= ↓ SHBG by 5.7 * – Each unit ↑WHR= ↓ SHBG by 7.1 *

Abbreviations: BIA, bioelectrical impedance analysis; CS, Cross-sectional; H-MR, hydrogen 1 [¹H]) magnetic resonance (MR) spectroscopy; L, Longitudinal; MRI, Magnetic resonance imaging; MFGRE, multiecho fast gradient-echo sequence; SAT, Subcutaneous adipose tissue; T, Total testosterone; VAT, Visceral adipose tissue; WHR, Waist-to-hip ratio

BACH, Boston Area Community Health; DPP, Diabetes Prevention Program; MMAS, Massachusetts Male Aging Study; NHANES, National Health and Nutrition Examination Survey

*P < 0.05

3.3.3.1. *Changes in visceral adiposity, subcutaneous adiposity and SHBG*

Many observational studies consistently show that adiposity is a major determinant of low SHBG, even overriding the effects of age and T. Changes in adiposity and SHBG occurred concurrently and is likely bidirectional [87, 169, 173, 175]. However, the extent to which changes in specific fat depots affect SHBG is poorly understood. The longitudinal studies assessing both SHBG and specific fat depots are sparse and are summarised in Table 4.

Table 4. Association between changes in visceral adiposity, subcutaneous adiposity and serum SHBG concentrations in men.

Reference (Year)	Study design	Sample size (Age, Years)	Body composition measures	Main findings
Kim C et al. (2017) [169]	CS & L (DPP)	246 (25-≥60)	CT scan as well as BMI	- SAT & VAT were not associated with SHBG on the cross-sectional analysis ($P>0.05$). - Changes in both VAT and SAT were inversely related to changes in SHBG ($P<0.02$).
Mongraw-Chaffin ML et al. (2015) [222]	L (MESA)	936 (44-84)	CT scans	In the multi-adjusted model, higher amounts of visceral fat were associated with lower SHBG in men ($P<0.05$).
Shi Z et al. (2013) [223]	L (MAILES)	1588 (35-80)	DXA Scans	The development of obesity (-0.55 nmol/L/y vs non-obese at both points, $P<0.05$) or central obesity (-0.55 nmol/L/y vs non-central obese at both points, $P<0.05$) was associated with a decrease in SHBG.
Gates MA et al. (2013) [171]	L (4.8 years) (BACH)	821 (30-79)	DXA scans	↑ Fat mass, BMI, WC & WHR = ↓ SHBG at baseline ($P<0.05$) but no correlation on follow up.
Rohrmann S et al. (2011) [218]	CS (NHANES III)	1265 (20-90)	BMI, WC and % body fat (BIA)	- SHBG is more strongly affected by abdominal than overall obesity. - Each unit change in body fatness was associated with ↑ in SHBG by 0.7 unit by BMI, 1.6 unit by WC and 0.9 by % body fat.
Peter A et al. (2010) [173]	Lifestyle intervention	118 (19-69)	Total body, visceral, and liver fat	- Liver fat, but not VAT or total body fat is negatively correlated with plasma SHBG levels - In a multivariate analysis, change in liver fat correlated with change in circulating SHBG, independently of sex, age, and changes in total body and visceral fat ($r=-0.29$, $P<0.002$). In

				this model, changes in total body and visceral fat did not correlate with changes in circulating SHBG anymore (both $P > 0.24$).
Nielsen TL et al. (2007) [224]	L (1 year) (OAS)	783 (20-29)	CT scan MRI	– In multiple regression analyses, CFM was independently correlated with reduced SHBG. LEFM correlated positively with SHBG. – Similarly, the SAT was independently related with reduced SHBG, while VAT was not. CFM, LEFM and SAT are independent contributors to the variation in SHBG.
Derby CA et al. (2006) [225]	L (8.9 years) (MMAS)	942 (40-70)	BMI WC & WHR	– $\uparrow$ BMI, WC & WHR at baseline = $\downarrow$ SHBG at baseline * – Also, $\uparrow$ BMI, WC & WHR at follow-up = $\downarrow$ SHBG at follow-up *
Mohr BA et al. (2006) [226]	L (8.8 years) (MMAS)	1009 (40-70)	BMI & WHR	– During 8.8 years period, 9% of men became overweight, and another 9% became obese. – Men who became overweight had significantly lower SHBG (T2 only=0.0007). – A similar trend was observed for obesity change (T2 only=0.0006).

Abbreviations: CS, Cross-sectional; CFM, central fat mass; DXA, dual-energy x-ray absorptiometry; L, Longitudinal; LEFM, lower extremity fat mass;

MRI, magnetic resonance imaging; SAT, Subcutaneous adipose tissue; VAT, Visceral adipose tissue

BACH, Boston Area Community Health; DPP, Diabetes Prevention Program; MMAS, Massachusetts Male Aging Study; MESA, Multi-Ethnic Study of Atherosclerosis; NHANES, National Health and Nutrition Examination Survey, OAS, Odense Androgen Study

* $P < 0.05$

3.3.3.2. *Effects of resistance training and weight reduction programme*

Several studies have shown that weight loss through caloric restriction and behavioural modification, metformin treatment in combination with lifestyle changes, or following bariatric surgery increases serum SHBG levels, and a few studies have also shown the associated improvement in insulin resistance (IR). Improvement in IR with resistance training is also associated with a rise in SHBG, although BMI was unchanged. The summary of the effect of resistance training and weight reduction programme on SHBG are shown in Table 5.

Table 5. Changes in SHBG levels undergoing dietary interventions or resistance training studies about body compositions (adiposity measures).

Reference (Year)	No.of participants (Age, years)	Study design	Body composition measurements	Intervention	Main findings
He Z et al. (2018) [205]	242 (17-65)	Exercise training	CT scans	20 weeks	– No association was detected between baseline SHBG and changes in adiposity traits in response to 20 weeks of exercise.
Wang FM et al. (2017) [227]	25 boys (11.8±2.1)	Interventional	Body composition analyser	Eight week weight reduction program	– No correlation between Δ SHBG and changes in anthropometric measurements in the boys after adjusting for age and Δ BFW ($P>0.05$). – A trend toward an increase in serum SHBG concentrations in boys ($P=0.39$) participated in a weight reduction program.
Drain JR et al. (2017) [228]	26 controls 25 experimental group	Interventional Case-control	DXA scan	12-week basic military training	– Men in the experimental group experienced significant increases in SHBG compared to control and main effect over time ($P < 0.01$). These changes were associated with lean and fat mass in men ($P < 0.05$).
Sarwer DB et al. (2015) [229]	32 (≥ 18)	Prospective cohort	BMI WC	Weight loss with bariatric surgery	– Men experienced significant increases from baseline for SHBG (P

		(Postoperative F/U assessments each year for four years)			<0.001) at all postoperative assessments. – The increases in SHBG remained significantly improved from preoperative levels at postoperative year 4.
Bekaert M et al. (2015) [230]	14 obese men (32-64)	Interventional	BIA The percentage of fat of body weight (fat %)	Two years post bariatric surgery	– SHBG levels increased from 27.9 to 52.4 nmol/L (P<0.001).
Hvid T et al. (2015) [231]	31 (21-39)	Interventional	DXA scan	Pre- & Post-endurance training	– Pre-training levels of SHBG were not associated with exercise-induced changes in BMI, FM or trunk FM in non-obese men.
Roberts CK et al. (2013) [212]	49 (36 RT group and 13 Control) (18-35)	Interventional [Resistance training (RT)] in overweight/obese men.	DXA scan	Men were randomised into an RT (12 weeks of training, 3/week) or control group (C, 12 weeks no training),	– Compared to C, Resistance training causes ↑SHBG * – In overweight/obese young men, RT increases SHBG.
Wang P et al. (2013) [213]	48 (24 continued to lose weight (WL) & 24 regained weight (WR))	Interventional	BMI WC Body fat %	An active WL phase of 8 weeks with a low-calorie diet and a follow-up phase of 26 weeks.	– Active weight loss causes ↑ SHBG (8 weeks); SHBG ↓during follow-up phase (26 weeks)* – Decrease of SHBG during the follow-up period was more pronounced in WR than that in WL (p<0.008).

	(37-53)				
Birkebaek NH et al. (2010) [232]	51 (7-15)	Interventional	BMI	A 10-week weight loss camp	– During weight loss, SHBG increased significantly in obese children and the changes in SHBG was independent of changes in BMI.
Maynar M et al. (2010) [233]	19 weight lifters (24±5)	Interventional (Strength training for four years)	Skinfold calliper	Supervised training for 20 weeks	– Resistance training causes ↑SHBG compared to pre-training.
Hawkins VN et al. (2008) [234]	102 (40-75)	Interventional	BMI DXA scan	12-month facility and home-based exercise program (Baseline, 3 and 12 months). Controls- no change in activity during the 12-month duration.	– Exercisers had significantly increased SHBG levels at 3 and 12 months after randomisation when compared with controls. – At 3 months, exercisers experienced a 14.3% increase in SHBG (P < 0.001) compared with a more modest, but still significant, 5.7% increase among controls (P = 0.04, exercisers vs controls P = 0.04). – Exercisers at 12 months maintained a consistent, significant 8.9% elevation of SHBG (P = 0.001) compared with a nonsignificant 4.0% elevation above baseline among controls (exercisers vs controls P = 0.13).

Niskanen L et al. (2004) [235]	58 (Obese) 46.3±7.5	Interventional	BMI & WC	Nine week rapid weight loss (very-low-calorie diet) and 12 months sustained weight maintenance	– SHBG concentrations increased by an average of 43% during the VLCD and decreased markedly during weight maintenance but were still higher than baseline at 12 months. – SHBG levels were increased until six months declined then after and remain stable until 12 month weight maintenance period.

Abbreviations: BIA, Bioelectrical impedance analysis; BFW, body fat weight; BMI, Body mass index; CT, Computed tomography; Δ, delta; DXA, Dual-energy x-ray absorptiometry scan; FM, Fat mass; F/U, follow up; RT, Resistance training; SHBG, Sex hormone-binding globulin; WC, Waist circumference; VLCD, Very low-calorie diet

3.3.4. Liver fat and SHBG

Recent data suggested that liver fat but not the total body or visceral fat as measured using magnetic resonance (MR) spectroscopy is significantly associated with SHBG levels [236] and consistent with the observation that elevated levels of fasting triglycerides were inversely related to levels of SHBG [174, 176, 237, 238]. Another study also reported a significant inverse correlation between MR measured liver fat content and serum SHBG levels, then again the relationship is independent of the changes in total body fat or abdominal fat mass [175]. Importantly, during lifestyle intervention with diet modification and increase in physical activity, an increase in SHBG was more strongly associated with a decrease in liver fat compared with visceral fat or total body fat [173]. As will be described in later sections, in human liver tissues, *SHBG* and *HNF4 α* mRNA expression decreased along with the elevated grade of hepatic steatosis [237]. Both *SHBG* and *HNF4 α* mRNA expression levels were negatively correlated with intrahepatic triglycerides [239]. Therefore, SHBG regulation is closely tied to liver fat, and factors that promote hepatic *de novo* lipogenesis. Liver fat, but not VAT or total body fat is negatively correlated with plasma SHBG concentrations [173]. Both *in vitro* and animal studies have shown that less SHBG is secreted into the circulation when fat accumulates in the hepatocytes as a result of dietary factors [87] and *de novo* lipogenesis [89, 240], during obesity development. Therefore, SHBG regulation is closely tied to liver fat, and factors that promote liver *de novo* lipogenesis [89, 161, 169, 173, 240].

3.3.4.1. Hepatic *de novo* lipogenesis and SHBG

In vitro studies using mainly HepG2 cells have demonstrated that *SHBG* gene expression is regulated by several transcription factors, among them, HNF-4 α and PPAR γ play an important role [29, 57]. For instance, monosaccharides-induced increased *de novo* lipogenesis and a build-up of cellular palmitate that decreases the production of HNF4- α , a key transcriptional factor that regulates the production of *SHBG* in hepatocytes [57,

94]. In this regard, it has been described that HNF-4 α is the most important transcription factor that activates SHBG expression [36, 92, 94], whereas PPAR γ has been identified as an inhibitor of SHBG expression [33, 95]. The latter could seem contradictory because it has been shown that thiazolidinediones, which activate PPAR γ , increase circulating SHBG levels. However, this effect is generally attributed to improved insulin resistance and glycaemic control [87]. Taken together, these data suggest that SHBG is highly indicative of *de novo* lipogenesis and is demonstrated in Figure 25. This lipogenic rate, in turn, determines and maintains the SHBG levels by regulating the transcription factors, HNF-4 α and PPAR γ [94].

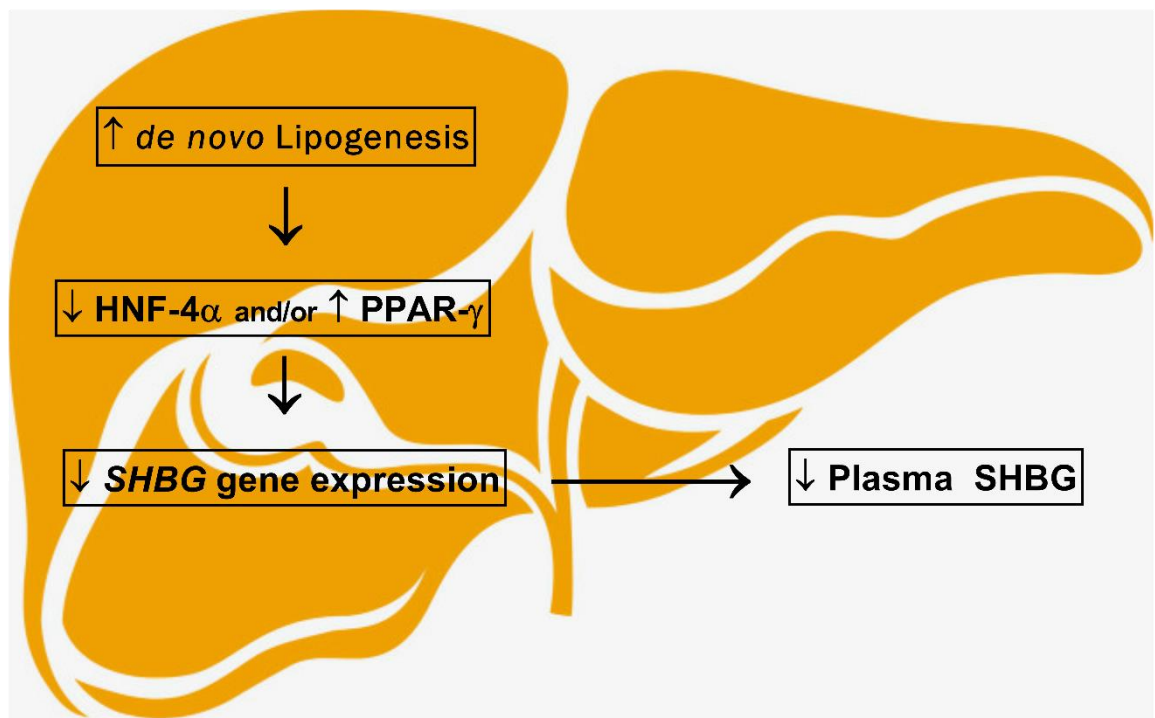


Figure 25. The proposed interplay between *de novo* lipogenesis and the molecular mechanisms of SHBG down-regulation.

Abbreviations: HNF4- α , hepatic nuclear factor 4 α ; PPAR γ , peroxisome-proliferator receptor γ ; SHBG, Sex hormone-binding globulin

3.3.5. Insulin and SHBG

It is widely believed that insulin represses hepatic SHBG production, but the evidence for this is largely circumstantial and not supported mechanistically.

3.3.5.1. *Effects of insulin on hepatic SHBG: in vitro*

In vitro studies have demonstrated that insulin might inhibit the production of SHBG in human hepatoma (Hep G2) cells [109, 241, 242], and by these findings, an inhibitory effect of insulin on SHBG secretion has been reported [243]. The inhibitory effect has been shown at the physiological concentration of insulin on the basal and stimulated SHBG production by Hep G2 cells [242]. In contrast with these findings, a recent elegant study by Selva *et al.* [94] demonstrated that insulin has no direct effect on hepatic *SHBG* expression and is not regulated by insulin. By contrast, elevated monosaccharides (glucose or fructose) and disaccharide (sucrose) play an essential role in the downregulation of SHBG production in the liver *in vivo* and *in vitro* [94]. More importantly, they demonstrated that this effect mediated by monosaccharides-induced increased *de novo* lipogenesis and a build-up of cellular palmitate that decreases the production of HNF4- α , a key transcriptional factor that regulates the production of *SHBG* in hepatocytes [57, 94] and is illustrated in Figure 22.

3.3.5.2. *Associations of insulin and SHBG: Clinical trial and population-based studies*

Few epidemiological studies investigating the relation between circulating SHBG and insulin have yielded conflicting results. It has been suggested that the degree of hyperinsulinemia is related to low concentrations of SHBG [220, 244, 245] and the lack of insulin in type 1 diabetes (T1D) may contribute to the increase in SHBG concentrations and that treatment with insulin may lower it [243, 245]. In a study among T1D male patients with mean insulin dose per kilogram of body weight, it is inversely related to SHBG concentrations [244]. Congruent to their findings, another study showed that total

insulin dose and insulin dose per kilogram was inversely related to SHBG, and not BMI [245]. A most recent study to estimate the effects of different routes of insulin administration on SHBG among T1D patients showed that portal insulin administration influences circulating SHBG.

Further, SHBG concentrations decreased significantly during continuous intraperitoneal insulin infusion (CIPII) treatment, and the difference in change between CIPII and subcutaneous (SC) insulin therapy was significant for SHBG among males [246]. Taken together, these results suggest an inhibitory effect of insulin on SHBG production as described above. However, the degree of hyperinsulinemia was not related to the reduced SHBG concentration in several other studies [91, 173, 175, 217].

Table 6. Associations of endogenous insulin and SHBG in men from population-based studies.

Reference (Year)	Study design	Sample size (Age, Years)	Confounding factors	Main findings
Abdella et al. (2017) [247]	CS	235 (19–49)	WHR	– Serum SHBG was not correlated with plasma insulin ($r=-0.404$, $P=0.11$).
Wang N et al. (2017) [248]	CS	4309 (18-93)	None	– Serum SHBG was inversely correlated with plasma insulin ($r=-0.332$, $P<0.05$).
Ye J et al. (2017) [249]	CS	1633 (29-50)	Age, BMI, total cholesterol, triglycerides, fT, and bT	– $\uparrow$ Insulin= $\downarrow$ SHBG concentration ($\beta = -0.261$, $p < 0.001$). – An inverse relationship remained associated (insulin: $\beta = -0.241$, $p < 0.001$) even after considering potential confounders.
Wang Q et al. (2015)[168]	CS	3697 at (15-74)	Age and BMI	– Prominent inverse associations were observed for insulin ($P < 0.002$).
Bonnet, F et al. (2013)[175]	CS	233 (27-77)	Age	– Serum SHBG was not correlated with fasting insulin ($r=-0.04$, $P<0.05$)
Simo R et al. (2012) [91]	CS	35 Obese men	BMI	– No correlation between serum SHBG and insulin ($r = 0.140$; $P = 0.199$).
Firtser S et al. (2012) [189]	L (the CRYFS)	1024 (24–45)	Age, BMI, smoking, alcohol consumption and physical activity.	– SHBG concentrations correlated inversely with plasma insulin, both cross-sectionally ($r=-0.37$), and longitudinally ($r=-0.39$), at $P<0.001$.

Vanbillemont et al. (2012) [217]	CS	677 (25–45)	Age & height + either: weight or whole body fat mass or truncal fat mass or appendicular fat mass	– The inverse relationship between SHBG and insulin ($P < 0.001$). – Observed associations between SHBG and insulin remain significant in all model of multi adjustment model, all $P < 0.01$.
Daka et al. (2011)[244]	CS	1361 (30–74)	Age, BMI, fasting glucose levels & cFT	– Fasting plasma insulin was significantly and inversely associated with SHBG concentrations ($\beta = -0.062$, $P = 0.02$) independent of age, BMI and fasting glucose levels but not when further adjusted for cFT ($\beta = -0.040$, $P = 0.30$).
Peter A et al. (2010)[173]	L (9-month lifestyle intervention)	90	Age and total body fat and liver fat	– SHBG concentrations were not associated with fasting insulinemia ($r = -0.07$ $P = 0.27$) in age, and total body fat adjustment model.
Vermeulen et al. (1996) [220]	L	250 non- obese (25-100) 50 Obese (25-62)	None	– SHBG concentrations were inversely correlated with insulin levels ($r = -0.69$, $P < 0.001$) in non-obese participants but not in obese men participants ($r = -0.18$, $P > 0.05$).
Strain et al. (1994) [250]	L	70	BMI	– A highly significant negative hyperbolic correlation between the fasting serum insulin and SHBG concentrations ($r = -0.607$; $P < 0.002$).

Abbreviations: BMI, Body mass index; bT, Bioavailable testosterone; CS, Cross-sectional; cFT, Calculated free testosterone; L, Longitudinal; r, correlation coefficient; SHBG, Sex hormone-binding globulin; WHR, Waist-to-hip ratio

CRYFS, Cardiovascular Risk in Young Finns Study

3.3.6. Cytokines and SHBG

SHBG is upregulated by adiponectin [93] whereas downregulated by a couple of proinflammatory cytokines (TNF α and IL-1 β) [29, 36, 91, 92]. There is epidemiologic evidence showing a correlation between the plasma levels of several cytokines and plasma SHBG levels in humans. Patients with low-grade chronic inflammatory diseases (i.e., obesity, diabetes, rheumatoid arthritis, or osteoarthritis), in this regard there is evidence that elevated circulating proinflammatory cytokines, exhibit low plasma SHBG levels [36, 92, 164]. Moreover, several epidemiologic studies have reported a positive relationship between adiponectin and SHBG plasma levels [93, 251].

3.3.6.1. *Effects of anti-inflammatory cytokines on hepatic SHBG: in vitro*

Adiponectin increases hepatic production of SHBG through upregulation of HNF-4 α levels via a reduction in the hepatic lipid content. The suggested molecular mechanism is mediated by AMPK and synergistic effect of the inhibition of *de novo* lipogenesis and the activation of β -oxidation, ultimately reducing the hepatic lipid content. This will cause a reduction in the hepatic fatty acid pool increasing HNF-4 α levels that in turn, increase SHBG production [93] and is illustrated in Figure 26.

Epidemiological studies have consistently shown that plasma SHBG levels correlate with plasma adiponectin levels in men is summarised in Table 7.

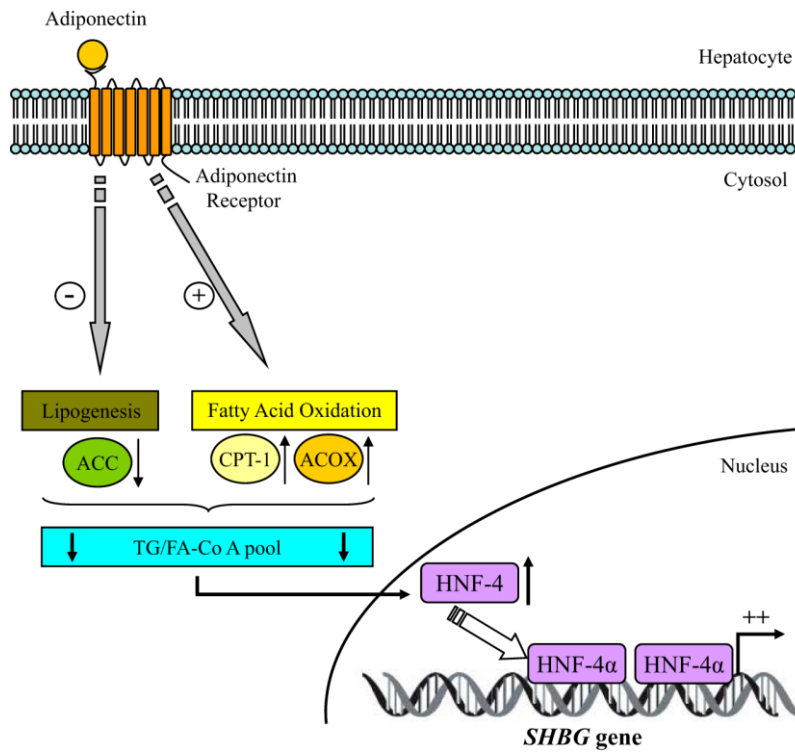


Figure 26. The mechanism by which adiponectin regulates hepatic SHBG production.

Reproduced, with permission, from Simo R et al. (2014) [93].

Abbreviations: ACOX, acyl-CoA oxidase; ACC, acetyl-CoA carboxylase; CPT I; Carnitine palmitoyltransferase I; FA-CoA, Fatty acid coenzyme A; HNF-4α, Hepatocyte nuclear factor-4 alpha; SHBG, Sex hormone-binding globulin; TG, Triglycerides

3.3.6.2. *Effects of pro-inflammatory cytokines on hepatic SHBG: in vitro*

TNF- α and IL1 β are an important pro-inflammatory cytokine, were shown to reduce SHBG production in HepG2 cells at both physiological and supraphysiological concentrations [36, 92]. TNF- α was shown to reduce SHBG production by decreasing HNF-4 α , and the effect is mediated via hepatocyte nuclear factor kappa B (NF- κ B) activation in HepG2 cells [91, 92]. Similarly, IL1 β was shown to reduce SHBG production via MAPK kinase (MEK)-1/2 and JNK MAPK pathways in HepG2 cells [36] and is demonstrated in Figure 22.

3.3.6.3. *Association of cytokines and SHBG: Clinical trial and population-based studies*

Although, there is a consistent positive relationship between plasma adiponectin and SHBG levels. There has been no consistent or replicated evidence for the association between circulating SHBG and inflammatory and pro-inflammatory cytokines. Moreover, the longitudinal studies assessing both SHBG and cytokines are not available. Epidemiological studies showing the associations of cytokines and SHBG in men is summarised in Table 7.

Table 7. Associations of circulating cytokines and SHBG in men.

Reference (Year)	Study design	Sample size (Age, Years)	Blood Marker used for analysis	Main findings
Anti-inflammatory				
Abdella NA & Mojiminiyi OA (2017) [247]	CS	235 (19-49)	Adiponectin	– A positive and significant association between SHBG concentrations and adiponectin levels ($\beta=0.03$, $P=0.02$) but did not persist after adjustment with WHR ($\beta=0.027$, $P=0.181$).
Simo R et al. (2014) [93]	CS	15 obese male underwent bariatric surgery	Adiponectin	– A positive and significant correlation ($r=0.612$, $P=0.015$) between SHBG concentrations and adiponectin levels. – Plasma adiponectin levels were independently and positively associated with circulating SHBG ($P = .045$) even after adjustment for BMI.
Yasui T et al. (2007) [251]	CS	154 (50-85)	Adiponectin	– Serum adiponectin concentrations showed a significant positive correlation with serum SHBG concentration and remained significant after adjustments for age and BMI.
Gannage-Yared M et al. (2006) [252]	CS	153 (≥ 50)	Adiponectin	– Serum Adiponectin was positively correlated with SHBG ($r=0.39$, $P<0.001$) even in aged and BMI adjusted model.
Inflammatory				
Brand JS et al. (2012) [253]	CS	2418 (40–78)	Total WBC and granulocyte count	– SHBG was inversely associated with total WBC count ($\beta=0.163$ per SD increase in SHBG), independently of age, BMI, physical activity, smoking and diabetes status (Multi-adjusted regression analysis) and is mainly accounted for by the granulocytes ($\beta=-0.132$).
Park B et al. (2018) [254]	CS	451 (≥ 50)	Total WBC count	– SHBG was independently and inversely associated with leukocyte count ($OR=1.70$, $P=0.03$) in the multi-adjusted regression model

				(age, smoking status, alcohol drinking, regular exercise, BMI, blood pressure, fasting plasma glucose, triglyceride, and HDL cholesterol level)
Flechner-Mors M et al. (2014) [237]	CS	787 (18-65)	CRP	– An inverse association between SHBG and CRP and persisted when SHBG was stratified as low SHBG (OR=1.58) and high SHBG (OR=0.62), both P<0.05 in multivariate analysis.
Zhang Y et al. (2013) [255]	CS	1989 (17–88)	CRP	– Higher SHBG was independently and inversely associated with CRP levels ($\beta = -0.116$) in the multi-adjusted regression model (age, waist circumference, triglycerides, HDL-cholesterol, fasting glucose, insulin, smoking status, hypertension, diabetes, family history of hypertension or diabetes and mutually SHBG and TT).
Tsilidis KK et al. (2013) [256]	CS	809 (>20)	Total WBC count CRP	– No associations were found between Total WBC count and SHBG levels in the multi-adjusted regression model (age, race/ethnicity, body mass index, waist circumference, cigarette smoking, diabetes, alcohol consumption, leisure-time physical activity and mutually SHBG and TT).
Liao CH et al. (2012) [257]	CS	255 (≥ 20)	hs-CRP	– SHBG was independently associated with hs-CRP in men ($r = -0.18$, $p = 0.009$) in multi-adjusted model (age, MetS components, insulin resistance, low-density lipoprotein-cholesterol, and serum sex hormone levels).
Laaksonen DE et al. (2003)[258]	CS	1896 (42-60)	CRP & Fibrinogen	– SHBG concentrations were positively associated with fibrinogen but inversely with CRP levels. – An observed association persisted only for CRP levels in age & BMI adjusted model.
Pro-inflammatory				
Simo et al. (2012) [91]	CS	35 Obese men	TNF- α -R1	– Serum SHBG was inversely correlated with plasma TNF- α -R1 ($r = -0.52$; $P < 0.001$),

				– SHBG was inversely correlated with plasma sTNF-α-R1 ($r = -0.79$; $P < 0.001$) – Plasma sTNF-α-R1 was independently correlated with SHBG concentrations in the multiple regression analysis and explained the 29.8% of SHBG variation.

Studies documenting longitudinal associations of circulating cytokines on plasma SHBG

None identified

Abbreviations: CS, Cross-sectional; CRP, C-reactive protein; hs-CRP, High-sensitivity CRP; OR, Odds ratio; r , correlation coefficient; sTNF- α -R1, Plasma soluble TNF- α -R1 levels; WBC, White blood cells

3.3.7. Sex-steroids and SHBG

3.3.7.1. Effect of androgens on SHBG: *in vitro*

Several *in vitro* studies have tested the effect of androgens (TT & DHT) on SHBG expression and secretion of SHBG by Hep G2 cells, but the results have been inconsistent [28, 29, 100, 109, 202, 241, 259-261]. Moreover, the molecular mechanism by which this occurs has not been elucidated. SHBG production in HepG2 hepatocarcinoma cells is inhibited by androgens including testosterone [28, 29, 100, 109, 202, 259] and DHT [262] in some studies, while several other studies have the stimulatory effect of androgens on SHBG production by Hep G2 cells [241, 260, 261]. Moreover, the biphasic effect of T was also reported that was characterised by inhibitory effect at low doses and an absence of effect at higher doses [24, 259].

SHBG in boys during puberty were initially thought to reflect a negative effect of androgen on SHBG production, but this was difficult to reconcile with a lack of any increase in plasma SHBG levels in men after orchiectomy [57, 100, 263].

3.3.7.2. Effect of exogenous androgens on SHBG

Exogenously administered androgens, even at low doses when taken orally, suppress SHBG [100, 264]. In contrast, testosterone supplementation via peripheral routes of administration (transdermal, depot implants) has minimal effect on plasma SHBG levels [1, 265]. However, androgen-mediated suppression of SHBG only occurs as a consequence of hepatic exposure to supraphysiologic androgen concentrations as seen with oral androgens due to first pass effects or transiently with high dose injectable testosterone formulations, especially short-acting testosterone esters [266].

3.3.7.3. *Androgens and SHBG: Clinical trial and population-based studies*

Measurements of plasma SHBG have also been used as a biomarker of androgen sensitivity [32]. Epidemiological evidence has consistently shown strong and positive associations between endogenous testosterone and SHBG in men and is summarised in Table 8.

Table 8. Associations of endogenous testosterone and SHBG in men.

Reference (Year)	Study design	Sample size (Age, Years)	Sub fraction of testosterone used for analysis	Confounding factors	Main findings
Abdella NA & Mojmiminiyi OA (2017) [247]	CS	235 (19-49)	TT FSH FAI SHBG (CLIA)	None	– A positive and significant correlation between TT concentrations and SHBG concentrations ($r=0.354$, $P=0.009$).
Wang N et al. (2017) [248]	CS	4309 (18-93)	TT (CLIA)	None	– SHBG was positively correlated with TT in middle-aged ($r=0.623$) and elderly men (0.709). Both at $P<0.05$.
Holt SK et al. (2014) [267]	CS (the UroEDIC)	641 (35-67)	TT cFT SHBG (LC-MS/MS)	Age, BMI, treatment arm, weighted HbA1c, and insulin dose.	– TT level was positively associated with SHBG ($\beta=6.40$ (5.91-6.89), $P<0.001$).
Papatheodorou SI et al. (2014)[268]	CS	1135 (≥ 20)	TT cFT (eCLIA)	None	– Circulating TT levels were positively associated with SHBG ($r=0.30$, $P<0.05$), whereas cFT levels were inversely associated with SHBG levels ($\beta=-0.28$, $P<0.05$).
Gong Y et al. (2013) [269]	CS	337 (60-90)	TT cFT bT (RIA)	Age, BMI, BP, plasma glucose, plasma, plasma urine acid, macrovascular disease	– Strong positive correlation between TT and SHBG ($r= 0.74$; $P<0.05$).

Bonnet, F et al. (2013)[175]	CS	233 (27-77)	TT (CLIA)	Age	– Strong and positive associations between serum SHBG and TT ($\beta=0.53$, $P<0.0001$).
Goto A et al. (2012) [176]	Case-control (Diabetes)	215 (50-79)	TT cFT (eCLIA)	None	– Circulating TT levels were strongly & positively associated with SHBG ($\beta=0.61$, $P<0.001$), whereas cFT levels were inversely associated with SHBG levels ($\beta=-0.37$, $P<0.001$).
Vanbillemonet et al. (2012) [217]	CS	677 (25-45)	TT cFT (RIA)	Body weight or whole body, truncal or appendicular fat mass	– Strong and positive associations between SHBG and TT ($\beta=0.61$, $P<0.001$), whereas FT levels were not associated with SHBG levels ($\beta=0.04$, $P=NS$).
Gomez JM et al. (2007)[270]	CS	134 (15-70)	TT (RIA)	None	– SHBG was positively associated with TT levels ($\beta=0.22$, $P=0.015$).
Aluja A & Garcia LF (2007) [271]	CS	89 (19-46)	TT bT (RIA)	Age	– Strong positive correlation between TT and SHBG ($r= 0.51$; $P<0.001$). – No significant correlation was found between bT and SHBG levels (0.03; $P>0.05$)
de Ronde W et al. (2005) [272]	CS	106 male neonates (1-6 months) 399 men (40-80)	TT (RIA) Non-SHBG-T	Age (in neonates) Age & BMI (men)	– SHBG was strongly and positively associated with TT levels ($\beta=0.02$, $r=0.45$, $P<0.001$) in neonates. – SHBG was strongly and positively associated with TT levels in men ($\beta=0.25$, $r=0.68$ $P<0.001$). SHBG was strongly and positively associated in all age groups in men.

de Ronde W et al. (2005) [110]	CS	399 (40-80)	TT (RIA) Non-SHBG-T	Age & BMI	– SHBG was strongly and positively associated with TT levels ($\beta=0.286$, $P<0.001$), β indicated every 1 $\mu\text{g/dl}$ change in SHBG.
Tsai EC et al. (2004) [273]	CS	221 (45-65)	TT bT fT (Fluoro immunoassay)	Age body fat (CT & DEXA)	– Strong and positive correlation between SHBG and TT ($r=0.63$, $P < 0.0001$). – No significant correlations between SHBG concentration and both bT ($r=0.07$) and fT ($r=0.1$).
Vermeulen A et al. (1996) [220]	CS	250 (25-100)	TT	NA	– Strong and positive correlation between SHBG and TT ($r=0.58$, $P < 0.001$).
Longcope C et al. (1990) [274]	CS	1640 (38-70)	TT cFT (RIA)	Age, BMI & cigarette smoking	– SHBG was positively associated with TT ($\beta=0.074$) levels, whereas cFT levels were inversely associated with SHBG levels ($\beta=-0.237$), all $P<0.001$.

Studies documenting longitudinal associations of serum testosterone and SHBG

None identified

Abbreviations: bT, Bioavailable testosterone; BP, Blood pressure; BMI, Body mass index; cFT Calculated free testosterone; CLIA, Chemiluminescent immunoassay; CS, Cross-sectional; FAI, free androgen index; FSH, Follicle stimulating hormone; HbA1c, Glycated haemoglobin; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; RIA, Radioimmunoassay; r, Correlation coefficient; SHBG, Sex hormone-binding globulin

3.3.7.4. *Effect of oestradiol on SHBG: In-vitro*

In vitro studies has demonstrated the stimulatory effect of E2 on SHBG production by Hep G2 cells [109, 241, 261, 262, 275], an effect most likely dependent on estrogen receptor α (ER- α) mediated upregulation of HNF4- α gene expression [24, 29] (Figure 22). In one of the most rigorous studies of this kind, it was reported that oestradiol stimulates the secretion of SHBG in a dose dependent manner [24].

3.3.7.5. *Oestradiol and SHBG: Clinical trial and population-based studies*

Although not entirely consistent, positive associations between endogenous E2 and SHBG in men has been reported and are summarised in Table 9.

Table 9. Associations of endogenous oestradiol and SHBG in men.

Reference (Year)	Study design	Sample size (Age, Years)	Sub fraction of testosterone used for analysis	Confounding factors	Main findings
Abdella NA & Mojiminiyi OA (2017) [247]	CS	235 (19-49)	E2 (CLIA)	None	– No associations between SHBG and E2 ($r=0.018, P>0.05$)
Wang N et al. (2017) [248]	CS	4309 (18-93)	TT (CLIA)	None	– SHBG was positively correlated with E2 in middle-aged ($r=0.159$) and elderly men (0.226). Both at $P<0.05$.
Papatheodorou SI et al. (2014)[268]	CS	1135 (≥ 20)	E2 cFE2 (eCLIA)	None	– Circulating E2 levels were not associated with SHBG ($r=0.04, P>0.05$), whereas cFE2 levels were inversely associated with SHBG levels ($r=-0.34, P<0.05$).
Bonnet, F et al. (2013) [175]	CS	233 (27-77)	17 β -Oestradiol (CLIA)	Age	– Positive associations between SHBG and E2 ($\beta=0.14, P<0.03$).
Vanbillemont et al. (2012) [217]	CS	677 (25-45)	E2 cFE2 (RIA)	Body weight or whole body, truncal or appendicular fat mass	– Circulating total E2 levels was strongly & positively associated with SHBG ($\beta=0.14, P=0.0003$), whereas cFE2 levels were inversely associated with SHBG levels ($\beta=-0.16, P=0.001$).
de Ronde W et al. (2005) [110]	CS	400 (40-80)	E2 (RIA) Non-SHBG-E2	Age & BMI	– SHBG was positively associated with E2 levels ($\beta=4.47, P<0.001$), β indicated every 1 $\mu\text{g/dl}$ change in SHBG.

			E2/TT Non-SHBG- E2: Non-SHBG-T		– SHBG was inversely associated with related to ratios E2/TT ($\beta=-0.016$, $P<0.001$).

Studies documenting longitudinal associations of blood Oestradiol and SHBG

None identified

Abbreviations: BMI, Body mass index; CLIA, Chemiluminescent immunoassay; CS, Cross-sectional; cFE2 Calculated free oestradiol; E2, Oestradiol;

RIA, Radioimmunoassay; r, Correlation coefficient; SHBG, Sex hormone-binding globulin; TT, Total testosterone

3.3.8. Thyroid hormones and SHBG

3.3.8.1. Effects of thyroid hormones on hepatic SHBG: *in vitro*

Thyroid hormones stimulate the production of SHBG in HepG2 hepatocarcinoma cells [96, 109, 241, 276, 277], as well as in a transgenic mouse model [96]. Importantly, because the human SHBG promoter lacks a thyroid hormone response element, the thyroid hormone effects are mediated by increased lipolysis (β -oxidation) and upregulation of HNF4- α gene expression [29, 96] and are illustrated in Figure 22.

Serum SHBG concentrations are increased in men with hyperthyroidism and decreased in those with hypothyroidism [278]. Values normalise when thyroid disorder was treated. For instance, hypothyroid men treated with T3 showed an increase in plasma SHBG levels [278, 279]. SHBG levels are reduced in hypothyroidism, which in men may be interpreted as testosterone deficiency [113, 278].

Epidemiological evidence has consistently shown strong and positive associations between endogenous thyroid hormones and SHBG in men. Associations between endogenous thyroid hormones and SHBG from population-based studies are summarised in Table 10.

Table 10. Associations of endogenous thyroid hormones and SHBG in men.

Reference (Year)	Study design	Sample size (Age, Years)	Sub fraction of thyroid hormone used for analysis	Main findings
Vanbillemont et al. (2012) [217]	CS	677 (25-45)	T3, fT3, T4 and fT4	- SHBG was positively associated with circulating T3 level ($\beta=0.03$, $P=0.02$) and T4 level ($\beta=0.14$, $P=0.001$) in age, height and weight-adjusted model. - No associations were found between circulating SHBG and fT3 level ($\beta=-0.02$, $P=NS$) & fT4 ($\beta=0.06$, $P=NS$) level.
Pascual-Figal et al. (2009) [280]	CS	104 (42-64)	T3 T4	- No correlation between SHBG and T3 level ($r=0.017$, $P=0.89$) as well as with T4 level ($r=-0.159$, $P=0.19$).
Wang, N et al. (2009) [248]		4206 (18-93)	T3 T4	- SHBG concentration was positively associated with T3 level ($r=0.143$) and T4 level ($r=0.200$), both at $P<0.05$.
Dumoulin SC et al (1995) [279]	CS	Euthyroid (111), hyper- (58) & hypothyroid (38) men	fT3 fT4	- SHBG levels are correlated strongly and positively with free fT3 concentration ($r=0.812$) as well as fT4 concentration ($r=0.693$) both at $P<0.01$.
Sarne DH et al. (1988) [281]	CS	Euthyroid (07), Hypothyroid (10), Hyperthyroid (5) (20-76)	T3 T4	- SHBG levels are correlated strongly and positively with free T3 concentration ($r=0.61$, $P<0.001$) as well as fT4 concentration ($r=0.66$, $P<0.001$).

Studies documenting longitudinal associations of thyroid hormones on plasma SHBG

None identified

Abbreviations: CS, Cross-sectional; f, free; r=Correlation coefficient; T3, Triiodothyronine; T4, Thyroxine

3.4. Genetic factors and SHBG

Prior studies have suggested that not only hormonal, metabolic and nutritional but also genetic factors play a significant role in SHBG variation [11, 57, 100, 105]. Based on the crystal structure of the N-terminal laminin G-like domain or evolutionary conservation of certain amino acids, several genetic polymorphisms have been identified in the human *SHBG* gene [10, 57]. Several naturally occurring single nucleotide polymorphisms (SNPs) are linked to differences in plasma SHBG levels are located in noncoding regions of the gene and possess sexual dimorphism [10, 57]. Several of these naturally occurring SHBG variants exhibit differences in their relative affinities for either androgens or estrogens, and their positions within the SHBG crystal structure [10], and are illustrated in Figure 27.

These SHBG variants (SNPs) have been identified with abnormalities in production or binding affinity for sex-steroids [10, 11, 282]. For example, the first of these to be identified SNP of SHBG (rs6259) located within exon eight was shown to be caused by an Asp327 to Asn substitution (D327N) in the mature SHBG polypeptide sequence. This substitution introduces an additional consensus site for N-linked glycosylation and an extra N-linked oligosaccharide per monomer, the use of which retards the plasma clearance of SHBG [57, 282]. The clinical significance of these naturally occurring SHBG variants is unknown, but in few genome-wide association studies (GWAS), SHBG SNPs are associated with T2D risk [238, 283-285] MetS risk [170, 286, 287] in men. The naturally occurring SHBG variants and their clinical significance are that have been described in men summarised in Table 12.

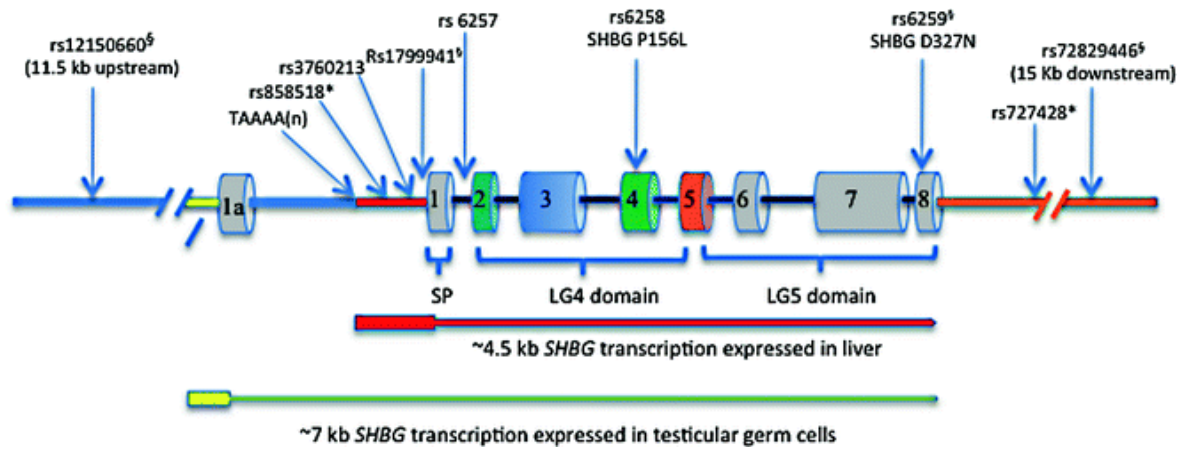


Figure 27. Schematic of the human SHBG gene and location of common polymorphism associated with individual differences in the production or function of plasma SHBG in men and women.

Those SNPs in linkage disequilibrium with each other are indicated with symbols (* or §), respectively.

Reproduced, with permission, from Hammond et al. (2017) [57].

Abbreviations: SHBG, Sex hormone-binding globulin; SNP, Single nucleotide polymorphism

Table 11. Non-synonymous SHBG polymorphisms linked to abnormalities in SHBG production or steroid-binding activity and their clinical significance.

Reference	Polymorphism/SNP (ID)	Location (Amino acids ^a)	Defect (Effect on production or steroid binding)	Clinical and physiological effects in men
Wu TS et al. (2014) [10]	rs373254168	T7N	Produced/Loss of O-glycosylation	-
Wu TS et al. (2014) [10]	rs143521188	T48I	Inefficient dimerisation/impaired Ca ²⁺ binding/reduced affinity for DHT	-
Wu TS et al. (2014) [10]	rs373769356	R123C	Reduced affinity for DHT/Increased affinity for E2	-
Wu TS et al. (2014) [10]	rs143269613	R123H	Reduced affinity for DHT/increased affinity for E2	-
Wu TS et al. (2014) [10]	rs368589266	R135C	Produced/Increased affinity for E2	-
Wu TS et al. (2014) [10]	rs145273466	L165M	Produced/Increased affinity for E2	-
Wu TS et al. (2014) [10]	rs372114420	E176K	Produced/Increased affinity for E2	-
Wu TS et al. (2014) [10]	rs146779355	G195E	Low secretion/reduced affinity for DHT No detectable SHBG in blood plasma	-
Pang XN et al. (2014) [287]	rs3760213	foot printed region (DNase1 footprint 6) *200 bp upstream	Higher levels of plasma SHBG	– Associated with a decreased risk for the MetS
Vos MJ et al. (2014) [111]	?	G195R	Translated but is not secreted, most likely because of a folding defect (in a man with no detectable SHBG in blood plasma)	– Fatigue, overt muscle weakness and low body weight, and other symptoms of hypoandrogenism
Ohlsson C et al. (2011) [284]	s6258	exon 8 P156L	A reduced binding affinity for testosterone	-

Power SG et al. (1992) [282]	rs6259	exon 8 D327N	Introduces an extra N-linked glycosylation site, the use of which retards the plasma clearance of SHBG	– Protective effect on T2D risk
Hogeveen KN et al. (2001) [30]	rs35785886	TAAAA(n) polymorphic repeat	Influence SHBG transcription through interactions with other downstream elements within the promoter including an SP1 binding site	– Greater risk for the MetS

Abbreviations: MetS, Metabolic syndrome; SHBG, Sex hormone-binding globulin; T2D, Type 2 diabetes mellitus

3.5. Influence of drugs on SHBG

Variety of drugs influence the expression of human *SHBG* and plasma SHBG levels [3, 11, 14, 288] and is summarised in Table 13.

Table 12. Summary of the effects of various drugs on hepatic SHBG production.

References	Drugs	Response	Mediator
Svalheim et al. (2015) [289]	Antiepileptic drugs (Phenobarbital, Phenytoin and Carbamazepine)	Increases <i>SHBG</i> expression Increases plasma SHBG levels	Mechanism unknown
Kapoor et al. (2008) [290]	Synthetic PPAR γ ligands (Thiazolidinediones, e.g. Pioglitazone, Rosiglitazone)	Increases <i>SHBG</i> expression Increases plasma SHBG levels	Indirect presumably via increased hepatic HNF4- α via reduced hepatic lipids and higher adiponectin levels (Reduces insulin resistance by ~30%)
Lonning et al. (1989) [291]	Rifampicin	Increases <i>SHBG</i> expression Increases plasma SHBG levels	Mechanism unknown
Browne-Martin & Longcope (2001) [292]	Tamoxifen	Increases <i>SHBG</i> expression Increases plasma SHBG levels	Mechanism unknown
Blick et al. (2012) [293]	Opioid analgesics (Morphine)	Increases plasma SHBG levels	Mechanism unknown
Nader et al. (2006) [294]	Mitotane (o'p'DDD)	Increases <i>SHBG</i> expression Increases plasma SHBG levels	Mediated by ER α mechanism unknown
Serin et al. (2001) [295]	Oral (but not transdermal) Oestradiol	Increases plasma SHBG levels	Mechanism unknown
Stanworth et al. (2009) [296]	Atorvastatin, but not Simvastatin	Decrease plasma SHBG levels	Mechanism unknown
Bonetti et al. (2008) [297]	Anabolic steroids	Decreases SHBG expression Decrease plasma SHBG levels	Mechanism unknown

Weickhardt et al. (2013) [298]	Tyrosine kinase inhibitors (e.g. Crizotinib)	Decreases SHBG expression Decrease plasma SHBG levels	Mechanism unknown
Cunningham et al. (1985) [299] Rao et al. (2013) [288]	Synthetic glucocorticoids* (Dexamethasone)	Decreases SHBG expression Decrease plasma SHBG levels	Mechanism unknown

Abbreviations: *ER* α , Oestrogen receptor alpha; *HNF-4* α , Hepatocyte nuclear factor-4 alpha; *PPAR* γ , Peroxisome proliferator-activated receptor gamma; *SHBG*, Sex hormone-binding globulin

3.6. Other miscellaneous factors

There is evidence for substantial racial and geographical differences in circulating SHBG concentrations on a global scale [172, 198, 300, 301]. Such variation could result in diversity in important health outcomes and understanding the causes of potential differences could yield new insights into racial and environmental influences on SHBG regulation.

ROLE OF SHBG AS AN INDICATOR OF METABOLIC RISK

4. Gender differences in metabolic disease risk

Gender differences exist in the risk factors, presentation, and outcomes of metabolic disorders chiefly T2D and CVD, suggesting the influence of sex hormones. Gender is a fundamental biological factor that plays a key role in the development of cardiometabolic disease and its complications, therefore, a better understanding of sex and gender-specific homeostasis in health and disease is inadvisable. Currently, there is evidence of much interesting sex and gender differences derived reports from both basic research and clinical studies. There have been tremendous advances in studying women's health issues and including women in drug trials and clinical studies. Ensuring consideration of male phenotype in cellular and animal model studies, as well as of an adequate proportion of men in clinical studies that promote sex-specific analysis, including sex differences among the a priori research questions, is important for future biomedical research and medicine and could contribute to better reproducibility of research.

Important physiological and pathophysiological sex differences of anthropometric, metabolic, and endocrine parameters are summarised in Figure 28. Increased liver fat, reduced androgens, impaired fasting glucose, metabolically unhealthy obesity and diabetes incidence are more pronounced in Male as compared to Female, and these factors were considered as major determinants for Plasma SHBG (Chapter 3.3).

Physiologic Sex-Differences

↓ HPA Axis Activity
 ↓ ARC POMC
 ↑ Central Insulin Sensitivity
 ↑ Food Intake
 ↑ Energy Expenditure
 ↓ BAT mass and activity
 ↓ Subcutaneous fat mass
 ↑ Visceral fat mass
 ↑ Liver fat
 ↑ Muscle mass
 ↓ Adiponectin & Leptin
 ↓ Peripheral Insulin Sensitivity
 ↑ Glucose oxidation during exercise
 ↑ FFA oxidation at rest
 ↑ Fasting glucose



↑ HPA Axis Activity
 ↑ ARC POMC
 ↑ Central Leptin Sensitivity
 ↓ Food Intake
 ↓ Energy Expenditure
 ↑ BAT mass and activity
 ↑ Subcutaneous fat mass
 ↓ Visceral fat mass
 ↓ Liver fat
 ↓ Muscle mass
 ↑ Adiponectin & Leptin
 ↑ Peripheral Insulin Sensitivity
 ↑ FFA oxidation during exercise
 ↑ FFA storage in TG at rest
 ↑ 2h Glucose (OGTT)



Normoglycemia

Sex-specific fetal programming

Pathologic Sex-Differences

↑ IFG Incidence
 ↑ Diabetes Incidence
 ↑ Diabetes at earlier Age
 ↑ Overweight Incidence
 ↑ Metabolically unhealthy obese
 ↑ Fatty liver
 ↓ Depression
 ↓ Androgens
 ↑ Erectile Dysfunction
 ↑ Neuropathy, Diabetic foot



↑ IGT Incidence
 ↓ Diabetes Incidence
 ↑ Diabetes at higher BMI
 ↑ Obesity Incidence
 ↑ Metabolically healthy obese
 ↓ Fatty liver
 ↑ Depression
 ↑ Androgens
 ↑ PCOS
 ↑ RR Cardiovascular disease



Hyperglycemia

Diab. Embryopathy & Fetopathy

Figure 28. Sexual dimorphism of metabolic disease risk.

Blue arrows indicate higher or lower levels or impact in men compared with women. Red arrows indicate higher or lower levels or impact in women compared with men. Fat mass: red, SAT, subcutaneous fat; orange, VAT, visceral fat; purple

Abbreviations: ARC POMC, Arcuate nucleus proopiomelanocortin; BAT, Brown adipose tissue; FFA, Free fatty acid; IFG, Impaired fasting glucose; HPA, Hypothalamus-pituitary-adrenal; OGTT, Oral glucose tolerance test; PCOS, Polycystic ovarian syndrome; RR, relative risk; SAT, Subcutaneous adipose tissue; VAT, Visceral adipose tissue

Reproduced, with permission, from Kautzky-Willer A et al. (2016) [302].

4.1. Disease-related effects of plasma SHBG in men

An emerging understanding of the complexity of SHBG synthesis and physiology has led to renewed interest in the protein's potential role in a wide range of metabolic disorders. A growing body of experimental evidence suggests that liver fat content is a major determinant of circulating SHBG concentrations in men [29, 173]. SHBG concentrations are lower with increasing obesity [87] and increase following weight loss, either by dieting or after bariatric surgery [29, 57, 213]. SHBG levels are reduced with insulin resistance [57, 138, 161] and in patients with T2D [53, 57] and NAFLD [89, 303] in men in most, but not in all, studies. Moreover, a low level of SHBG is associated with an increased CVD risk factors [188], and developing T2D [168, 177, 304], as well as the CVD [191, 305] that accompanies these conditions. Furthermore, SHBG genetic variation has been proposed to contribute to the development of T2D [168, 238, 285].

In contrast, SHBG can be markedly increased with advanced liver disease [3, 54], hyperthyroidism [29, 54], Kwashiorkor, [193, 209] and anorexia nervosa [210].

Epidemiological studies are an important component to studying the effects of circulating SHBG in humans, by allowing examination of the effect of SHBG exposure on the outcome (e.g. CVD or T2DM), while controlling for the effects of other related factors. However, we do not know whether low SHBG is a risk factor or rather a risk marker for metabolic risk or whether it is causally linked to these metabolic risk, predominantly T2D and CVD. Additional research is needed to determine whether low SHBG is an early predictor of these metabolic risk or whether it should be considered as an additional component of the metabolic risk assessment. Furthermore, what is the novel impact of these findings on future research or clinical interventions?

4.1.1. SHBG and Obesity

Obesity is defined as a state of having excess body fat [306] and is measured by BMI, WC, WHR, body fat distribution and abdominal fat mass [307, 308]. A predominantly upper body/visceral fat distribution in obesity is closely associated with the risk of cardiometabolic disease, whereas increased body fat over the hips and thighs is protective [309]. Men with obesity, particularly when central, is associated with low concentrations of SHBG [169, 173] while lean have highest SHBG levels [235, 310]. The mechanism by which obesity is associated with lowered SHBG has not been fully elucidated in human. However, a recent Saez-Lopez and her colleagues using the human *SHBG* transgenic mice models to studies the SHBG expression and regulation during obesity development. These data provide potential molecular mechanisms and transcription factors causing the SHBG down-regulation during obesity development, which involved changes in liver *HNF-4 α* and *PPAR γ* mRNA and protein levels [87] and is illustrated in Figure 29. Furthermore, these results were confirmed using human liver biopsies. Importantly, the authors concluded that this model resembles what occurs in human obese subjects because plasma SHBG concentrations were reduced in obese mice when compared with lean mice [87].

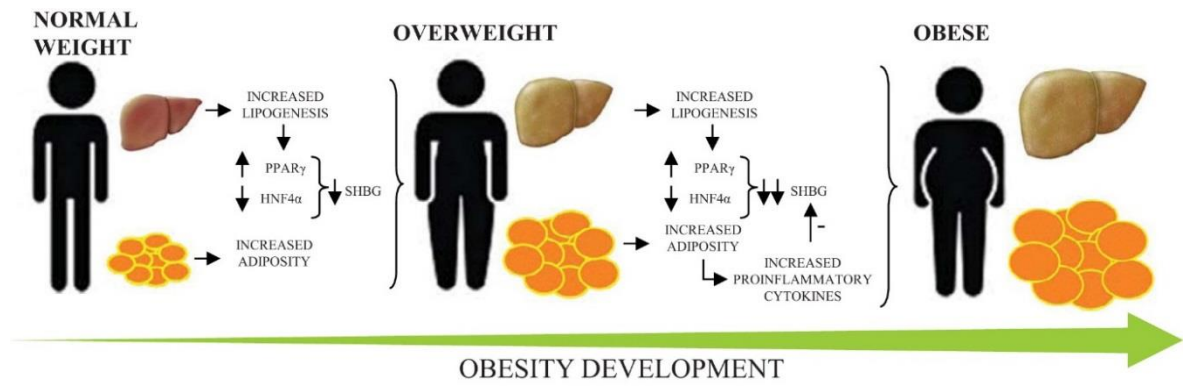


Figure 29. The proposed interplay between ectopic liver fat and SHBG and the molecular mechanisms of SHBG down-regulation during obesity development.

Abbreviations: HNF4- α , hepatic nuclear factor 4 α ; PPAR- γ , peroxisome proliferator-activated receptor γ ; SHBG, Sex hormone-binding globulin

Reproduced, with permission, from Saez-Lopez C et al. (2015) [87].

4.1.2. SHBG and insulin resistance

Insulin resistance (IR) is a hallmark of T2D [311] and associated diseases, and is defined as a state, wherein, three primary metabolic tissues that are sensitive to insulin, skeletal muscle, liver, and adipose tissue become less sensitive to insulin and its downstream metabolic actions under normal serum glucose concentrations [312, 313]. Accurate evaluation of insulin resistance is, of course, possible by clinical examinations such as the hyperinsulinemic euglycemic clamp, which is regarded as the “gold standard” for determining insulin resistance, or by a modified insulin suppression test [314]. However, the complicated nature of these techniques, which need to be performed in a clinical setting and carry the inherent potential danger of hypoglycaemia, limits their routine use. A commonly used alternative indices are the Homeostatic Model Assessment (HOMA), and more recently, the Quantitative Insulin Sensitivity Check Index (QUICKI) [314-316].

Low SHBG is linked with the presence of IR in men. SHBG levels increase when IR improves or with weight loss [213, 231] or following treatment with insulin-sensitising drugs, e.g. rosiglitazone in obese men [290]. There is evidence that IR assessed by HOMA, was related inversely to *SHBG* mRNA and *HNF4 α* mRNA as well as to circulating SHBG levels [24, 161]. Prospective studies have shown that low baseline SHBG concentrations are associated with future IR in adult men that remained robust even after adjustment for baseline adiposity, insulin and testosterone levels [168, 177, 238]. Other cross-sectional studies have also consistently demonstrated that low SHBG is strongly associated with indices of IR in men [138, 178, 317]. In contrast, a positive correlation was observed between SHBG and insulin sensitivity measured by the clamp or QUICKI [168, 173, 177, 246, 318, 319]. Accordingly, SHBG has emerged as a biomarker for IR. Association between serum SHBG levels and IR are summarised in Table 13. However, the mechanism for this association remains controversial.

Table 13. Association between serum SHBG concentrations and insulin resistance.

Author	Study characteristics	Study population	Method of measuring IR	Sex steroids included	Sex assay	steroid	Confounding factors	Main findings
Wang FM et al. (2017) [227]	CS	25 boys (11.8±2.1)	HOMA-IR	NA	NA		None	– No associations between SHBG and HOMA-IR index ($r=-0.07$).
Joyce KE et al. (2017) [177]	L (9.8 years) (the Cardiovascular Health Study)	791 (≥ 65)	HOMA-IR	SHBG, TT, fT DHT & LH	LC-MS/MS		Alcohol consumption, current smoking, BMI, and mutual adjustment for TT, fT, DHT	– SHBG was inversely associated with HOMA-IR when adjusted for demographics, alcohol – Consumption, smoking status, and BMI ($P=0.01$). – With further adjustment for DHT, the association with SHBG was attenuated by approximately one-third ($P=0.11$).
Ye J et al. (2017) [249]	CS	1633 (29-50)	HOMA-IR & HOMA- β	TT, fT, bT & SHBG	eCLIA		Age, BMI, total cholesterol, triglycerides, fT, and bT	– After adjusting for potential confounders, the reduced plasma concentrations of SHBG was associated with insulin resistance (HOMA-IR: $\beta = -0.229$,

							p < 0.001; HOMA- β : β = - 0.220, p < 0.001).
Abdella NA & Mojiminiyi OA (2017) [247]	CS	235 (19-49)	HOMA-IR	TT E2 SHBG	CLIA	None	– No associations between SHBG and HOMA-IR index (r=-0.478, P=0.061).
Li J et al. (2017) [320]	CS	1461 (≥ 20)	HOMA-IR	TT E2 fT 3 α -diol G	eCLIA	Age, ethnicity/race, education level, family income to poverty ratio, smoking, and physical activity	– SHBG is inversely associated with IR, SHBG (RERI = 5.71, 95% CI = 0.77, 10.64) about IR.
Wang N et al. (2017) [248]	CS	4309 (18-93)	HOMA-IR	TT E2 SHBG	CLIA	None	– SHBG was negatively correlated with HOMA-IR (r=-0.339, P<0.05).
Wang Q et al. (2015) [168]	L (6.0 years) (the Young Finns study)	1377 (24-39)	HOMA-IR	TT SHBG	CLIA	Baseline age, BMI, glucose, insulin and TT	– Associations of baseline SHBG with 6-year HOMA-IR index indicated that SHBG is inversely associated with future insulin resistance (β =-0.08, 95% CI:-0.13, -0.03, P=<0.002).
Hvid T et al. (2015) [231]	I	31 (21-39)	Glucose clamp technique	TT SHBG TT/SHBG ratio	Fluoro immunoassays	None	– SHBG was inversely correlated with HOMA-IR before training (r = -0.38; p<0.05) but not

							after (r = -0.32; p>0.2) resistance training.
Tanabe M et al. (2015)[321]	CS	249 (46-60)	HOMA-IR	TT aFT CFT bFT SHBG	CLIA & RIA	None	– SHBG showed weak inverse correlations with HOMA-R (r=-0.179,P<0.01)
Tong G et al. (2014) [318]	CS	63 (28-76)	HOMA-IR	SHBG	-	Age	– SHBG was inversely correlated with HOMA-R (r=-0.451; P <0.001).
Bonnet F et al. (2013) [175]	CS	233 (27-77)	HOMA-IR dysmetabolic men	TT E2 SHBG	eCLIA RIA	Age	– No associations between SHBG and HOMA-IR index (β = -0.08, P=0.19).
Zhang J et al. (2013) [322]	CS	2361 (20-73)	HOMA-IR	TT SHBG	eCLIA	Age	– SHBG were inversely correlated with HOMA-IR (r=-0.153 p<0.001).
Wang P et al. (2013) [213]	I	48 (37-53)	HOMA	TT SHBG	CLIA	None	– SHBG was inversely correlated with HOMA-IR (r=-0.48 p<0.01).
Simo R et al. (2012) [91]	CS	35 Obese men (Age not mentioned)	HOMA-IR	SHBG	NA	BMI	– No correlation between serum SHBG and HOMA-IR (r = -0.110; P = 0.314) was observed.

Vanbillemont G et al. (2012) [217]	CS	677 (20-45)	HOMA-IR	TT fT E2 f E2 SHBG	RIA	Age, BMI + weight or whole body fat mass or truncal fat mass or appendicular fat mass	– Inverse associations were found between SHBG and HOMA-IR index in each model, P<0.01 for all.
Daka B et al. (2011) [244]	CS	1361 (30-74)	HOMA-IR	TT E2 fT SHBG	CLIA	None	– A strong inverse association was found between SHBG and HOMA-IR (p<0.001).
Gannage-Yared MH et al. (2011) [323]	CS	201 (18-30)	HOMA-IR	TT DHEAS SHBG	CLIA	Age, smoking, BMI, WC	– SHBG was inversely associated with HOMA- IR (β =-0.009, p=0.026)
Li C et al. (2010) [178]	CS	1226 (≥20)	HOMA-IR	TT cFT BT SHBG	eCLIA	Age, race, smoking status, alcohol intake, physical activity level, LDL cholesterol, and CRP	– Inverse correlation between SHBG and HOMA-IR (r=-0.25; P<0.0001).
Yeap BB et al. (2009) [324]	CS	2470 (70-80)	HOMA2-IR	TT fT SHBG	CLIA	Age, BMI, WC, HDL, TG and hormone levels	– SHBG correlated inversely with log HOMA2-IR (r=-0.24; P<0.001) in the univariate model. – SHBG was not independently associated with

							HOMA2-IR ($\beta=-0.009$, $P=0.49$).
Danielson KK et al. (2008) [325]	CS	23 (10-32)	HOMA2	TT fT SHBG	RIA	Sex, pubertal stage, body composition, and race,	– SHBG levels were not significantly associated with HOMA2, A 1-unit increase in HOMA2 was associated with a - 4.6 nmol/L (95% CI -11.0 to 1.9) ($P=0.15$) decrease in SHBG.
Yasui T et al. (2007)[251]	CS	154 (50-84)	HOMA-IR	TT cFT bT E2 DHEAS SHBG	eCLIA RIA	Age, BMI	– Inverse correlation between SHBG and HOMA-IR ($r=-0.209$; $P=0.019$).
Rajala UM et al. (2007)[326]	CS	346 No stated	QUICKI	TT SHBG	No stated	BMI, total testosterone, LDL cholesterol, triglycerides, hS-CRP, alcohol consumption, smoking, physical exercise, and education.	– Significant positive correlation of QUICKI with SHBG ($r= 0.364$, $P < 0.001$) in multi-adjusted model.
Osuna JA et al. (2006) [219]	CS	70 (20-62)	HOMA-IR	TT fT E2 SHBG	RIA	Not accounted	– SHBG concentration showed a negative correlation with

							HOMA-IR ($r=-0.266$; $p < 0.05$).
Gannage-Yared MH et al. (2006) [252]	CS	153 (≥ 50)	QUICKI	SHBG only	NA	Not accounted	– QUICKI was positively correlated with SHBG ($r= 0.33$, $P=0.001$).
Tsai EC et al. (2004)[273]	CS	221 (45-65)	HOMA-IR	TT SHBG	Fluoro immunoassay	Age body fat (CT & DXA) TT	– Age-adjusted HOMA-IR was inversely correlated with SHBG ($r=-0.39$; $P < 0.0001$). – SHBG was inversely associated with HOMA-IR ($\beta= - 0.015$; $P < 0.001$). – Inverse associations with HOMA-IR ($\beta=-0.009$; $P < 0.001$) was also independent of all measures of body fat and TT.
Okubo M et al. (2000)[317]	CS	203 ≥ 67	HOMA-IR	SHBG	-	Age, BMI, and WHR.	– SHBG was significantly correlated with HOMA-R after adjustment for age only. – SHBG was not significantly correlated with HOMA-R in men after adjustment for age and

							– BMI and even after additional adjustment for WHR.

Abbreviations: β , Standardized regression coefficients; bT, Bioavailable testosterone; BMI, Body mass index; cFT, Calculated free testosterone; CI: confidence interval; CLIA, Chemiluminescent immunoassay; CT, Computed tomography; CS, Cross-sectional; DHT, 5 α -dihydrotestosterone; DHEAS, Dehydroepiandrosterone sulfate; DXA, Dual-energy x-ray absorptiometry scan; E2, Oestradiol; cFT, Calculated free testosterone ; HR: hazard ratio; HDL, High-density lipoprotein; hS-CRP, High sensitivity C-reactive protein; HOMA-IR, Homeostasis model assessment-insulin resistance; I , Interventional; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; L, Longitudinal; QUICKI, Quantitative insulin sensitivity check index; r, Correlation coefficient; RIA, Radioimmunoassay; RERI, relative excess risk due to interaction SHBG, Sex hormone-binding globulin; TT, Total testosterone; WHR, Waist-to-hip ratio

4.1.3. SHBG and non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver disorders worldwide [327]. NAFLD is characterised by an increase in hepatic triglyceride content with or without inflammation and fibrosis. NAFLD covers a spectrum of liver abnormalities ranging from simple non-alcoholic fatty liver (NAFL) to advanced pathologies, including non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma [327, 328]. Among these, hepatic steatosis (fatty liver) alone is referred to as NAFL, and NASH is defined as a more serious process with inflammation and hepatocyte damage (steatohepatitis); typically, NASH is accompanied by peri cellular fibrosis, which may progress to cirrhosis (Figure 30) [328, 329]. Patients with only NAFL carry a very low risk of adverse outcomes, whereas the presence of NASH increases the risks of liver and possibly non-liver-related outcomes compared to those of patients with NAFL alone [237, 328]. Adverse hepatic outcomes related to NASH may include cirrhosis, liver failure and hepatocellular carcinoma, whereas non-liver-associated adverse outcomes are primarily related to increased cardiovascular disease and malignancy [328, 330]. Metabolic disorders, such as lipid accumulation, insulin resistance, and inflammation, have been implicated in the pathogenesis of NAFLD, potentially arises from a dysregulation of adipose lipolysis and liver *de novo* lipogenesis [328], but the mechanisms underlying the pathogenesis and progression of NAFLD are still incompletely understood.

NAFLD is associated with clinical states such as obesity, insulin resistance, and T2D [327, 328]. Patients with NAFLD are twice as likely to die of cardiovascular disease as liver disease, which is largely related to shared risk factors including obesity, hypertension and T2D [328, 331].

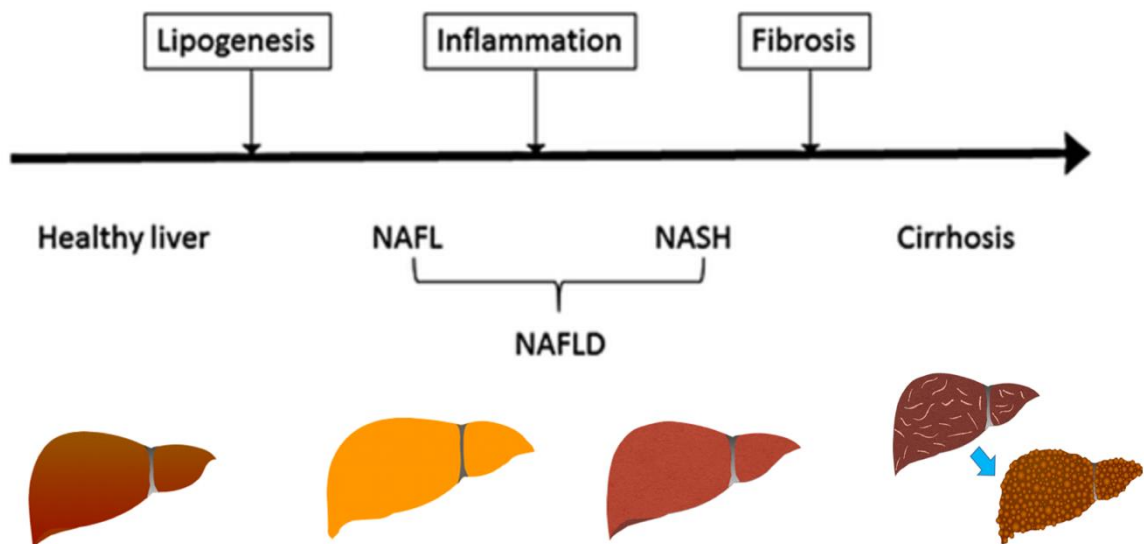


Figure 30. Schematic of progression from fatty liver to cirrhosis.

Abbreviations: NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease.

Reproduced, with permission, from Kim SM et al. (2017) [332].

Apart from the increase in lipogenesis that drives fat accumulation in the liver, there is extensive evidence supporting a central role of SHBG in the development of NAFLD [89]. Most recently, Saez-Lopez and group using HepG2 cells and human SHBG transgenic mouse models of developing NAFLD and hepatic steatosis have shown that SHBG modulates hepatic lipogenesis and reveals how the reduction of *SHBG* expression is associated with NAFLD development through an increase in hepatic lipogenesis [89]. They reported that in a healthy liver, there are normal SHBG plasma levels that determine a lipogenic rate by regulating PPAR γ through the ERK-1/2 MAPK pathway. This lipogenic rate, in turn, determines and maintains the SHBG levels by regulating the HNF-4 α and PPAR γ transcription factors. In NAFLD development, SHBG levels are reduced by elevated proinflammatory cytokines and genetic factors and nutritional factors that decrease the signalling through the ERK-1/2 MAPK pathway, increasing PPAR γ protein activating lipogenesis. This increase in lipogenesis results in a further reduction of SHBG production by downregulating the HNF-4 α transcription factor, entering a cycle that will result in an unstoppable process of constant fat accumulation and reduction of SHBG production [89, 240]. These data provide a potential mechanism by which fatty liver causes a reduction in plasma SHBG. The molecular mechanism suggesting downregulation of SHBG mediated by increased *de novo* lipogenesis in NAFLD development is illustrated in Figure 31. The Saez-Lopez and group also reported that obesity-prone db/db mice with transgenic overexpression of SHBG were protected against high fat diet-induced hepatic steatosis, even the following orchietomy. The author concluded that SHBG might contribute to rather than be solely a consequence of hepatic fat accumulation [89].

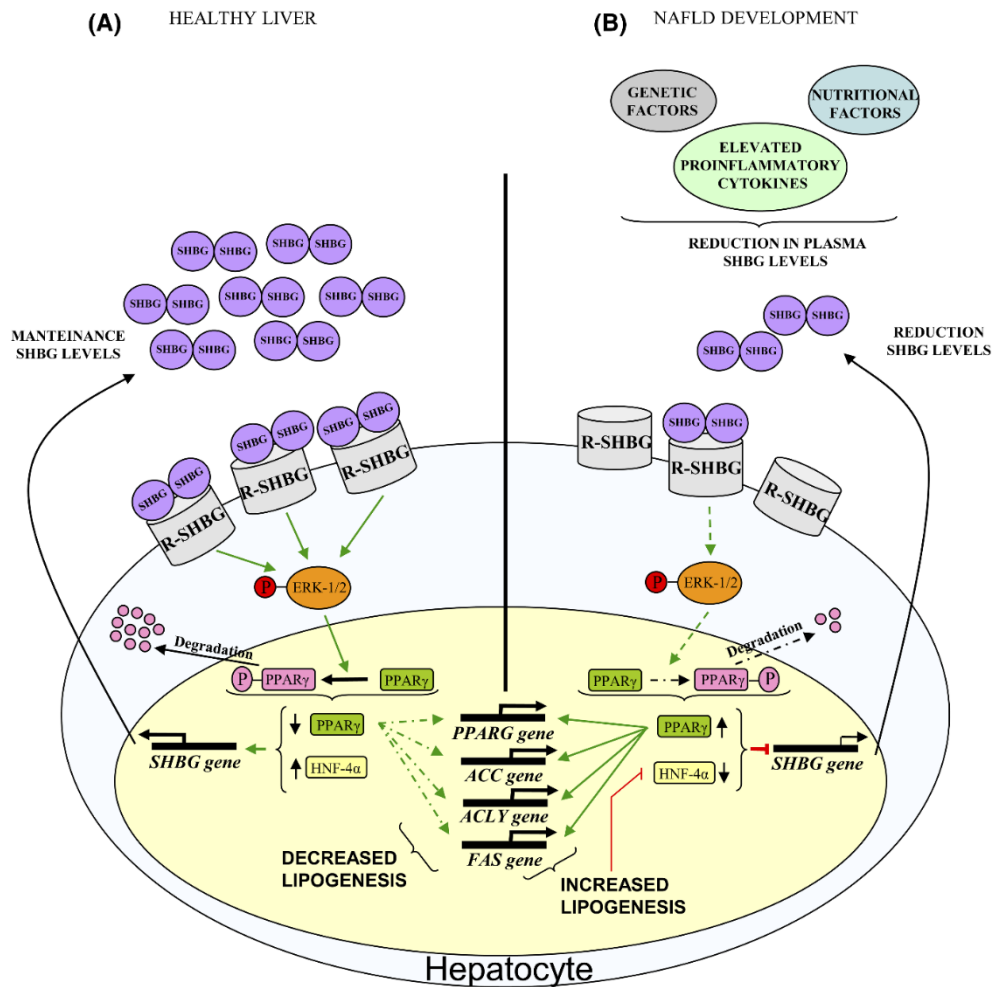


Figure 31. A central role of SHBG in the development of NAFLD compared with a healthy liver.

Abbreviations: ACC, Acetyl-CoA carboxylase; ACLY, ATP citrate lyase; FAS, Fatty acid synthase; ERK-1/2, Extracellular-signal regulated kinase; HNF-4 α , Hepatocyte nuclear factor-4 alpha; NAFLD, Non-alcoholic fatty liver disease; PPAR γ , Peroxisome proliferator-activated receptor gamma; P, Phosphorylated; RSHBG, Sex hormone-binding globulin-receptor; SHBG, Sex hormone-binding globulin

Reproduced, with permission, from Saez-Lopez C et al. (2017) [89].

The strong relationship between SHBG and risk of development of NAFLD raises the intriguing question of whether SHBG is merely a biomarker of NAFLD or could play an active role in the development and progression of NAFLD and visceral fat accumulation is an important area of future investigations.

Based on several observational studies, men with NAFLD had significantly lower SHBG levels compared with those without hepatic steatosis [237, 303, 329]. These studies consistently have shown an inverse association between low SHBG levels and NAFLD and are summarised in Table 14. Moreover, increased intrahepatic fat accumulation and insulin resistance is a common link explaining the low circulating SHBG levels in this disease. No prospective studies are examining the relationship between SHBG and NAFLD.

Table 14. Associations between serum SHBG and NAFLD: Populations based studies.

Author	Study characteristics	Study population (Age)	Outcome definition	Sex steroids included	Sex steroid assay	Confounding factors	Main findings
Luo J et al. (2018) [239]	CS	903 (40-75)	USG criteria NAFLD	TT DHEAS SHBG	CLIA	Adjusted for age, household income, WHR, trunk fat, current smoking and drinking, physical activity, hypertension, diabetes, serum glucose, HOMA-IR, TG, HDL-C, ALT, UA, TT and DHEAS levels	– SHBG concentrations were inversely associated with the presence of NAFLD (OR 0.42, 95% CI 0.23, 0.77).
Jaruvongvanich V et al. (2017)[333]	Meta-analysis	13,721 men	USG criteria NAFLD	TT SHBG	Immunoassay	-	– Men, with NAFLD, had lower circulating SHBG (mean difference -8.72 nmol/L; 95% CI -16.7 to -0.5), compared to controls.
Hua X et al. (2017) [334]	CS	608 (45-72) 302 cases	USG criteria NAFLD MetS-IDF	SHBG, TT	eCLIA	Age, sex, duration of diabetes, smoking status, alcohol use, BMI, WC, GGT, Triglycerides, HDL-C, HOMA-IR and TT.	– Lower serum SHBG is associated with a higher prevalence of NAFLD. – Presence of NAFLD was 6.36 (95% CI 4.87–8.31; P < 0.001) with per one SD

							decrease in SHBG concentrations. – Low SHBG is prevalent in NAFLD than in non-NAFLD patients, regardless of the MetS status.
Wang et al. (2016) [335]	CS	2689 (18-93) 1454 cases	USG criteria NAFLD	TT SHBG	CLIA	Age, abdominal obesity, diabetes, SBP, HDL-C, LDL-C, triglycerides and TT	– Low concentrations of SHBG was associated with an elevated risk of NAFLD, (OR = 3.42, 95% CI 2.41, 4.87) in the multivariable adjusted model.
Lazo M et al. (2015) [174]	CS	2,899 men (45-84)	CT Fatty liver	TT cFT bT E2 & SHBG	RIA	Age, race/ethnicity, WHR, hypertension, total and HDL cholesterol, current smoking, and HOMA-IR.	– High SHBG was associated with a reduced prevalence of fatty liver (ORs 0.46; 95% CI 0.27, 0.77; P=0.002) after multivariable adjustment, including HOMA-IR.
Flechner-Mors et al. (2014) [237]	CS	787 (18-65) 287 cases	USG criteria NAFLD	SHBG	-	Age, BMI, HDL-C and CRP	– Fatty liver was shown to exert a significant influence on serum SHBG concentrations in men.

							Men with grade 1 fatty liver had (ORs 1.96, 95% CI 1.28, 3.02; p=0.0022) for low SHBG concentrations.
Hua et al. (2014) [303]	Case-control study	80 cases 160 control (20-78)	USG criteria NAFLD in T2D patients	SHBG TT	CLIA	Age, BMI, fasting C-peptide and TT	- SHBG is independently associated with NAFLD in men (OR= 0.91; 95% CI, 0.86–0.96, P = 0.001). - ORs of NAFLD decrease with increased quartiles of serum SHBG levels.
Shin et al. (2011) [336]	CS	154 (48-66)	NAFLD in T2D patients	SHBG	CLIA	Age, BMI, WC, hypertension, triglycerides, ALT, GGT, CRP, HOMA-IR, anti-diabetic medications, E2, & TT	- SHBG concern decreased gradually with increasing fatty liver disease severity, OR= 0.09 95% CI 0.03, 0.26. - Likelihood of having high-grade NAFLD also decreased gradually according to increasing SHBG tertile OR=0.14 95% CI 0.03–0.57.

Studies documenting longitudinal associations of serum SHBG and NAFLD.

None identified

Abbreviations: ALT, Alanine transaminase; *bT*, Bioavailable testosterone; BMI, Body mass index; *cFT*, Calculated free testosterone; CI: Confidence interval; CLIA, Chemiluminescent immunoassay; CT, Computed tomography; CS, Cross-sectional; DHT, 5 α -dihydrotestosterone; DHEAS, Dehydroepiandrosterone sulfate; DXA, Dual-energy x-ray absorptiometry scan; E2, Oestradiol; *cFT*, Calculated free testosterone ; GGT, Gamma-glutamyltransferase; HR: hazard ratio; HDL, High-density lipoprotein; HOMA-IR, Homeostasis model assessment-insulin resistance; I , Interventional; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; L, Longitudinal; *r*, Correlation coefficient; RIA, Radioimmunoassay; SHBG, Sex hormone-binding globulin; TG, Triglyceride; TT, Total testosterone; UA, Uric acid; WC, Waist circumference; WHR, Waist-to-hip ratio

4.1.4. SHBG and diabetes

Diabetes mellitus (diabetes) is a metabolic disorder of multiple aetiologies characterised by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism subsequently from defects in insulin secretion, insulin action, or both [337]. There are two main types of diabetes: type 1 and type 2. Type 1 diabetes (T1D, childhood-onset diabetes) is characterised by a lack of insulin production. Type 2 diabetes (T2D, adult-onset diabetes) is caused by the body's ineffective use of insulin and is largely the result of excess body weight and physical inactivity [311]. T2D is the most common form of diabetes, affecting 85-90% of all people with diabetes [311, 338]. New figures indicate that the number of people living with diabetes is expected to rise from 425 million adults (20-79 years) in 2017 to 629 million by 2045 [339]. T2D diabetes is associated with 3-fold greater risk for CVD accounting up to 80% of the 200 million global death with diabetes [337, 338]. The prevalence of particularly T2D rises with age, the greatest number between 40 to 59 years and is higher in men than in women [311, 339].

Although there was a classic assumption that hepatic production of SHBG is downregulated by insulin [109, 243], it has been demonstrated that excessive intake of monosaccharides glucose and fructose leads to lower human SHBG production by hepatocytes and not by insulin [94]. Also, it has been reported that SHBG is not related to fasting insulinemia or insulin secretion [91, 173, 175]. SHBG might, therefore, represent an early and sensitive biomarker for an impaired glycaemic control. However, it has been reported that administration of diazoxide (oral hyperglycaemic drug) in healthy men [243] and rosiglitazone (insulin-sensitiser) in hypogonadal men with T2D [290], increases SHBG concentrations. SHBG has emerged as one of the multiple genetic and environmental factors that potentially contribute to the pathophysiology of diabetes [53, 238, 325]. The complex biological mechanisms that explain the association between circulating SHBG concentrations and the risk for diabetes are not

fully understood. All these findings suggest that conditions unrelated to insulin signalling pathways or altered systemic insulin levels such as T1D and IR in nondiabetic individuals could, therefore, affect SHBG and their dependent physiological processes. SHBG concentrations are lower in men with T2D and also predict the onset of these adverse metabolic states. IR is an important mediator of this relationship. Understanding the relationship between these adverse metabolic states and SHBG may, therefore, have clinical implications.

4.1.4.1. SHBG and type 1 diabetes (T1D)

Although the relation between SHBG and diabetes has long been recognised, the literature on the associations of SHBG with T1D is scarce. Concentrations of SHBG are elevated in T1D patients and has been consistently shown by few epidemiological studies investigating the relation between SHBG and T1D [244, 245, 310, 325, 340] and are summarised in Table 15. These studies were limited by their cross-sectional design, small samples (<500), or insufficient adjustment for potential confounders, including TT. Another major limitation in all of the previous studies is the TT.

Epidemiological studies consistently show that circulating SHBG concentrations are lower in T2D patients than non-diabetic individuals, but the causal nature of this association is controversial. To date, no large prospective cohort study has examined the association of T1D with SHBG in healthy men.

Table 15. Association between SHBG level and T1D in men.

Author	Study characteristics	Study population	Sex steroids included	Sex steroid assay	Main findings
Kelsey MM et al. (2016)[310]	CS	70 (12-19)	TT FAI E2 DHEAS SHBG	CLIA	– ↑SHBG in T1D (50.4±6.1) than Obese men (33.7±10.6) and men with T2D (49.32±2.83) and similar concentration compared to Normal lean men (72.9±9.0) (P<0.001 for all).
Ng Tang Fui M et al. (2013) [341]	CS	240 (34-69)	TT cFT SHBG	GC-MS	– ↑SHBG in T1D than T2D and non-diabetes (P<0.001).
Daka B et al. (2011) [244]	CS	1385 (30-74)	TT cFT SHBG	Immunoassay	– Men with T1D exhibited higher concentrations of SHBG (56.0 nmol/l) than both T2D (38.1 nmol/l) and non-diabetic subjects (37.6 nmol/l), P<0.001 for all.
Maric C et al. (2010) [342]	Case control	T1D (n=297) Healthy control (n=96)	TT cFT E2 CFE2 SHBG	Immunoassay	– No difference in SHBG concentrations compared to healthy controls (P=0.12).
Tomar R et al. (2006) [245]	CS	50 T1D (23-58) 50 T2D (28-51)	TT cFT bT SHBG	RIA	– ↑SHBG in T1D than T2D (49.32±2.83 vs 20.44±1.68) (P<0.001).
van Dam EW et al. (2003) [340]	Case-control	52 men 53 age & BMI matched healthy control (30-45)	TT cFT FAI E1 E2 A4 DHEAS SHBG	eCLIA	– T1D had ↑ SHBG compared to healthy controls (42.0 vs 26.0 nmol/L) (P<0.001).

Studies documenting longitudinal associations of serum SHBG and T1D.

None identified

Abbreviations: A4, Androstenedione; bT, Bioavailable testosterone; cFE2, Calculated free oestradiol; cFT, Calculated free testosterone; CS, Cross-sectional; DHEAS, Dehydroepiandrosterone sulfate; eCLIA, Electrochemiluminescent immunoassay; E1, Oestrone; E2, Oestradiol; FAI, Free androgen index; GC-MS, Gas chromatography-tandem mass spectrometry; RIA, Radioimmunoassay; SHBG, Sex hormone-binding globulin; TG, Triglycerides; TT, Total testosterone; T1D, Type 1 diabetes mellitus; T2D, Type 2 diabetes mellitus

4.1.4.2. SHBG and type 2 diabetes (T2D)

While the inverse relationship between insulin resistance and reduced plasma SHBG concentrations is incontrovertible, likely, the risk of T2D associated with low concentrations of SHBG is due in part to an inverse association between both markers of insulin resistance, impaired glycaemic control and plasma SHBG [29, 54, 100]. SHBG might, therefore, represent an early and sensitive biomarker for an impaired glycaemic control. The rising incidence of T2D in men is well recognised and typically depends on obesity, insulin resistance, and ageing, in keeping with the suggestion that these adverse metabolic factors are important determinants of plasma SHBG [29, 57]. Even though the observational associations between circulating SHBG and adverse metabolic factors were prominent, the Mendelian randomisation analysis provided only limited evidence of a causal role of SHBG on the risk for T2D [283, 304]. Nevertheless, two other mendelian randomisation analyses suggest that SHBG may have a causal role in the risk of T2D [168, 238].

SHBG concentrations are lower in men with metabolic syndrome (MetS) [180-183] and T2D [53, 176, 343] and also predict the potential role of SHBG in the development of T2D onset of these adverse metabolic states, in most, but not in all, studies [177, 184-186, 304, 344]. Moreover, these studies were limited by their by cross-sectional design, population studied (either middle-aged or older men), small incident T2D (<150), or insufficient adjustment for potential confounders such diabetes risk factors and TT. The findings of prospective observational studies reporting an association between plasma SHBG and T2D in men is summarised in Table 16. Thus, a large, well-designed cohort of middle-aged to older men, adequately powered studies and making an adequate adjustment for several other potential explanatory factors contributing to the relationship of SHBG and incident T2D, including adiposity, behavioural and metabolic confounders are needed to understand the basis of the relationship between SHBG and risk of development of T2D.

It also remains unclear whether SHBG is merely a biomarker of T2D, coexisting with diabetes because of in-common risk factors or a consequence of the diabetes or whether it is causally linked to T2D.

Table 16. Association between SHBG concentrations and T2D in men.

Reference	Study characteristics	Study population (Age)	Outcome (Incident/cases)	Sex steroids included (assay method)	Confounding factors	Major findings (multi-adjusted model unless otherwise mentioned)
Joyce KE et al. (2017)[177]	L 9.8 years (the CHS)	852 (≥ 65)	Incident T2D (112)	SHBG,TT, ft DHT & LH (LC-MS/MS)	Age, race, site, alcohol consumption, current smoking status, BMI + cFT/ DHT.	– SHBG was not associated with risk of diabetes in initial models (HR 0.88 95% CI 0.62, 1.24; P=0.46) or with adjustment for other androgens (HR 1.09, 95% CI 0.74, 1.59; P=0.66).
Li J et al. (2017) [320]	CS (the NHANES)	1461 (≥ 20)	T2D cases (174) & IR 54.7% of the 1461 male participants were overweight or obese	TT E2 ft 3α-diol G (eCLIA)	Age, ethnicity/race, education level, family income to poverty ratio, smoking, and physical activity	– SHBG is inversely associated with T2D, SHBG (RERI = -0.07, 95% CI -1.64, 1.49) about T2D.
Karagoz A et al. (2016) [345]	L 5.7 years (the TARF study)	943 (mean age 55 ± 11)	Incident T2D (102)	TT SHBG (eCLIA)	Age, systolic BP, total cholesterol, waist girth +TT	– SHBG concentration was not associated with T2D risk (RR for 1-SD increment (1.7 fold) was 0.87, 95% CI 0.71, 1.02).
Hu J et al. (2016) [346]	L (5.0 years) Nested Case-control (the EIMDS)	145/145 (35-80)	Incident T2D (145)	SHBG,TT, E2 & DHEAS (eCLIA)	BMI, systolic BP, diastolic BP, current smoking, alcohol use, hypertension history,	– The risk of T2D decreased with increasing SHBG tertile (OR 0.30 [95% CI 0.10–1.02] and OR 0.25

					exercise frequency, family history of T2D, FPG, TC, HDL-C, and LDL + combined effect of sex steroids.	[95% CI 0.11–0.66]; <i>P</i>trend = 0.041). When serum lipids were further adjusted, the relationship of SHBG with T2D remained. – There were no significant effects of SHBG and TT combinations on the risk of T2D while the combined effects of SHBG and E2 on the risk of T2D in men were notable.
Zhu H et al. (2016) [347]	CS	2654 (18-82)	T2D cases (243)	SHBG,TT & fT (CLIA)	Age, residence area, economic status, WC, LDL-C, HDL-C, TG, systolic BP, HOMA-IR + (FT and SHBG) or (SHBG and TT).	– Increasing quartiles of SHBG was inversely associated with the presence of both prediabetes and T2D (<i>P</i> for trend<0.003). – Associations between SHBG and the presence of both prediabetes and diabetes were attenuated but remained significant after additional adjustment for TT or cFT.
Holmboe et al. (2016) [184]	L 18.9 years (the MONICA)	5350 (30-70)	Incident T2D (211) CVD	SHBG, T, free T, E2, LH, FSH, LH to T ratio	BMI, study, alcohol consumption, and exercise with age as the underlying time scale.	– The outcome was stratified based on Smokers and Non-smokers.

						– SHBG in the highest quartile had a decreased risk of developing T2D (non-smokers: HR 0.30, 95% CI 0.14, 0.63; P=0.01 vs. smokers: HR 0.40, 95% CI 0.20, 0.78; P=0.01).
Mather KM et al. (2015) [304]	L 3.2 years (the DPP)	969 (≥25)	Incident T2D (211)	SHBG, TT, fT, DHT, DHEA/S, E1, E1S & E2	Age, race/ethnicity, smoking, alcohol consumption, leisure activity, TT, E2, WC, inverse fasting insulin & insulinogenic index.	– SHBG concentration was not associated with diabetes risk (HR=1.03 per SD difference in SHBG, P=0.68). – No differences in diabetes incidence by SHBG quartile trend.
Salminen, M et al. (2014) [348]	L Nine years (the Lieto study)	430 (≥64 years)	Incident T2D (30)	SHBG, TT, fT & LH (Immunoassay)	BMI, FBG, CVD & TT	– SHBG was not associated with the incidence of T2D (HR 1.00, 95% CI 0.97–1.02; p=0.64).
Soriguer F et al. (2012) [349]	L Six years at T2 11.0 years at T3 (the Pizarra study)	T2 (341) T3 (227)	Incident T2D T1 to T2 (11.3%) T1 to T3 (15.6%)	TT bT SHBG (Immunoassay)	Age and obesity or WC	– SHBG concentration was not associated with incident diabetes in 6 years (OR for 1-SD increment was 0.43, 95% CI 0.09, 1.95; P=0.27). – SHBG was inversely associated with incident T2D in 11 years (OR for 1-SD increment was 0.31,

						95% CI 0.09, 1.00; P=0.05).
Goto A et al. (2012) [176]	Case-control (the Saku cohort study)	215/215 (51-79)	T2D cases (215)	SHBG TT, fT (eCLIA)	Smoking status, physical activity, history of hypertension, family history of diabetes, alcohol use, BMI, fatty liver index, of fasting insulin and TT.	– SHBG concentration is not associated with T2D (OR= 1.22, 95% CI 0.56,2.67, P=0.54).
Vikan T et al. (2010) [185]	L (9.1 years) (the Tromsø study)	1454 (Mean age 59)	Incident T2D (76)	SHBG,TT, fT & E2 (Immunoassay)	Age, HDL-cholesterol, systolic BP, WC & TT.	– A significant decrease in risk for diabetes by 1 SD increase in SHBG, HR 0.55 (95% CI 0.39–0.79), after adjustment for age, HDL-cholesterol, and systolic BP but not after additional adjustment for WC. – As for SHBG, there was a decrease in the risk of diabetes in both the third, HR 0.38 (95% CI 0.18–0.81), and the fourth quartile, HR 0.37 (95% CI 0.17–0.82), than in the lowest quartile of SHBG in the multivariate analysis; however, no significant risk was observed after adding WC.

						– When modelled as continuous variables with HR per SD increase, there was still a predictive effect of SHBG (HR 0.58, CI 0.37 – 0.92) when TT was included in the same multivariate model before but not after adjustment for WC.
Lakshman et al. (2010) [186]	L 7.0 years (the MMAS)	1128 (40-70)	Incident T2D (54)	SHBG,TT & fT (Immunoassay)	Age, BMI, high blood pressure, smoking, alcohol intake, and physical activity + TT	– The risk of developing T2D per SD decrease in SHBG was (HR= 1.93, 95% CI, 1.38, 2.70, $p = .0001$) independent of age & BMI. – The highest risk of developing T2D was associated with the lowest quartile of SHBG (HR= 3.97, 95% CI, 1.9, 8.36, $p = .0019$). – In the multi-adjusted model, SHBG was strongly associated with T2D (HR= 1.95, 95% CI, 1.34, 2.82, $p = .0004$), associated with a 1 SD decrease in SHBG.
Ding EL et al. (2009) [238]	L (8.0 years) Nested case-control	170/170	Incident T2D (170)	SHBG only	Age, self-reported race, fasting status, BMI, smoking status, degree	– Low circulating concentrations of SHBG are a strong predictor of the

	(the PHS II)				of exercise, alcohol use, presence or absence of multivitamin use, hyperlipidaemia, family history of diabetes & BP.	risk of T2D (ORs for the highest vs. the lowest quartile, 0.10; 95% CI, 0.03, 0.36; P<0.001); these findings remained highly robust in multiple sensitivity analyses.
Colangelo LA et al. (2009) [350]	CS (the MESA)	3156 (45-84)	T2D cases (137)	SHBG, TT, bT, E2 & DHEA	Age, BMI, waist circumference, and in the pooled analysis, ethnicity	– SHBG in the highest quartile had a decreased risk of developing T2D OR=0.35, 95% CI 0.24, 0.49; P=<0.0001.
Selvin E et al. (2007) [351]	CS	1413 (≥ 20)	T2D cases (101)	SHBG, TT, fT, bT, E2, E2/T ratio	Age, race/ethnicity, BMI, WHR, & TT or E2.	– SHBG was higher in men with T2D but not after age & race/ethnicity adjustment. – SHBG was not associated with diabetes status (P=0.42).
Startberg et al. (2004) [352]	CS (the Tromsø study)	1419 (25-84)	T2D cases (55)	SHBG & TT (Immunoassay)	Age & WC	– ↓SHBG in T2D than non-diabetic men (43.7 vs 51.8; P<0.001).
Laaksonen et al. (2004) [344]	L 11.0 years (the KIHDRFS)	702 (42-60)	Incident T2D (57)	SHBG, TT & fT (Fluoro immunoassay)	Age, presence of CVD, smoking, alcohol consumption, socioeconomic status, WHR, diabetes, BP with medication.	– Low SHBG concentrations independently predict the development of T2D in middle-aged men. ORs for developing T2D in the lowest fourth of

						concentrations of SHBG at baseline is (OR=2.74, 1.42, 5.29).
Stellato RK et al. (2000) [353]	L (7.0 years) (the MMAS)	1030 (40-70)	Incident T2D (54)	SHBG,TT & cFT (RIA)	BMI, hypogonadism, hypertension, heart disease, depression, dominance + TT.	– ORs for incident diabetes of 1.89 for a one SD decrease in SHBG concentrations (15.8 nmol/l), with 95% CI 1.14, 3.14; $P = 0.014$.
Haffner SM et al. (1996) [354]	L (5.0 years) Nested case-control (the MRFIT)	176/176 (35-57)	Incident T2D (176)	SHBG,TT & c FT (RIA)	Age, diastolic BP, TC, reported the number of cigarettes smoked per day, fasting glucose, glucose 1 hour after a 75-g load, BMI, & insulin + TT.	– Lower concentrations of SHBG at baseline was associated with subsequent development of T2D (RR=0.61, 95% CI 0.37, 0.99) and was stronger with further adjustment for TT, (RR=0.55, 95% CI 0.32, 0.94).
Tibblin G et al. (1996) [355]	L 13.0 years	659 (67-80)	Incident T2D (35)	SHBG,TT c FT & LH (RIA)	BMI, WHR, TT, cFT & SHBG	– SHBG concentration was lower in men with newly discovered diabetes than in the nondiabetic and IGT groups ($P < 0.05$). – In multivariate analyses, low SHBG was not an independent predictor for the development of diabetes ($P = 0.053$).

Haffner, SM et al. (1993) [356]	L Eight years (the SAHS)	56 (25-64)	Incident T2D (20)	SHBG only	None considered	– SHBG is not associated with the incidence of NIDDM.

Abbreviations: *bT*, Bioavailable testosterone; *BP*, Blood pressure; *BMI*, Body mass index; *CVD*, Cardiovascular disease; *CLIA*, Chemiluminescent immunoassay; *CI*, confidence interval; *DHEAS*, Dehydroepiandrosterone sulfate; *DHT*, 5 α -dihydrotestosterone; *E2*, Oestradiol; *E1*, Oestrone; *E1S*, Oestrone sulphate; *cFT*, Calculated free testosterone ; *FBG*, Fasting blood glucose; *HbA1c*, Glycated haemoglobin; *HR*, hazard ratio; *HDL*, High-density lipoprotein; *IR*, Insulin resistance; *LC-MS/MS*, Liquid chromatography-tandem mass spectrometry; *LDL*, low density lipoprotein; *LH*, Luteinizing hormone; *NIDDM*, Non-insulin dependent diabetes mellitus; *ORs*, Odds ratios; *RIA*, Radioimmunoassay; *RERI*, Relative excess risk due to interaction; *RR*, Relative risk; *SHBG*, Sex hormone-binding globulin; *TT*, Total testosterone; *TC*, Total cholesterol; *TG*, Triglycerides; *T2D*, Type 2 diabetes; *WC*, Waist circumference; *WHR*, Waist-to-hip ratio

CHS, Cardiovascular Health Study; *DPP*, Diabetes Prevention Program; *EIMDS*, Environment, Inflammation and Metabolic Diseases Study; *KIHDRFS*, Kuopio Ischemic Heart Disease Risk Factor Study; *MMAS*, Massachusetts Male Aging Study; *MONICA I, II and III*, three Danish Monitoring Cardiovascular risk factor surveys; *MESA*, Multi-Ethnic Study of Atherosclerosis; *MRFIT*, Multiple Risk Factor Intervention Trial; *NHANES*, National Health and Nutrition Examination Survey; *PHS II*, Physicians' Health Study II of men; *SAHS*, San Antonio Heart Study

4.1.5. SHBG and cardiovascular disease (CVD) risk factors

CVD comprises diseases of the heart and vascular system, including atherosclerosis, cerebrovascular disease, and peripheral artery disease (PAD) [357]. The incidence of CVD varies by gender. Men, on average, develop CVD about ten years earlier than women [358]. Therefore, several traditional risk factors are associated with a higher risk for CVD events, including older age, male sex, high blood pressure, current smoking, abnormal cholesterol levels, diabetes, obesity, and physical inactivity (Figure 32) [357, 359].

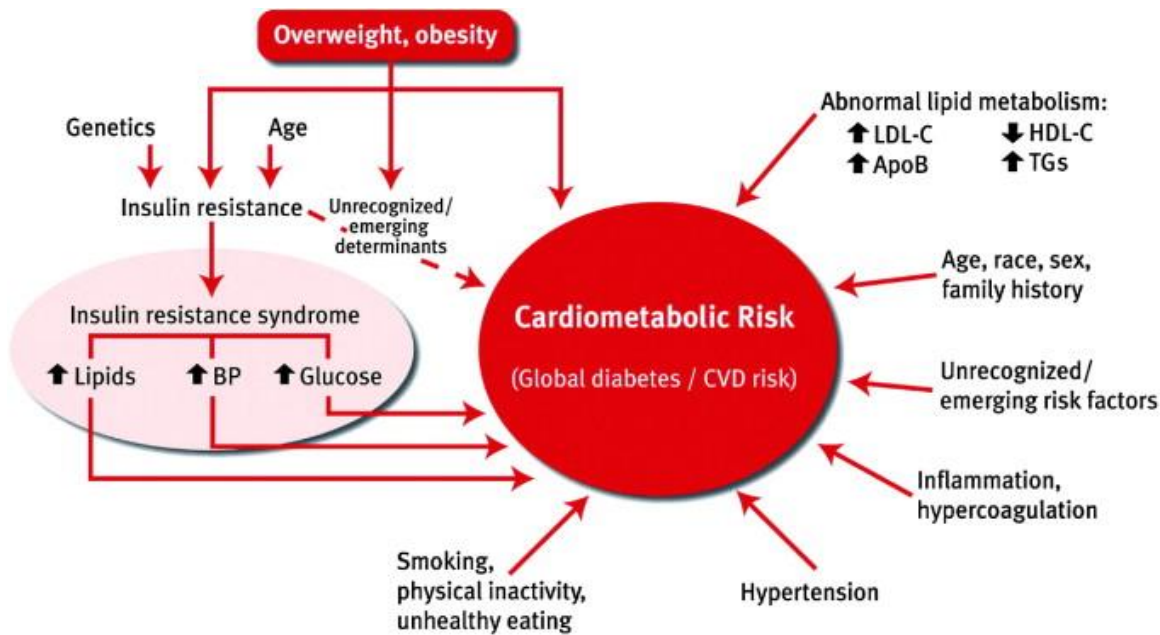


Figure 32. Traditional and emerging CVD risk markers that contribute to global cardiometabolic risk.

Reproduced, with permission, from Brunzell JD et al. (2008) [359].

Abbreviations: Apo B, apolipoprotein B; BP, blood pressure; CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride

Risk factors can be combined in many ways to classify a person's risk for a CVD event as low, intermediate, or high. Several calculators and models are available to quantify a person's 10-year CVD event risk [357]. Accurate identification of persons at high risk for CVD events, particularly nonfatal myocardial infarction (MI) or stroke, and CVD death provides the opportunity for more intensive risk factor management to reduce the likelihood of such an event. Also, identifying persons at low risk may allow for a reduction in interventions with a low benefit to risk ratio for those not likely to benefit [357, 359, 360].

Although the role of SHBG in the aetiology of CVD remains unclear, many epidemiological studies have previously investigated a negative relationship between SHBG concentrations and multiple CVD risk factors, such as blood pressure, BMI, WC, visceral adiposity, dyslipidaemia, IFG/IGT, CRP, and MetS, defined by a clustering of such clinical and biochemical risk factors [178, 180, 183, 188-190, 323, 361]. There is consistent observational evidence that low serum SHBG concentrations are associated with multiple CVD risk factors, including hypertension, abdominal obesity, circulating lipids, insulin resistance, dysglycemia, thrombosis, and inflammatory markers. SHBG levels were also frequently associated with all of the individual components of the MetS in prospective studies. Associations between SHBG and CVD risk factors from prior population studies are summarised in Table 17. These studies showed that metabolic perturbations were generally stronger for serum triglycerides and WC–SHBG was inversely and strongly associated with elevated triglycerides and increase WC, both emerging biomarkers of the risk of CVD and T2D.

Table 17. Association between SHBG and CVD risk factors: Populations based studies.

Author	Study characteristics	Study population	Primary Endpoint*	Sex steroids included (assay method)	Confounding factors	Main findings (multi-adjusted model unless otherwise mentioned)
Gagnon SS et al. (2018) [214]	CS	846 (18-48)	CVD risk factors	TT SHBG (CLIA)	None	– SHBG was inversely associated with BMI ($\beta = -0.435$; $p < 0.001$). – SHBG was inversely associated with triglycerides ($\beta = -0.291$; $p < 0.001$) and SBP ($\beta = -0.096$, $p = 0.003$), and positively associated with HDL-C ($\beta = 0.158$; $p < 0.001$).
Liu C et al. (2017)[362]	CS	614 (≥ 40)	MetS	TT cFT SHBG (CLIA)	None	– SHBG was inversely correlated with: WC ($r = -0.245$, $p < 0.001$), FBG ($r = -0.115$, $p = 0.005$), TG ($r = -0.359$, $p < 0.001$), and positively with HDL-C ($r = 0.244$, $p < 0.001$).
Moon H et al. (2017) [363]	CS	1098 (≥ 30)	MetS	TT cFT SHBG (eCLIA)	Age, smoking, household, alcohol, exercise and cFT.	– ORs for MetS was 0.50, 95% CI 0.36, 0.68 for 1SD $\uparrow$ SHBG.

						– SHBG was inversely associated with all components except BP.
Jaspers L et al. (2016) [187]	L (3.0 years) (the RS)	1647 (≥45)	CVD risk factors (7 metrics)	TT E2 SHBG & FAI (LC-MS/MS & RIA)	Age, cohort, ethnicity, education, marital status, prevalent cancer/COPD, WHR and E2 / TT.	– OCH was associated with higher SHBG levels (OR = 2.56, 95% CI 1.45, 4.49). – SHBG remained significant for after adjustment for all confounders (OR= 2.12, 95% CI 1.20, 3.73).
Onat A et al. (2015) [364]	L (10.1 years) (the TARF)	868 (36-60)	Incident MetS=117	TT SHBG (eCLIA)	Age, abdominal obesity, smoking status, GGT, CRP + TT.	– SHBG concentrations were inversely associated with MetS (RR of each 1.75 fold increase in SHBG: 0.57; 95% CI: 0.62-0.93; P < 0.05).
Wang Q et al. (2015)[168]	CS (the NFBC & the YFS)	3697 (24-39)	CVD risk factors (circulating lipids and metabolites)	TT & SHBG (NMR metabolomics data)	Age, BMI, insulin, and TT.	– Higher circulating SHBG is favourably linked with a wide range of cardiometabolic risk factors, including lipoprotein lipids and subclasses, fatty acids, amino acids and inflammation-linked glycoproteins. – These associations remained robust after

						adjustment for baseline adiposity, insulin and TT levels. (detailed in Figure 24)
Antonio L et al. (2015) [180]	L (the EMAS)	3369 (40-79)	Incident MetS (n=289)	TT,cFT ,E2, fE2,E2/T SHBG (LC-MS/MS)	Age, centre, alcohol intake, smoking, physical activity, general health + TT.	– SHBG was independently associated with incident MetS (OR=1.78 95 %CI 1.48, 2.13). – Association between SHBG and incident MetS was attenuated, but remained significant (OR =1.33 95 %CI 1.05, 1.68)), after additional adjustment for TT.
Tanabe M et al. (2015) [321]	CS	249 (46-60)	MetS	TT aFT CFT bFT SHBG (CLIA & RIA)	None	– Low SHBG concentration was observed in subjects with MetS compared with those without MetS (41.4 ± 16.0 vs. 50.0 ± 16.3 $P < 0.001$) – As the number of MetS increased, SHBG values gradually decreased significantly.
Yang HY (2015) [365]	CS	3600 (20-89)	MetS	TT SHBG (CLIA)	Age, smoking and drinking status, BMI and TT.	– SHBG values gradually declined with the addition

						of MetS characteristics ($P < 0.05$). – A significant correlations between serum SHBG and each MetS characteristic (except for SBP). – Multi-adjusted model also showed inverse associations between serum SHBG and MetS, OR=0.96 (0.95–0.97, $P < 0.01$).
Canoy D et al. (2014) [188]	CS (the NFBC)	2716 (≥31 years)	CVD risk factors	TT, cFT & SHBG (CLIA)	Smoking, education, physical activity, insulin, HOMA-IR, WC, BMI & TT	– Higher SHBG was associated with decreasing BP, total cholesterol, triglycerides and CRP, and increasing HDL-cholesterol (all P for trend <0.05). – SHBG was unrelated to LDL-cholesterol.
Hsu B et al. (2014)[181]	CS & L (2 years) (the CHAMP study)	CS (n=1299) L (n=578) (≥70 years)	Incident MetS=106	TT, DHT, cFT, E1, E2, SHBG (LC-MS/MS)	Age, BMI and smoking status	– In cross-sectional analyses, for each 1-SD reduction in SHBG concentration, men had adjusted odds ratios for prevalent MetS at baseline of 1.16 (95% CI, 1.10 –1.28; $P<0.05$) for SHBG.

						– In the longitudinal analyses, low concentration of SHBG was predictive of incident MetS (ORs for each 1-SD reduction in SHBG was 1.09 (95% CI, 1.00 –1.18; P<0.05).
Brand JS et al. (2014) [182]	Meta-analysis (20 observational studies)	17,625 (Mean age 30.2-77.0 years) Incident Met S (n=584)	MetS	TT, cFT & SHBG (Varies-eCLIA, RIA & fluoroimmunoassay)	Age, smoking, alcohol consumption, physical activity, BMI and HOMA-IR.	– Men with low concentrations of SHBG were more likely to have prevalent MetS (ORs per quartile decrease was 1.73 (95% CI 1.62-1.85) and incident MetS (HRs per quartile decrease was 1.44 (95% 1.30-1.60). – Associations of SHBG with MetS in men vary according to individual MetS components, being primarily mediated through abdominal obesity, hypertriglyceridemia and hyperglycaemia.
Bonnet F et al. (2013) [175]	CS	233 (27-77)	CVD risk factors dysmetabolic men	TT E2 SHBG (eCLIA & RIA)	Age, smoking & BMI + TT/E2 or + VAT/ intrahepatic fat/ GGT	– SHBG is inversely associated with both glucose and lipid concentrations independent of TT/E2.

						– SHBG is inversely associated with both glucose and lipid concentrations in dysmetabolic men and that this association is related to intrahepatic but not to visceral fat content.
Zhang J et al. (2013) [322]	CS	2361 (20-73)	MetS	TT SHBG (eCLIA)	Age, smoking, BMI, HOMA-IR + TT	– SHBG is inversely associated with MetS (OR=0.99 95% CI 0.97–1.00; p=0.033). – SHBG remained inversely associated with MetS after further adjusting for TT.
Pang XN et al. (2013)[366]	CS	437 (45-94)	MetS	TT cFT SHBG (CLIA)	None	– SHBG is inversely correlated with BMI, WC, WHR, diastolic BP, FPG & TG while positively correlated with HDL-C ($P < 0.05$ for all). – As the number of MetS components increased, the concentrations of SHBG lower further.
Haring R et al. (2013) [361]	CS & L (5 years)	CS (n=1906)	Incident MetS (n=328)	TT cFT SHBG (CLIA)	Age, smoking, BMI, HbA1c & non-	– In cross-sectional multivariable models, baseline SHBG was not

	(the SHIP)	L (n=1435) (20-79)			response weights + TT	associated with prevalent MetS (OR per SD decrease: 1.13; 95% CI: 0.98, 1.30, p=0.08). – In longitudinal multivariable models, baseline SHBG concentrations (OR per SD decrease: 1.47; 95% CI: 1.17, 1.86, p=) associated with incident MetS. – Change analyses for each MetS component showed that decreasing SHBG concentrations were associated with increased WC and elevated TGs; however, SHBG change was not associated with incident MetS until adjustment for TT.
Chin KY et al. (2013) [367]	CS	332 (≥40)	MetS	TT cFT bT SHBG (CLIA)	Age, smoking, drinking status & BMI	– SHBG was inversely correlated with diastolic BP ($\beta=-0.114$, P=0.04) TGs ($\beta=-0.242$; P<0.001), WC ($\beta=-0.381$; P<0.001) and positively correlated with HDL-C ($\beta=0.263$; P<0.001).

						– SHBG was inversely and independently associated with MetS ($\beta=-0.262$; $p<0.05$).
Hong D et al. (2013) [368]	CS	2172 (21-79)	MetS	TT cFT SHBG (RIA)	Age, smoking status, alcohol intake, exercise & BMI	– Compared with the highest quartile, ORs of lowest quartile of SHBG for MetS: 6.34; 95% CI: 2.29, 17.58; $P<0.001$). – SHBG was inversely associated with WC, fasting glucose, TG, and BMI.
Kweon SS et al. (2012) [369]	CS (the Namwon Study)	4081 (45-74 years)	MetS	TT, cFT SHBG (CLIA)	Age, education level, marital status, smoking status, alcohol consumption, heart disease, cerebrovascular disease, CRP & HOMAIR	– Men with MetS had lower SHBG concentrations than those without MetS (59.11 ± 23.03 vs. 46.61 ± 18.20; $P<0.001$). – SHBG levels were inversely associated with all MetS components, except low HDL-C. – SHBG levels were inversely associated with MetS (OR of each standard deviation increase in logarithmic value, 0.57; 95% CI, 0.52-0.61) and were linear across quartiles of SHBG.

Firtser S et al. (2012) [189]	CS & L (6.0 years) (the CRYFS)	CS (n=1024) L(n=768) (24-45)	CVD risk factors	TT cFT SHBG (RIA)	Age, BMI, smoking, alcohol consumption and physical activity	– Higher concentrations of SHBG was associated with favourable cardiovascular risk profile characterised by lower levels of triglycerides, and systolic BP, and higher levels of HDL-C (P<0.05).
Liao CH et al. (2012) [370]	CS	237 (20-88)	MetS	TT cFT bFT E2 DHEAS (CLIA)	Age & smoking	– SHBG was significantly associated with MetS (OR per 1 SD decreased: 0.948; 95% CI: 0.913, 0.985, $p = 0.006$). – Increasing numbers of MetS components was associated with decreasing levels of SHBG. – SHBG was inversely associated with BP, blood glucose and TGs.
Gannage-Yared MH et al. (2011) [323]	CS	201 (18-30)	CVD risk factors	TT DHEAS SHBG (CLIA)	Age, smoking, BMI, WC	– SHBG was inversely associated with WC, BMI, hs-CRP and TGs (P for all <0.05).
Brand JS et al. (2011)[371]	Meta-analysis (32 studies)	10 537 men	MetS	TT cFT SHBG (Varies-eCLIA, RIA & fluoro immunoassay)	Age, BMI & diabetes status	– MetS is associated with lower SHBG concentrations.

		(mean age varies from 14-89)				Higher SHBG concentrations were associated with a reduced risk estimate (0.29, 95% CI 0.21–0.41; P<0.05).
Bhasin S et al. (2011)[183]	CS & L (the FHS)	CS1 (n=1407) CS2 (n=1887) L(n=618)	MetS	TT cFT SHBG (LC-MS/MS)	Age, smoking, BMI, HOMA & TT	- SHBG is independently associated with the risk of MetS (ORs for 1-SD decrease in SHBG and prevalent Met S: 1.41; 95% CI: 1.18, 1.69 and incident MetS: 1.53; 95% CI: 1.15, 2.04. - SHBG was inversely associated with elevated BP, elevated WC, and low HDL-C while TGs in CS analysis was not associated but inversely with elevated TGs in longitudinal analysis.
Li C et al. (2010) [178]	CS (the NHANES III)	1226 (≥20)	MetS	TT cFT bT SHBG (eCLIA)	Age, race, smoking status, alcohol intake, physical activity level, LDL cholesterol, CRP & HOMA-IR.	- Low concentrations of SHBG was independently associated with an increased likelihood of having MetS (OR: 2.17; 95% CI: 1.32, 3.56). - SHBG was also significantly associated with abdominal

						obesity ($P < 0.001$ for linear trend) and a high concentration of TGs ($P < 0.001$).
Maggio M et al. (2010)[372]	CS (the InCHIANTI)	452 (65-96)	MetS	TT, cFT, DHEAS, E2, fE2 SHBG (RIA)	Age, smoking, alcohol consumption, physical activity, IL-6 & insulin +TT/E2/DHEAS	– Men with MetS components (≥ 3) have lower SHBG concentration compared to men MetS components (≤ 3) (83.6 vs. 104; $P < 0.001$).
Yasui T et al. (2008)[373]	CS	174 (61-75)	CVD risk factors	TT cFT bT E2 DHEAS SHBG (eCLIA & RIA)	Age & BMI	– SHBG concentrations are not correlated with any lipid profiles parameters (all $P > 0.05$).
Kupelian V et al. (2008)[374]	CS (the MMAS)	1885 (30-79)	MetS	TT cFT SHBG (eCLIA)	Age, race/ethnicity, physical activity, smoking, and alcohol consumption	– A strong inverse associations ORs for 1-SD decrease in SHBG and prevalent Met S: 2.46; 95% CI: 1.95–3.1; $P < 0.05$) and largely consistent across quartiles. – SHBG was associated with elevated TGs, elevated WC and low HDL (< 40 mg/dL).
Chubb SA et al. (2008)[375]	CS (the HIMS)	2502 (≥ 70)	MetS	TT cFT SHBG (CLIA)	Age, TT, SHBG and the squares of TT & SHBG	– Men with MetS had lower SHBG concentrations than those without MetS

						(36.8±14.0 vs. 45.5±17; P<0.001). – Low SHBG concentration was inversely associated with MetS, with ORs of one-unit decrease: 1.77; 95% CI: 1.53, 2.06; P<0.001). – Low SHBG was also associated with increased for each component and decrease HDL-C (<1.03 mmol/L).
Rodriguez A et al. (2007) [376]	L (5.8 year) (the BLSA)	417 (20-94)	Incident MetS (n=99)	TT FTI DHEAS SHBG (RIA)	Age & BMI	– SHBG concentrations were inversely related to the risk for development of MetS with HR per 1SD decrease: 0.58; 95% CI: 0.44-0.78; P<0.0003).
Onat A et al. (2007)[377]	CS	307 (52-68)	CVD risk factors	TT cFT SHBG (eCLIA)	Age, smoking, HOMA-IR & TT.	– SHBG was inversely associated with waist girth (β=0.99) & TG (β=0.99); P=<0.05 for both). – Low SHBG concentration is associated with MetS (P<0.05).
Kupelian V et al. (2006)[378]	CS &	1709 (40-70)	MetS	TT cFT SHBG (RIA)	Age, BMI, MetS score, smoking, & self-reported health	– SHBG are strongly predictive of MetS with RR per 1SD increase: 1.20;

	L (the MMAS)		Incident MetS (n=300)			95% CI: 95% CI: 1.03, 1.40; P<0.05). – Associations between SHBG and MetS were significantly stronger among men with a BMI ≤ 25 1.65 (95% CI, 1.12–2.42), but not in men with a BMI ≥ 25. – Nonobese men with low SHBG were at 2- to 4-fold increased risk of developing MetS over 15 yr of follow-up.
Kalme T et al. (2005)[190]	CS	335 (70-89)	MetS & CVD risk factors	TT & SHBG (Immunofluorometric)	Age	– Low SHBG was associated with an increased prevalence of abnormal glucose tolerance and the MetS: OR= 1.36; 95%CI: 1.22, 1.50). – An inverse correlation between SHBG and components of MetS and CVD risk factors including 2-hour glucose, systolic BP and BMI, whereas positive correlations with HDL-C (P<0.05 for all).

Bataille V et al. (2005)[379]	CS	352 (50-59)	CVD risk factors	TT E2 SHBG (RIA)	Age, SBP, BMI (or WHR), smoking, alcohol intake, physical activity and insulin	– SHBG was inversely associated with TGs ($\beta=-0.216$) and ApoA1 ($\beta=-0.270$). – SHBG was positively associated with HDL-C ($\beta=0.385$). Conversely, there was no association between SHBG and LDL-C.
Laaksonen DE et al. (2004)[344]	L (11.0 years) (the KIHDRFS)	702 (42-60)	Incident MetS (n=147)	TT cFT SHBG (Fluoroimmunoassay)	Age, prevalent CVD, smoking, alcohol consumption, socioeconomic status, BMI (or WC), insulin, glucose, triglycerides; and systolic BP and use of BP medication.	– Low SHBG concentrations independently predict the development of MetS: OR 1.67; (95% CI: 1.05, 2.65; $P<0.05$) for the lowest fourth of concentrations of SHBG.
Laaksonen DE et al. (2003)[258]	CS	1896 (42-60)	MetS	TT cFT DHEAS SHBG (Fluoroimmunoassay)	Age, smoking, alcohol consumption, cardiovascular disease and adulthood socioeconomic status	– Low SHBG concentrations were strongly associated not only with components of the MetS but also with the MetS itself, independently of BMI: OR 1.66; 95% CI: 1.03, 2.67. – A linear trend for this association: OR 1.86; 95% CI 1.30, 2.69) for upper vs lower third.

Gyllenberg J et al. (2001)[380]	CS	508 (41-72)	CVD risk factors	TT FAI E2 SHBG (RIA)	Age, smoking, systolic BP & body fat mass	– An inverse association between SHBG and cardiovascular risk factors, primarily the plasma lipids, VLDL-C, & TGs, but no association with TC and LDL-C. Whereas, there were positive associations between SHBG and HDL-C.

Abbreviations: bT, Bioavailable testosterone; β , Standardized regression coefficients; bT, Bioavailable testosterone; BP, Blood pressure; BMI, Body mass index; CVD, Cardiovascular disease; CLIA, Chemiluminescent immunoassay; CI: confidence interval; DHEAS, Dehydroepiandrosterone sulfate; DHT, 5 α -dihydrotestosterone; E2, Oestradiol; E1, Oestrone; cFT, Calculated free testosterone ; FBG, Fasting blood glucose; HR: hazard ratio; HDL, High-density lipoprotein; HOMA-IR, Homeostasis model assessment-insulin resistance; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; LDL-C, Low density lipoprotein Cholesterol; MetS, Metabolic syndrome; ORs, Odds ratios; OCH, Optimal cardiovascular health; RIA, Radioimmunoassay; RR, Relative risk; SHBG, Sex hormone-binding globulin; TT, Total testosterone; TC, Total cholesterol; TGs, Triglycerides; VLDL-C, Very low density lipoprotein Cholesterol; VAT, Visceral adipose tissue; WC, Waist circumference; WHR, Waist-to-hip ratio

BLSA, Baltimore Longitudinal Study of Aging; CHAMP, Concord Health and Ageing in Men Project; CRYFS, Cardiovascular Risk in Young Finns Study; FAMHES, Fangchenggang Area Male Health and Examination Survey; FHS, Framingham Heart Study; HIMS, Health in Men Study; InCHIANTI, Invecchiare in Chianti study; KIHDRFS, Kuopio Ischemic Heart Disease Risk Factor Study; MMAS, Massachusetts Male Aging Study; NHANES,

*National Health and Nutrition Examination Survey; NFBC, Northern Finland Birth Cohort; RS, Rotterdam Study; SHIP, Study of Health in Pomerania;
TARF, Turkish Adult Risk Factor study ; YFS, Young Finns study*

4.1.6. SHBG and CVD

CVD comprises diseases of the heart and vascular system, including coronary heart disease, stroke, ischemic heart disease (IHD), heart failure, cardiomyopathy, cerebrovascular disease, peripheral vascular disease (PAD) etc. [337, 357]. An estimated 17.5 million people died from CVD in 2012, and by 2030, more than 23.3 million people will die annually from CVD [337, 359].

Although it is well known that there is a firm relationship between low SHBG concentrations and adverse CVD risk, the role of SHBG on clinical CVD events is still undefined in men. A few prospective studies have investigated the association between SHBG and clinical CVD events in men [191, 305, 381, 382]. However, limitations have been in their outcome measurement by either using single or composite outcomes or including CVD or population examined, i.e. only elderly participants. Studies are also diverse in their outcome measurement IHD (myocardial infarction (MI)) [184, 383-385], and ischemic stroke [383, 385, 386], heart failure [280], and few solely focused on ischemic stroke events [184, 324, 386], measures of vascular calcification [387, 388], subclinical measures of CVD [389], marker of endothelial dysfunction [390] and atrial fibrillation [391] and subclinical ischemic disease [389]. The studies reporting the associations between SHBG and CVD events in men are summarised in Table 18.

While low SHBG concentrations have been associated with increased CVD but single events in observational studies of community-dwelling men [184, 345, 383-386], only four studies has assessed this association specifically in men with composite CVD [191, 305, 381, 382], with inconclusive findings.

Table 18. Associations between SHBG levels and CVD events in men.

Reference	Study characteristics	Study population (Age)	Outcome (Incident/cases)	Sex steroids included (assay method)	Confounding factors	Major findings (multi-adjusted model unless otherwise mentioned)
Wang A et al. (2018)[382]	L 6.2 years (ORIGIN trial)	5553 (mean age 63.5 years)	CVD (1028) 80% had previously established diabetes	TT cFT SHBG (MAP 250+ panel on the LUMINEX)	Age, LH levels, previous CVD, previous diabetes diagnosis, use of metformin, use of statins, systolic BP, HbA1c, LDL, BMI and smoking	One-SD increase in SHBG predicted CVD events in the model adjusted for age (HR 1.10, 95% CI 1.04–1.17; $p < 0.01$) as well as additional risk factors (HR 1.07, 95% CI 1.00–1.14; $p = 0.03$).
Karagoz A et al. (2017) [345]	L 5.75 years (the TARF study)	943 (mean age 55 ± 11 years)	CHD (128)	T SHBG (eCLIA)	Age, systolic BP, total cholesterol, waist girth, testosterone, and SHBG.	- SHBG did not predict CHD (RR=0.99, 95% CI 0.84, 1.17) when SHBG was included as a continuous variable. - High SHBG predicted increased CHD risk with HtgW with the ‘healthy’ category, served as a referent.
Holmboe SA et al. (2016) [184]	L 15.7 years (MONICA I, II and III study)	5350 (30–70 years)	IHD (659) Stroke (147)	SHBG, T, free T, E2, LH, FSH, LH: T	-NA	SHBG was not associated with stroke SHBG ($P = 0.086$).

				(time-resolved fluoroimmunoassay)		Men in the higher age-standardised SHBG quartile had a lower risk of IHD compared with men in the lowest quartile but only significant for nonsmoking men (HR 0.67, 95% CI 0.46–0.99).
Chan YX et al. (2016) [191]	L 14.9 years (the BHS study)	1804 (17-97 years)	Composite CVD events [CHD, Stroke, CHF and PAD] (234)	TT, cFT, DHT and E2 (LC-MS/MS)	Age, smoking, vigorous exercise, alcohol, BMI, diabetes, CVD history, COPD history, non-skin cancer history, SBP, hypertension, lipids medication, cholesterol, HDL, (log) triglycerides, (log) CRP, (log) creatinine.	- When analysed as a continuous variable, SHBG was inversely associated with CVD events in the age-adjusted model (HR = 0.85, 95% CI 0.76–0.94; P = 0.003), however, this was not significant after adjustment for other risk factors (HR = 1.03, 95% CI 0.92–1.15; P = 0.623). - No associations were seen when SHBG was analysed in quartiles.

Shores MM et al. (2014) [381]	L 8.9 years (the study) CHS	1032 Men aged 66-97 years.	CVD event 436(174 MIs & 105 Strokes)	TT cFT DHT SHBG (LC-MS/MS)	Age, race, clinic site, smoking status, alcohol consumption systolic BP, antihypertensive use, HDL cholesterol, BMI, WC & diabetes.	– SHBG was not associated with incident CVD in fully adjusted analyses.
Yeap BB et al. (2014) [383]	L 6.6 years (the study) HIMS	3690 70-89 years	Stroke (300)	TT,DHT, E2, SHBG & LH (LC-MS/MS)	Age, education, smoking, BMI, WHR, hypertension, dyslipidemia, diabetes and creatinine, prevalent CVD and cancer	– SHBG was not associated with incident stroke HR 1.31, 95% CI 0.85-2.01.
Shores MM et al.[386] (2014)	L Ten years (the study) CHS	1032 66–97 years	114 (incident ischemic stroke)	SHBG, TT, fT, DHT, fDHT (LC-MS/MS)	Age, ever smoked, pack-years (ln), systolic blood pressure, antihypertensive medications, atrial fibrillation, total and HDL cholesterol, lipid-lowering medications, high creatinine, fasting glucose (ln), use of insulin or oral hypoglycaemic medications and TT.	– In age-adjusted models, SHBG had a nonlinear relationship with stroke risk (P = 0.053 for both the linear and quadratic terms and P = 0.007 for the quadratic term). – SHBG has a positive association with incident ischaemic stroke (P for trend=0.04). However, when total T was added to the model, the risk

						estimates were attenuated, and SHBG was not associated with incident ischaemic stroke.
Soisson V et al. (2013) [384]	Case-cohort of L study 4.0 years (the French 3C study)	495 >65 years	112 (CHD)	TT, E2 and SHBG (RIA)	Study centre, BMI, diabetes, hypertension, hypercholesterolemia and smoking status	– No significant association of serum SHBG with IAD was detected in the multi-adjusted model. – Adjusting testosterone - IAD event association for SHBG made no substantial change to the results.
Ohlsson C et al. (2011) [305]	L 5.1 years (the MrOS Study)	2416 (69-81 years)	Major CV event [MI, CHD, Stroke TIA] (485)	TT cFT E2 SHBG (GC-MS/MS)	Age, Osteoporotic Fractures in Men study site, morning sample & T	– SHBG concentrations were inversely associated with the risk of CV events, HR Per-quartile increase in SHBG is 0.90, 95% CI 0.83, 0.98, P= 0.012. – In analyses that included both testosterone and

						SHBG, high testosterone but not SHBG predicted nonfatal CVD events, HR Per-quartile increase in SHBG is 0.94, 95% CI 0.86,1.03, P= 0.17.
Hyde Z et al. (2011) [385]	L 5.1 years (the HIMS study)	3637 (70-88 years)	IHD [Angina, MI and coronary revascularisation] (458)	TT, fT, SHBG & LH (CLIA)	Age, WHR, prevalent IHD, hypertension, dyslipidaemia, smoking status, diabetes and Charlson's weighted co-morbidity index.	– SHBG was not associated with IHD events.

Abbreviations: BMI, Body mass index; CVD, Cardiovascular disease; CRP, C-reactive protein; CHD, Coronary heart disease; CI: confidence interval; COPD, Chronic obstructive pulmonary disease; DHT, 5 α -dihydrotestosterone; E2, Oestradiol; cFT, Calculated free testosterone ; HbA1c, Glycated haemoglobin; HR: hazard ratio; HDL, High-density lipoprotein; HtgW, Hypertriglyceridemia; IAD, Ischemic arterial disease; IHD, Ischemic heart disease; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; LDL, low density lipoprotein; MAP 250+ panel on the LUMINEX 100/200, customized Human Discovery Multi-Analyte Profile (MAP) 250+ panel on the LUMINEX 100/200 platforms; LH, Luteinizing hormone; MI, Myocardial infarction; ORs, Odds ratios; SHBG, Sex hormone-binding globulin; TT, Total testosterone; TIA, Transient ischemic attack; WHR, Waist-to-hip ratio

BHS, Busselton Health Study; CHS, Cardiovascular Health Study; HIMS, Health in Men Study; MrOS, Osteoporotic Fractures in Men; MONICA I, II and III, Danish Monitoring Cardiovascular risk factor surveys; ORIGIN, Outcome Reduction with an Initial Glargine Intervention trial; TARF, Turkish Adult Risk Factor study

Similarly, the association of low SHBG concentrations and CVD related mortality has been investigated in a few population-based studies, which demonstrated conflicting results. Low SHBG has been suggested as a marker for increased CVD mortality risk in two studies [190, 392], while four studies have demonstrated no association [385, 392-395] and one study have demonstrated positive association [396], between SHBG and CVD mortality. Table 19 summarise the earlier studies reporting the associations between SHBG and CVD related mortality in men.

Table 19. Associations between SHBG levels and CVD related mortality in men.

Reference	Study characteristics	Study population (Age)	CVD related mortality	Sex steroids included (assay method)	Confounding factors	Major findings (multi-adjusted model unless otherwise mentioned)
Ramachandran S et al. (2018) [397]	L 4.3 years (the BLAST study)	362 men with T2D (58-73 years)	44 men died	TT cFT SHBG (eCLIA)	Age, BMI, TG, HDL- C, HbA1c, Statin & TT	– SHBG is associated with mortality in men with T2D (HR= 1.03, 95% CI: 1.01, 1.04; P=0.002). – This association was mediated by age; it was only found in men aged >66 years (HR= 9.37, 95% CI: 2.68, 32.79; P<0.001).
Tint AN et al. (2016) [398]	L 7.6 years (Austin Health)	531 men (Mean age 66 years)	175 men died	TT SHBG (Immunoassay)	-	– SHBG was not significantly associated with cardiovascular disease (CVD)-related mortality (HR 1.015 (95% CI 0.999, 1.031), P=0.052).
Shores MM et al. (2014) [381]	L 10.8 years (the CHS study)	1032 (66-97 years)	777 men died	TT cFT DHT SHBG (LC-MS/MS)	Age, race, clinic site, smoking status, alcohol consumption, systolic BP, antihypertensive use, HDL cholesterol, BMI, WC & diabetes.	– SHBG was not associated with CVD mortality in fully adjusted analyses.

Goodman-Gruen D et al. (1996) [392]	L 19 years (the RBS)	760 (50-82 years)	235 CVD death 134 IHD death	SHBG only	Age, systolic BP, cholesterol, HDL-cholesterol, smoking status, alcohol consumption, fasting blood glucose & BMI.	– SHBG levels were not associated with risk of fatal CVD (RR 0.99 (95% CI 0.99,1.01) or IHD (HR 1.00 (95% CI 0.99,1.00) – The quintile of baseline SHBG was unrelated to CVD or IHD mortality rate.
Holmboe SA et al. (2015) [394]	L 18.5 years (the MONICA I-II & Inter99)	5323 (30-70 years)	428 CVD related deaths	T, E2, LH, FSH, SHBG (Immunofluorometric assay)	BMI, study, alcohol consumption, & exercise	– No associations were observed for SHBG about CVD mortality.
Menkes A et al. (2010) [399]	L 9.0 years (the NHANES III)	1114 (≥20 years)	42 CVD death	TT bT cFT E2 FE2 SHBG E2: TT TT: SHBG E2: SHBG (eCLIA)	Age, race-ethnicity, smoking status, household income, education, alcohol consumption, exercise, per cent body fat + TT	– Men with low SHBG concentrations had a lower risk of death from CVD than men with high concentrations HR= 0.24 (95% CI: 0.05, 1.24). – In the multivariable-adjusted model, HRs of CVD death for a decrease in SHBG concentrations equal to the difference between the 90th and 10th percentiles was 1.73 (95% CI: 1.22, 2.43).

Kalme T et al. (2005) [190]	L 8.0 years (Seven Countries Study)	335 men (70-89 years)	140 CVD, CHD and Stroke deaths	SHBG only	abnormal glucose tolerance & prevalent CVD	– Low SHBG indicated increased CVD mortality RR=1.65 (1.06 –2.58) ;P= 0.026 and CHD mortality RR=1.70 (1.01–2.88) ;P= 0.048 – Observed association persisted after adjustment for: ○ age and BMI, SHBG RR=1.80 (1.12–2.88) ;P= 0.015 for CVD and RR= 1.85 (1.06 –3.22) ;P= 0.030 for CHD mortality ○ abnormal glucose tolerance, RR=1.78 (1.13–2.82) ;P= 0.014 and RR=1.81 (1.05–3.13) ;P= 0.033. ○ MetS, RR=1.60 (1.01–2.56); P= 0.047 and 1.73 (1.00 –3.00); P= 0.051. – But not after adjustment for prevalent CVD and also after adjustment for MetS for CHD mortality.
Hyde Z et al. (2012) [396]	L 5.1 years	3637 (70-88 years)	207 CVD deaths	TT, fT, SHBG & LH (CLIA)	Age, WHR, hypertension,	– Higher SHBG and LH levels were significantly associated with reduced

	(the study)	HIMS				dyslipidaemia, diabetes, smoking status, Charlson's weighted comorbidity index, prevalent CVD, and number of cancer diagnoses	survival in both univariate and multivariate models

Abbreviations: BMI, Body mass index; CVD, Cardiovascular disease; CRP, C-reactive protein; CHD, Coronary heart disease; CI: confidence interval; COPD, Chronic obstructive pulmonary disease; DHT, 5 α -dihydrotestosterone; E2, Oestradiol; cFT, Calculated free testosterone ; HbA1c, Glycated haemoglobin; HR: hazard ratio; HDL, High-density lipoprotein; HtgW, Hypertriglyceridemia; IAD, Ischemic arterial disease; IHD, Ischemic heart disease; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; LDL, low density lipoprotein; MAP 250+ panel on the LUMINEX 100/200, customized Human Discovery Multi-Analyte Profile (MAP) 250+ panel on the LUMINEX 100/200 platforms; LH, Luteinizing hormone; MI, Myocardial infarction; ORs, Odds ratios; SHBG, Sex hormone-binding globulin; TT, Total testosterone; TIA, Transient ischemic attack; WHR, Waist-to-hip ratio

BLAST study, an acronym taken from the UK cities and towns of Birmingham, Lichfield, Atherstone, Sutton Coldfield, and Tamworth; CHS, Cardiovascular Health Study; HIMS, Health in Men Study; MrOS, Osteoporotic Fractures in Men; MONICA I, II and III, Danish Monitoring Cardiovascular risk factor surveys; NHANES III, Third National Health and Nutrition Examination Survey; RBS, Rancho Bernardo Study

POTENTIAL ROLE OF SHBG IN PROSTATE CANCER

5. SHBG and prostate cancer

5.1. Prostate

The prostate is a walnut-sized organ that is an integral part of the male reproductive system. The prostate is located in the pelvis and is surrounded by the rectum, the bladder, the periprostatic and dorsal vein complexes and neurovascular bundles that are responsible for erectile function, and the urinary sphincter that is responsible for passive urinary control. The prostate is composed of branching tubuloalveolar glands arranged in lobules surrounded by fibromuscular stroma. The acinar unit includes an epithelial compartment made up of epithelial, basal, and neuroendocrine cells and separated by a basement membrane, and a stromal compartment that includes fibroblasts and smooth muscle cells, which helps to push semen into the urethra so that it can be expelled from the body during ejaculation [400, 401]. The epithelial cells of prostate manufacture a fluid that contains large amounts of prostatic acid phosphatase (PAP) and PSA, a kallikrein-like serine protease that degrades proteins to promote liquefaction of semen. Both prostate epithelial cells and stromal cells express ARs and depend on androgens for growth. T, the major circulating androgen, is converted by the enzyme 5 α -reductase to DHT in the gland [400, 401]. SHBG plays an important role in PCa pathophysiology since it modulates transport and availability of androgens. SHBG, which binds to these androgens and prevents their diffusion across cell membranes, is known to regulate their action. There is also evidence of a direct action of SHBG in PCa cells, demonstrating its interaction with these cells and the subsequent intracellular signalling [83, 84, 114], are detailed in Chapter 2.4.

5.2. Diseases of the prostate

Prostatic diseases are benign or malignant disorders that affect the prostate primarily. Benign prostatic hyperplasia (BPH) and prostatitis are the most common benign disease while PCa is a most common malignant disease of the prostate. These three disorders are the most frequent type of prostate disease [400, 402] and are illustrated in Figure 33.

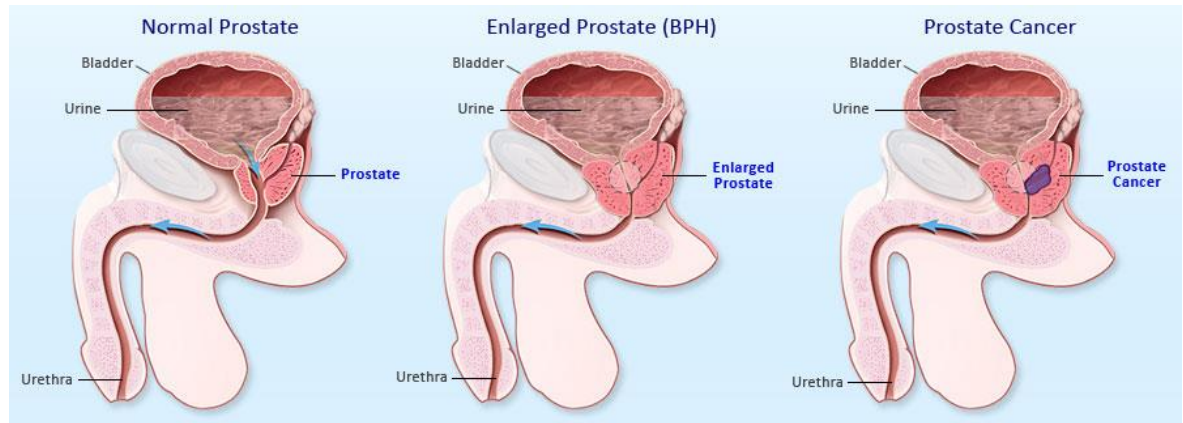


Figure 33. A most frequent type of prostate disease.

Source <http://amitghose.com/awareness-prostatecancer.html>

5.2.1. Benign prostatic hyperplasia (BPH)

BPH is a non-cancerous growth (enlargement) of the prostate gland to the point that it begins to compress the urethra, which slows or blocks the flow of urine that can still cause symptoms that require medical or surgical treatment. BPH is accompanied by a significant increase in the proliferation rate of epithelial cells in the hyperplastic acini, but they do not tend to progress to malignancy [403]. BPH starts after the age of 40, more than 50% of men over age 50, and more than 80% of men over the age of 80 have BPH [401, 404].

5.2.2. Prostatitis

Prostatitis is the inflammation (swelling) of the prostate gland, which is characterised by symptoms of painful or difficult urination and discomfort around the anus, scrotum and the perineum. Depending on the cause, prostatitis can be acute (caused by a bacterial infection) or can be chronic (non-bacterial inflammation). Prostatitis is the most common prostate disease in younger and middle-aged men (≤ 50), but it may also occur in older men.

5.2.3. Prostate Cancer (PCa)

PCa is the most commonly occurring non-cutaneous cancer, and the fifth leading cause of cancer deaths in men, worldwide [405]. An estimated 1.1 million newly diagnosed cases of PCa were recorded worldwide in 2012, accounting for 15% of the all new cancers diagnosed in men [405, 406]. In Australia alone, approximately 21,000 newly diagnosed cases of PCa occur annually and of these around 15% will progress to the high-risk disease within 7 to 10 years of diagnosis and on to incurable, metastatic disease within a further five years [407]. In 2016, PCa was the 3rd most common cause of cancer death in Australia. It was also the second most common cause of cancer death among males in 2016. It is estimated that it will remain the third most common cause of cancer death in Australia and the second most common cause of male cancer death

in 2018. In 2016, there were 3,248 deaths from PCa in Australia. In 2018, it was estimated that this would increase to 3,500 deaths. In 2018, it was also estimated that the risk of a male dying from PCa by his 85th birthday would be 1 in 31 [408]. In 2018, the incidence rate of PCa among males was expected to increase from the age group 35-39 until age group 65-69. It is also estimated that the age-standardised mortality rate will be 25 deaths per 100,000 males. The age-standardised mortality rate from PCa is expected to increase with age, from age group 50-54 [408].

5.3. Screening and diagnosis of PCa

The digital rectal examination (DRE) and testing blood levels of serum PSA are the most useful screening methods for the detection of PCa [409, 410]. Most patients are diagnosed after presenting with abnormal DRE or elevated PSA, followed by a multicore biopsy sampling and algorithm for diagnostics evaluation is illustrated in Figure 34. Pathological evaluation of tissue obtained by needle biopsy (multicore biopsy) or radical prostatectomy (RP) biopsy is essential to confirm a PCa diagnosis and to grade cancer [411].

PCa is the most common male malignancy, and in the current era of PSA screening, nearly 90% of PCa are clinically localised at the time of their diagnosis. However, a significant percentage of men develop rapidly progressing aggressive PCa leading to metastasis and death from the disease many others will have indolent cancers that are cured with initial therapy or may be safely observed [409, 412].

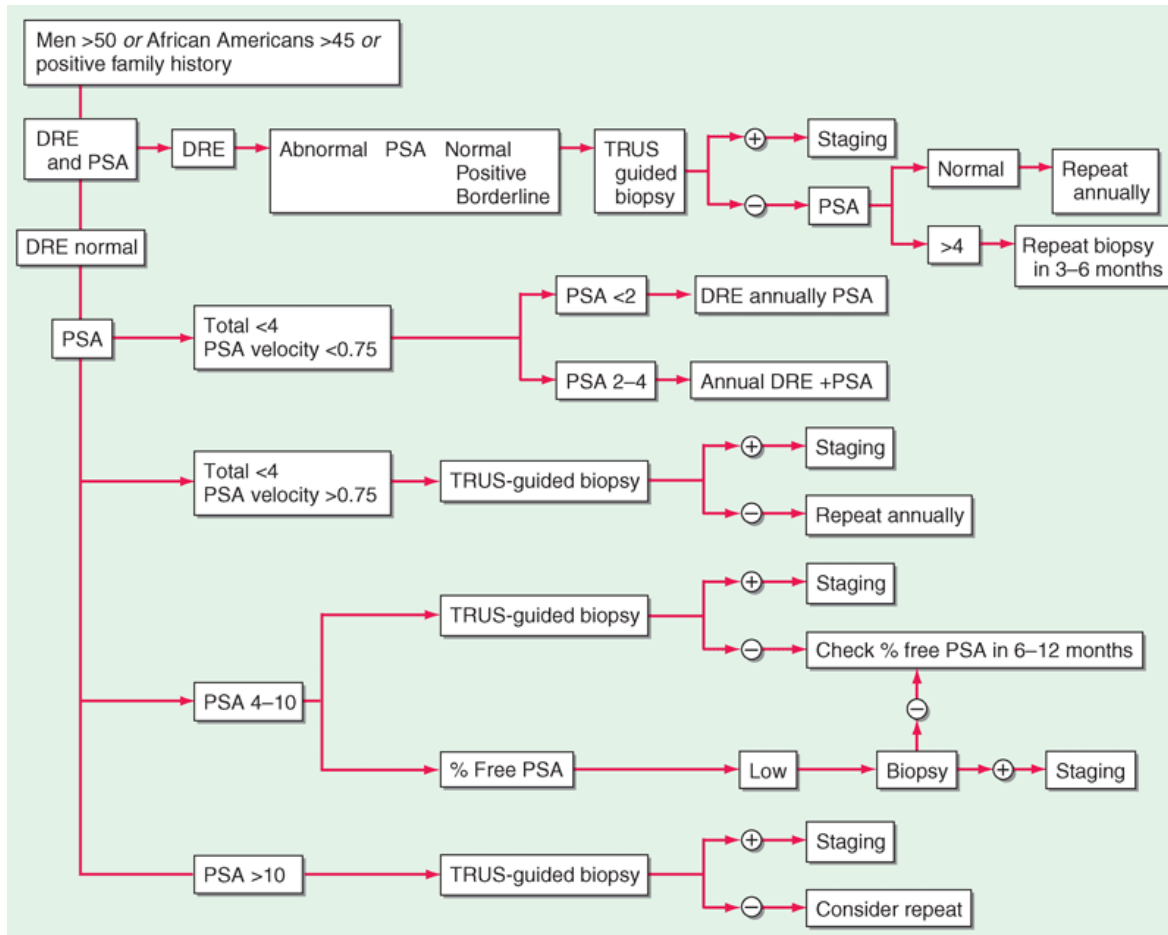


Figure 34. Schema for diagnostic evaluation of PCa in men.

Adopted from Harrison's principles of internal medicine 19e; McGraw-Hill Companies, Inc.: www.accessmedicine.com

Abbreviations: DRE, digital rectal examination; PCa, Prostate Cancer; PSA, prostate-specific antigen; TRUS, Trans rectal ultrasonography

Biopsy

Prostate biopsy remains the gold standard for diagnosing PCa. Histopathological assessment of biopsy tissue is the mainstay of diagnosing PCa, and in the present era, PCa is diagnosed through histological examination of needle-biopsy specimens [411]. The trans rectal ultrasonography (TRUS) guided multicore biopsy technique is the most advanced and standard mode of prostate biopsy sampling [409, 413]. The mode of prostate biopsy, in particular, the zones in which different prostatic diseases predominate, is key to disease management [409, 410]. Most carcinomas develop in the peripheral zone, which occupies 70% of the gland, and cancers in this location may be palpated during a DRE whereas BPH tends to be a process that occurs in the transitional zone [409, 414].

5.3.1. PCa: grading and staging

Grading

Grading is an important part of the evaluation of PCa. Approximately 98% of PCa cases are glandular in origin; the Gleason scoring system or the Gleason score (GS) (also called the Gleason sum) is the most commonly used grading system used for pathological evaluation of PCa (prostatic adenocarcinoma). The GS of biopsy material is a key parameter and plays a vital role in diagnostic evaluation, risk stratification, prognostication, and management decisions regarding PCa [411]. This system for prostatic adenocarcinoma was developed by Donald Gleason in 1966 and is one of the strongest prognostic factors and is integral to the management decisions for urologists [409, 415].

This system is based on the histological appearance of PCa cells, specifically, the extent of glandular differentiation and the pattern of growth in the stroma. Normal tissue has an ordered pattern of growth, but in cancer tissue, the pattern is not ordered because of the unpredictable way cancer cells grow [409, 410]. When malignant cells

are confined to the prostate acini, and the basal cell layer remains at least partially intact, it is defined as prostatic intraepithelial neoplasia (PIN). High-grade PIN is an established precursor to adenocarcinoma, which is distinguished by an absence of the basal epithelial layer [416]. The GS is used to show how abnormal or different cancer tissue is when compared with normal tissue and consist of primary and secondary grades. The two most common patterns of growth seen in the biopsy samples are each given a number from 1 (most differentiated) to 5 (least differentiated). Each specimen is assigned a GS based on their most prevalent (primary) and second-most prevalent (secondary), histologic grades. The combination of the two grades gives the total score (ranging from 2 to 10, with most cancers falling between 6 and 8) [410, 416]. For example, if the GS is written as $3+4=7$, it means that primary grade 3 and the secondary grade is 4, and they are added for a GS of 7. The greater the difference from the normal tissue pattern, the higher the GS, the more aggressive cancer acts in the body [409, 410], and the pattern is illustrated in Figure 35.

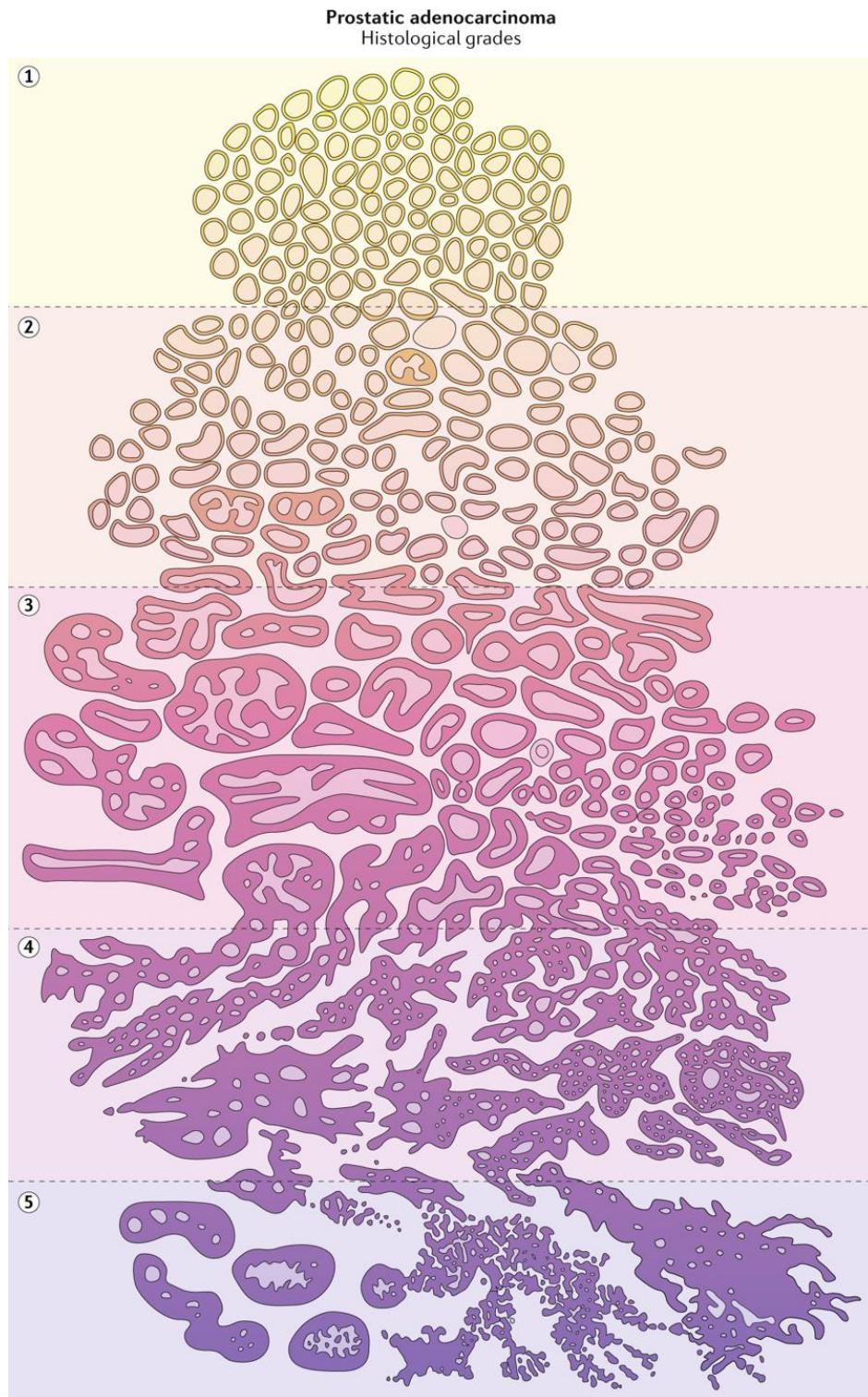


Figure 35. Prostate tumour grading and scoring system developed by Donald Gleason and is a surrogate for tumour aggressiveness.

Reproduced, with permission, from Sathianathan NJ et al. (2018) [409].

Despite the current standard practice of multicore biopsy sampling, the most aggressive area of the tumour is frequently under-represented or over-represented. Indeed, 25–50% of cases of PCa need to be either upgraded or downgraded from their initial biopsy score to a more accurate surgical GS after analysis and grading of prostatectomy tissue or biopsy core [417]. Furthermore, the GS system is vulnerable to sampling variation (i.e., failing to sample the area with the highest GS), incomplete sampling, and discordance in biopsy interpretation and scoring (GS) by pathologist [417]. It has been reported that the GS in TRUS guided multicore biopsy technique misses 21% to 28% of PCa and undergrades 14% to 17%. A much more complete and accurate assessment of tumour volume and grade is recognised after RP when the true extent of primary, secondary and tertiary GS is known; this is defined as the pathological GS [409, 411].

Aggressiveness

The decision of which subsequent treatment to pursue in clinically localised PCa and predicting whether localised PCa will become metastatic depends on the aggressiveness of the tumour. Aggressive PCa, defined by the progression of the localised disease to metastasis after therapy and accounts for almost two-thirds of PCa related mortality [405]. Current clinical parameters do not stratify aggressive PCa from indolent disease (clinically localised PCa). The histological grades as determined by a pathologist, including GS in biopsy or biopsy cores, are currently the optimal method to detect tumour aggressiveness (Figure 30), but none has proven to be definitive [418]. The key challenges of biopsy-based determination of PCa aggressiveness include tumour heterogeneity, biopsy-sampling error, and variations in biopsy interpretation [417].

Staging

A staging system is a standard method for the cancer care team to describe cancer's size and the extent of its growth within or beyond the prostate, i.e. how far cancer has spread. Staging is largely based on the tumour-node-metastasis (TNM) system that was

first implemented in 1978. The most widely used staging system for PCa is the American Joint Committee on Cancer (AJCC) TNM system, which was most recently updated in January 2018 [419].

The TNM system for PCa is based on five key pieces of information:

- The extent of the main (primary) tumour (T category). There are two types of T categories for PCa:
 - the clinical T category (written as cT) is the urologist's best estimate of the extent of disease, based on the results of the physical exam (including a DRE) and prostate biopsy, and any imaging tests;
 - the pathologic T category (written as pT) is based on the pathological evaluation and is likely to be more accurate than the clinical T.
- Whether cancer has spread to nearby lymph nodes (N category);
- Whether cancer has spread (metastasised) to other parts of the body (M category);
- The PSA level at the time of diagnosis;
- The Gleason score.

Numbers or letters after T, N, and M provide more details about each of these factors. Higher numbers mean the cancer is more advanced. Once the T, N, and M categories have been determined, this information is combined (along with the GS and PSA level if they are available) in a process called stage grouping to get the overall stage of cancer and is illustrated in Figure 36.

The main stages of PCa range from I (1) through IV (4). Some stages are split further (A, B, etc.). As a rule, the lower the number, the less cancer has spread. A higher number, such as stage IV, means cancer has spread more. And within a stage, an earlier letter means a lower stage. Although each men's cancer experience is unique, cancers with

similar stages tend to have a similar outlook and are often treated in much the same way [419].



Figure A. T4 tumor invading adjacent structures other than seminal vesicles, such as bladder, rectum, levator muscles, and/or pelvic wall.

ANATOMIC STAGE/PROGNOSTIC GROUPS ¹					
Group	T	N	M	PSA	Gleason
I	T1a–c	N0	M0	PSA <10	Gleason ≤6
	T2a	N0	M0	PSA <10	Gleason ≤6
	T1–2a	N0	M0	PSA X	Gleason X
IIA	T1a–c	N0	M0	PSA <20	Gleason 7
	T1a–c	N0	M0	PSA ≥10<20	Gleason ≤6
	T2a	N0	M0	PSA ≥10<20	Gleason ≤6
	T2a	N0	M0	PSA <20	Gleason 7
	T2b	N0	M0	PSA <20	Gleason ≤7
	T2b	N0	M0	PSA X	Gleason X
IIB	T2c	N0	M0	Any PSA	Any Gleason
	T1–2	N0	M0	PSA ≥20	Any Gleason
	T1–2	N0	M0	Any PSA	Gleason ≥8
III	T3a–b	N0	M0	Any PSA	Any Gleason
IV	T4	N0	M0	Any PSA	Any Gleason
	Any T	N1	M0	Any PSA	Any Gleason
	Any T	Any N	M1	Any PSA	Any Gleason



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Definitions

Primary Tumor (T)

CLINICAL

- TX** Primary tumor cannot be assessed
- T0** No evidence of primary tumor
- T1** Clinically inapparent tumor neither palpable nor visible by imaging
- T1a** Tumor incidental histologic finding in 5% or less of tissue resected
- T1b** Tumor incidental histologic finding in more than 5% of tissue resected
- T1c** Tumor identified by needle biopsy (for example, because of elevated PSA)
- T2** Tumor confined within prostate²

- T2a** Tumor involves one-half of one lobe or less
- T2b** Tumor involves more than one-half of one lobe but not both lobes
- T2c** Tumor involves both lobes
- T3** Tumor extends through the prostate capsule²
- T3a** Extracapsular extension (unilateral or bilateral)
- T3b** Tumor invades seminal vesicle(s)
- T4** Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall (Figure A)

Pathologic (pT)³

- pT2** Organ confined
- pT2a** Unilateral, one-half of one side or less
- pT2b** Unilateral, involving more than one-half of side but not both sides
- pT2c** Bilateral disease
- pT3** Extraprostatic extension
- pT3a** Extraprostatic extension or microscopic invasion of bladder neck⁴
- pT3b** Seminal vesicle invasion
- pT4** Invasion of rectum, levator muscles, and/or pelvic wall

Regional Lymph Nodes (N)

CLINICAL

- NX** Regional lymph nodes were not assessed
- N0** No regional lymph node metastasis
- N1** Metastasis in regional lymph node(s)

PATHOLOGIC

- pNX** Regional nodes not sampled
- pN0** No positive regional nodes
- pN1** Metastases in regional node(s)

Distant Metastasis (M)⁵

- M0** No distant metastasis
- M1** Distant metastasis
- M1a** Nonregional lymph node(s)
- M1b** Bone(s)
- M1c** Other site(s) with or without bone disease

Notes

- ¹ Tumor found in one or both lobes by needle biopsy, but not palpable or reliably visible by imaging, is classified as T1c.
- ² Invasion into the prostatic apex or into (but not beyond) the prostatic capsule is classified not as T3 but as T2.
- ³ There is no pathologic T1 classification.
- ⁴ Positive surgical margin should be indicated by an R1 descriptor (residual microscopic disease).
- ⁵ When more than one site of metastasis is present, the most advanced category is used. pM1c is most advanced.
- ⁶ When either PSA or Gleason is not available, grouping should be determined by T stage and/or either PSA or Gleason as available.

Figure 36. The American Joint Committee on Cancer TNM system for PCa staging.

Reproduced, with permission, from the American Joint Committee on Cancer (AJCC) TNM system, 7e [419].

Abbreviations: M, Distant metastasis; N, Regional lymph nodes; PSA, Prostate-specific antigen; T, Primary tumour; TNM, Tumour-node-metastasis system

5.4. PCa: Risk stratification

Risk stratification depends on an accurate prostate biopsy. Multiple risk stratification systems are widely used to improve prediction of PCa aggressiveness, such as the D'Amico classification system, the Cancer of the Prostate Risk Assessment score, the National Comprehensive Cancer Network guidelines, the Epstein criteria with subsequent modifications, and the pathological grading system of the International Society of Urological Pathology (ISUP) in 2005 and with subsequent modification [409, 410, 417] as shown in Table 20. All those systems are accepted by the 2016 World Health Organization (WHO) classification of tumours, which serves as the international standard for pathologists [410]. All those systems relies on best currently available clinical and pathological parameters (such as GS, serum PSA levels, clinical and pathological staging to stratify patients into risk groups indicating their risk of disease recurrence after curative treatment [409], three of current risk stratification guidelines are summarized in Table 20 [409, 410]. Although they highlighted the GS as the single most powerful variable in risk assessment [417], these tools still do not adequately predict the outcome. Even though systematic prostate biopsy (TRUS guided multicore biopsy technique) remains the standard of care, this approach misses 21% to 28% of PCa and undergrades 14% to 17% [410]. This D'Amico risk group classification is commonly used to guide clinical management. For instance, according to D'Amico risk group classification of low-risk, intermediate-risk, and high-risk disease, early detection (low-risk) are crucial for the successful management of PCA, with 10-year reoccurrence rate of 17% whereas the 10-year reoccurrence rate with high risk is 71%. Although PCa progresses very slowly, patients usually miss appropriate treatment due to the absence of apparent symptoms at an early stage. Moreover, when detected early, PCa survival rates are better than 98% while diagnosed late, and those survival rates drop below 26% [416].

Subsequent modifications were recommended by Epstein *et al.* in 2016 (Epstein *et al.*, 2005) [409] and accepted by the WHO and the ISUP in 2005 [409], which were universally accepted in the urological field and accepted by the WHO [409]. Further risk stratification using molecular features could potentially help distinguish indolent from aggressive PCa.

Table 20. Risk stratification schema for PCa [409].

Category	D'Amico	NCCN	ISUP
Very low risk	NA	• Stage T1c, and • Gleason ≤ 6, and • PSA < 10 ng/ml, and • PSA density < 0.15 ng/ml/ml, and • $\leq$ Two cores positive, and • $\leq 50\%$ of cancer in each core	Grade 1 cancer: Gleason score of 3 + 3 Only individual, discrete, well-formed glands
Low risk	• Gleason ≤ 6, and • PSA < 10 ng/ml, and • Stage T2a or lower	• Stage T2a or lower, and • Gleason ≤ 6, and • PSA < 10 ng/ml	Grade 2 cancer: Gleason score of 3 + 4 Predominantly well-formed glands with a lesser component of poorly formed, fused, or cribriform glands
Intermediate risk	• Gleason 7, or • PSA 10–20 ng/ml, or • Stage T2b	Favourable intermediate risk • Stage T2b, or • Gleason 3 + 4, or • PSA 10–20 ng/ml	Grade 3 cancer: Gleason score of 4 + 3 Predominantly poorly formed, fused, or cribriform glands with lesser component of well-formed glands
		Unfavourable intermediate risk • Primary Gleason grade 4, or • $\geq 50\%$ cores positive, or	Grade 4 cancer: Gleason scores of 4 + 4, 3 + 5, and 5 + 3 Only poorly formed, fused, or cribriform glands or well-formed glands plus area lacking glands

		• Multiple intermediate- risk factors	
High risk	• Gleason 8–10, or • PSA >20 ng/ml, or • Stage T2c or higher	• Stage T2c, or • Gleason 8–10, or • PSA >20 ng/ml	Grade 5 cancer: Gleason scores of 4 + 5, 5 + 4, and 5 + 5 Lacks gland formation (or with necrosis) with or without poorly formed, fused, or cribriform glands
		Very high risk • Stage cT3-4, or • Node-positive disease	

Abbreviations: ISUP; International Society of Urological Pathology; NCCN; National Comprehensive Cancer Network; NA, not applicable; PSA, Prostate-specific antigen

5.5. Overview of sex steroids and PCa

The developing prostate is reliant on the androgens (T, DHT) and its reduced metabolite, DHT and the importance of these androgens, in the development and progression of PCa is extensively documented [147, 420, 421]. T is transported to prostate in the blood bound to hepatic protein, including SHBG. Free T freely diffuse through plasma membranes of prostate cells where T is converted to the more androgenic DHT by the enzyme 5 α -reductase. Binding of DHT to the AR which resides intracellularly, induces dissociation of androgen-AR complex and translocates to the nucleus, where it activates transcription of androgen-responsive genes activation (or repression) of target genes leads to biological responses including growth, proliferation and differentiation of both the prostate stroma and epithelium and the production of PSA and is illustrated in Figure 37 [420, 422-424]. SHBG has shown a pivotal effect on the development of PCa by regulating androgen [420]. The androgen dependence of the prostate is highlighted by androgen deprivation, which leads to the induction of apoptosis *in vitro* and *in vivo* [420]. Androgens are thought to play a role in prostate carcinogenesis by altering the balance between cell proliferation and apoptosis. Although androgens are essential for the normal development and growth of the prostate, prolonged administration of T has been shown to generate PCa in rodents [422], and androgens enhance the growth of several human PCa cell lines [416, 425].

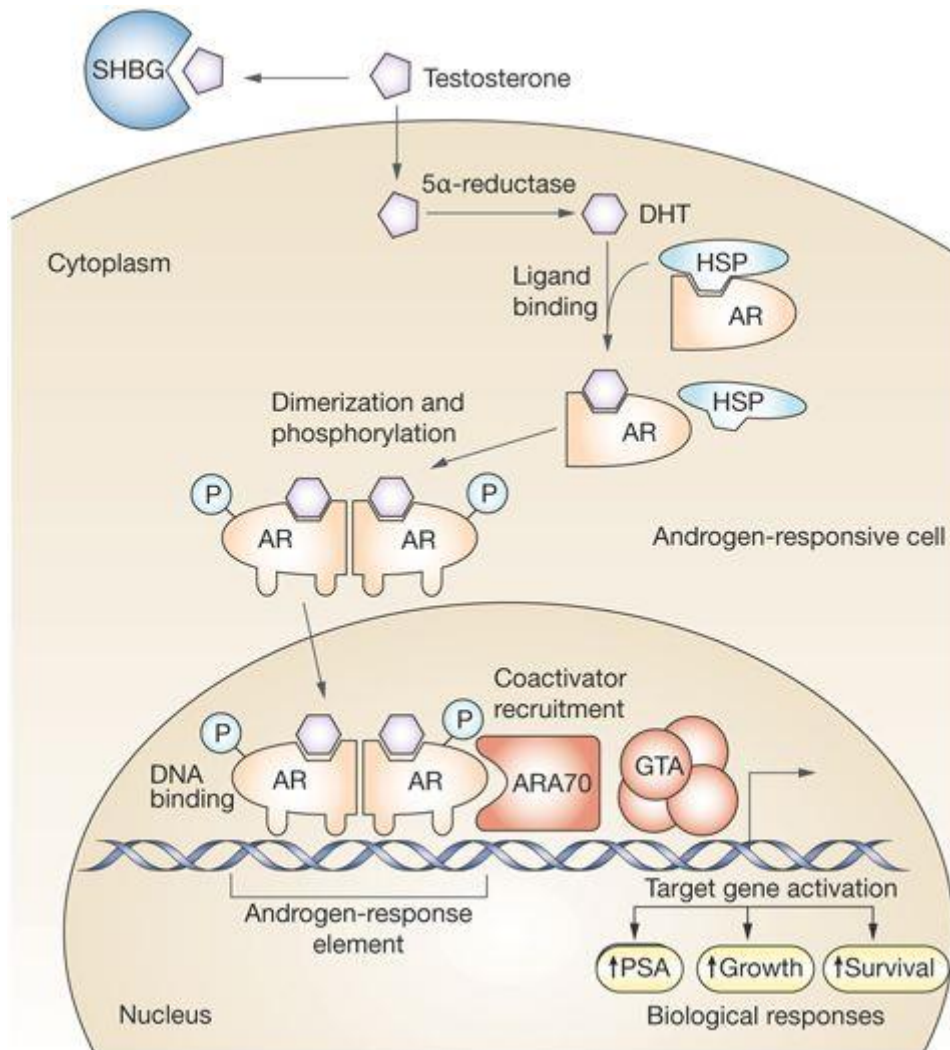


Figure 37. Transport and action of Androgen in Prostate.

Reproduced, with permission, from Harris WP et al. (2009) [420].

Abbreviations: AR, Androgen receptor; ARA70, Androgen receptor-associated protein 70; DHT, Dihydrotestosterone; GTA, General transcription apparatus; HSP, Heat-shock protein; P, Phosphorylated; PSA, Prostate-specific antigen; SHBG, Sex hormone-binding globulin.

Several findings in the literature support the idea that E2 also plays an important role in the modulation of androgens action as well as in the growth, differentiation, and homeostasis of normal prostate tissues. Prostate tissue expresses both ER α and ER β [150, 426]. E2 are also involved in prostate carcinogenesis through binding to the pre receptor (RSHBG) bound SHBG. The binding of E2 to RSHBG-SHBG complex induced PSA production, an effect which could be blocked by antiandrogens and not anti-estrogens [114, 139, 150, 426]. However, it is also suggested that the same signalling cascade in breast cancer cells has a different final effect, i.e. it doesn't exert an anti-proliferative effect [128].

5.5.1. Clinical progression of PCa and its aggressiveness

Although PCa is typically diagnosed in its early stages, and the disease exhibits a relatively indolent course, a significant percentage of men develop rapidly progressing aggressive PCa. Moreover, approximately half of the newly diagnosed PCa will have a localised disease that has a very low risk of progression [405, 427]. Definitive treatments for the clinically localised disease are surgery and radiation, but patients with the low-risk disease or a shorter life expectancy can be managed with active surveillance, which consists of PSA assays and repeats biopsies at regular intervals until there is evidence of disease progression [416]. Patients who progress may develop locally advanced or metastatic disease, which is initially treated primarily with ADT. Since the growth of PCa is driven by androgens (i.e. T, DHT), removal of endogenous androgens and blocking the AR, the cell signalling protein that facilitates androgen action, collectively termed ADT, is the mainstay treatment for aggressive (i.e. locally invasive or metastatic) PCa [428]. However, almost all advanced PCa progresses to castration-resistant disease (CRPC) after a period of ADT and resistance to ADT is inevitable [416, 429, 430]. The clinical progression of PCa is illustrated in Figure 38.

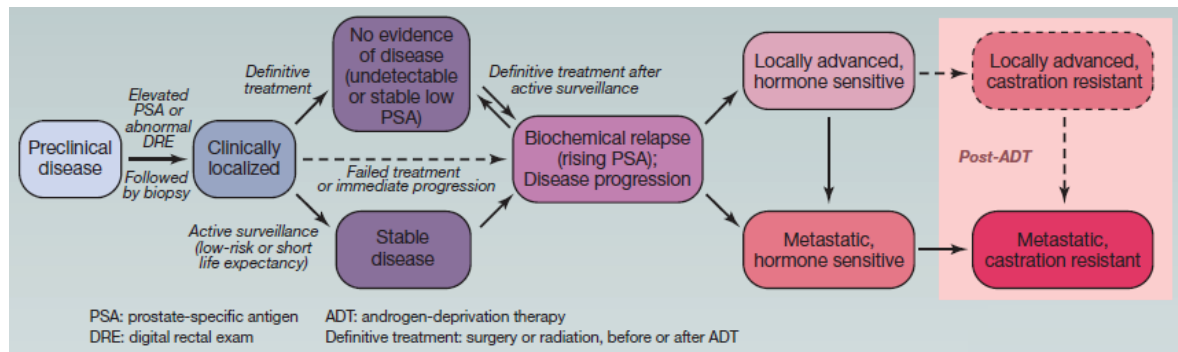


Figure 38. *Clinical Progression of Prostate Cancer.*

Reproduced, with permission, from Barlow LJ et al. (2013) [416].

Abbreviations: ADT, Androgen-deprivation therapy; DRE, Digital rectal examination; PSA, Prostate-specific antigen

Although surgical or chemical castration can initially be effective delaying disease progression, the majority of patients eventually develops CRPC and is lethal. Although only about one in seven of all newly diagnosed PCa will progress to metastatic disease over a lifetime [405] almost all advanced PCa progresses to CRPC within few months of ADT [429-431]. Once inevitable progression is established, it remains essentially incurable, and the mean survival of CRPC is 7-16 months [428, 429] and is illustrated in Figure 39.

The failure of ADT and consequent onset of CRPC is almost always associated with inappropriate maintenance of AR signalling that results in increased levels, or activity, of the AR and enhanced bioavailability of androgen, often synthesised intra-tumorally [432]. Although CRPC is no longer dependent on androgen stimulation, AR is still expressed in tumour cells, and the disease becomes highly aggressive [433]. Accordingly, there is an urgent need to (i) determine who to treat, i.e. which PCa is likely to be aggressive as opposed to which can be safely observed, and (ii) identify mechanisms by which resistance to ADT occurs and strategies to overcome these mechanisms.

Although changes in nuclear AR signalling are likely to be involved in the progression of these tumours, there may be a possible role for SHBG, a protein that transport and mediates the action of androgens and an emerging alternative role as a membrane-based steroid signalling pathway.

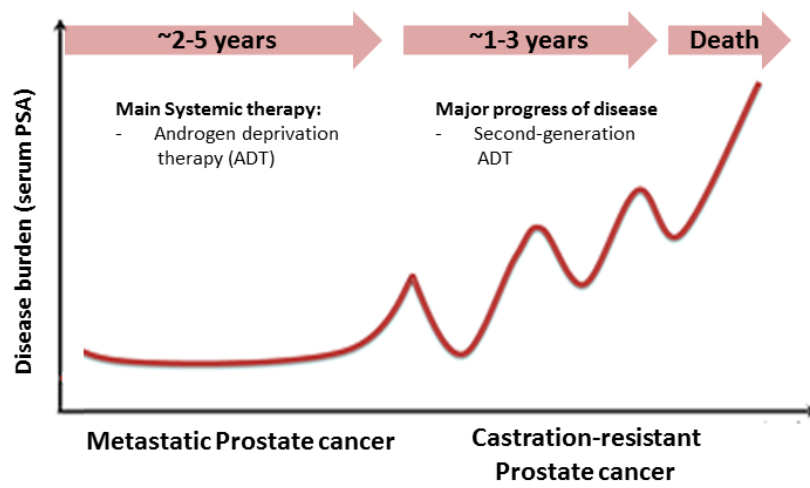


Figure 39. PCa progression.

Abbreviations: ADT, Androgen deprivation therapy

5.6. SHBG as a novel marker of PCa behaviour (aggressiveness)

SHBG is a dimeric protein that functions to transport androgens and other sex steroids in the circulation and, as such, is likely to be a critical determinant of androgen action in hormonally responsive tissues such as the prostate, breast or ovary [83, 434] as illustrated in Figure 37. Along with modulation of action of androgens and its metabolism [65, 83, 434], it is progressively evident that SHBG might also have various other physiological roles as described earlier [83]. The developing prostate is reliant on the androgens, T and its reduced metabolite, DHT that has a higher affinity for SHBG. Human SHBG binds DHT with about five times greater affinity than T and about 20 times higher affinity than E2 [57]. Also, there is evidence that SHBG accumulates in the cytoplasm of specific cell types where it may influence the intracellular actions of androgens or estrogens [65, 434].

Moreover, growing evidence from *in vitro* studies suggests that SHBG is upregulated in LNCaP prostate cancer cells upon DHT stimulation [83, 146]. Furthermore, SHBG is internalised and plays a role in regulating intracellular hormone action, indicating an important role of SHBG in maintaining cell stemness, which may have clinical consequence [65, 146, 434]. SHBG may also have T-independent actions; it has been reported to regulate the growth of PCa cells and to interact with ER α and ER β [145].

As described earlier (Chapter 2.4), SHBG also interacts directly with plasma membrane receptors on the surface of prostate epithelial cells and participates directly in cellular signalling pathways [84, 147]. SHBG is most abundant in luminal epithelial cells of the prostate, whereas stromal cells from prostate explants possess the greatest SHBG binding ability, i.e., contain a specific receptor (RSHBG) on prostate cell membranes [83, 142, 147]. SHBG binds to RSHBG, forming an SHBG-RSHBG complex that causes a rapid and sustained increase in cAMP then activation of cAMP-dependent PKA within the cancer cells [131, 136, 137, 139, 145, 151, 152]. Details regarding the

downstream effects of steroid signalling through SHBG exist, but are not extensive. To date, downstream effects reported in prostate cell lines include PSA induction, increased apoptosis, and regulation of cell growth; however, the overall biology of this pathway is not well understood [84, 142].

A landmark study by Hryb and colleagues [75], demonstrated that human prostate tissues express both *SHBG* mRNA and protein, as do PCa cell lines, suggesting that SHBG is locally produced and might have paracrine function [75]. Further, for more than a decade, it has been known that SHBG is expressed in prostate, predominates in luminal epithelial cells with weak expression in stromal cells [74, 75, 435]. SHBG has also been shown to modulate the androgen [142, 434] and oestrogen [148] action and metabolism in PCa cells. These findings are particularly interesting in light of reports that PCa cells have the capacity of *de novo* intratumoral androgen synthesis during the progression of PCa [436, 437]. Therefore, an increased SHBG expression in PCa might serve as a reservoir for locally produced androgens that can be accessed by the AR, thus cancer progresses despite the limited supply of androgens and become aggressive. This assumption is also supported by the experiments using PCa epithelium and stroma that imply a paracrine function of SHBG as an ARs coactivator [75, 152]. Based on this vital information, the presence of SHBG transcripts in human PCa cells is of interest because low levels of intracellular SHBG may amplify the biological activities of low concentrations of androgens, and this may be particularly important under conditions of androgen withdrawal.

Furthermore, a growing body of evidence shows that SHBG may directly bind to a high-affinity cell surface receptors (RSHBG) on prostate epithelial cells and activate intracellular signalling on its own [84, 128], as described thoroughly in Chapter 2.4. However, the cell surface receptors for SHBG (RSHBG) has not been isolated or fully characterised. Therefore, we do not know whether SHBG has an independent role in

regulating prostate growth or function. Specific downstream effects of the SHBG-RSHBG complex merit further investigation since they may help to clarify the underlying mechanisms linking SHBG to PCa.

5.6.1. Plasma SHBG and PCa risk

Compared with T, epidemiological data from adult men investigating the association between circulating SHBG levels and PCa risk are relatively scarce. Moreover, these data on the associations between SHBG and PCa has been somewhat inconsistent, with a number of studies finding that high circulating concentrations of SHBG are associated with an increased PCa risk [438-441], others have reported to the contrary, few studies showed modestly inversely associated [442] while others showed no association with PCa risk [443-446] and is summarized in Table 21. As described in an earlier section (Chapter 2.3), SHBG is also synthesised in PCa epithelial cells but is not secretory, if any, the proportion of prostate-produced SHBG that reaches the plasma has not been determined [84, 85, 146]. Thus, the proportion of plasma SHBG is exclusively originating from the liver. Plasma SHBG levels are not associated with PCa risk; if anything, the plasma SHBG would be associated with the worst tumour outcome. The literature is now strongly in favour of the neutral effect of plasma SHBG on PCa risk. Although plasma SHBG levels may not show a relationship with PCa, locally produced SHBG may offer a surrogate marker to investigate associations between SHBG and PCa, thus require further investigation.

Table 21. Serum SHBG and PCa risk: Population-based studies.

Author	Study characteristics	Outcome	Study population	Confounding factors	Main findings
Lee JK et al. (2015)[438]	CS	Preoperative SHBG who underwent RP	Preoperative who underwent RP and biochemical recurrence. (n=307)	Age, PSA, TT, fT, BMI, prostate volume, clinical stage, and biopsy GS	– In multivariate analysis, higher SHBG was found to be an independent predictor of with postoperative BCR free survival adverse pathologic features (OR= 2.50 95% CI 1.21, 5.19; P=0.014).
Shim M et al. (2014)[439]	Case control	Men with PCa before and after RP	Men with PCa (n=699) Aged matched control (n=700)	None considered	– Low SHBG is an independent predictor of PCa differentiation (OR= 0.53 95% CI 0.38, 0.74; P<0.001).
Garcia-Cruz E et al. (2013) [440]	CS Prostate biopsy owing to PSA elevation or abnormal DRE (n=279)	PCa	PCa cases (n=84)	Age, DRE, fT, BT & TT.	– SHBG concentrations was associated with an increased risk of PCa (OR = 3.27 95% CI 1.52, 7.04, P<0.002). – SHBG concentrations were significantly high in biopsy positive PCa compared to biopsy negative PCa (P=0.008).
De Nunzio C et al. (2013) [443]	CS	PCa Abnormal finding on DRE and/or an	(n=740) PCa cases (n=275)	None considered	– SHBG concentrations was not associated with an increased risk of PCa (OR= 1.00 95% CI 0.99, 1.09, p=0.77) or high grade disease (aggressiveness)

		elevation PSA (> 4ng/mL)			(OR=1.00 95% CI 0.99, 1.02), p=0.71).
Sawada N et al. (2010) [444]	Nested case-control study	PCa	JPHC Study PCa cases (n=201) Control (n=402) (40-59 years)	Smoking status, alcohol intake, marital status, BMI, intake of green tea, miso soup + mutual adjustment for TT and SHBG.	– SHBG was not associated with a risk of PCa OR =1.38 95% CI 0.69, 2.77, P trend = 0.23).
Salonia A et al. (2009) [447]	CS	Nodal Metastatic PCa	One hundred sixty-eight consecutive patients who had undergone radical retropubic prostatectomy with ePLND for clinically localised PCa. (48-77 years)	Age, PSA, clinical stage, and primary and secondary Gleason grade Smoking status, alcohol intake, marital status, body mass index, and intake of green tea and miso soup; mutual adjustment for TT and SHBG	– SHBG is more accurate than any established variable in predicting for LNI at ePLND. More importantly, SHBG alone was more accurate than the combination of patient age, PSA level, clinical stage, and Gleason grade in predicting for LNI (OR 1.11; p<0.001).
Lee SE et al. (2008) [448]	CS	Extra-prostatic extension of tumour Aggressive PCa	288 consecutive patients who were scheduled to undergo RRP for clinically localised prostate cancer	Age, BMI, Serum PSA level, biopsy Gleason score, and clinical stage	– SHBG was not shown to be a significant predictor for high pathological Gleason score (≥7) in multivariate analysis (HR=1.3 95% CI 0.780,2.224, p=.0.303)
Roddam AW et al. (2008) [442]	Case control	Pooled analysis of 18	(the Endogenous Hormones and PCa Collaborative Group)	Age, BMI, marital status, educational status,	– SHBG was statistically significantly and inversely related to PCa risk (RR = 0.86, 95% CI 0.75, 0.98), comparing

		prospective studies	Incident PCa (n=3550) and control subjects (n=5815)	smoking, and alcohol consumption	the highest fifth with the lowest fifth), and evidence of a statistically significant dose-response relationship was also found ($P_{\text{trend}} = 0.01$).
Weiss JM et al. (2008) [445]	Nested case-control	Incident PCa	(PLCO cancer screening trial) Incident PCa (n=727) Control (n=889) (55-74 years)	BMI, family history and TT	– There were no associations of SHBG concentrations with PCa risk (Q4 vs. Q1: [OR=0.45 95% CI 0.23, 0.89], $P_{\text{trend}} = 0.06$) and PCa aggressiveness.
Kristal AR et al. (2008)[446]	Nested case-control study	Incident BPH	PCa Prevention Trial Incident BPH (n=708) Control (n=709) (≥ 55 years)	Age at baseline, race, body mass index, smoking status, alcohol consumption, and insulin-like growth factor binding protein 3, TT & E2.	– No differences in SHBG concentrations between cases and controls. – No associations of SHBG concentrations with PCa risk.
Grasso M et al. (1990) [441]	CS	PCa and BPH cases	PCa (25), BPH (17), Normal (14) (47-85 years)	None considered	– Higher concentrations of SHBG was found among PCa patients compared to healthy control or patients with BPH ($P < 0.01$ for both). – No apparent correlations between SHBG concentrations and the clinical stages of PCa.

Abbreviations: BPH, Benign prostate hyperplasia; BCR, Biochemical recurrence; BMI, Body mass index; CS, Cross-sectional; DRE, Digital rectal examination; ePLND, extended pelvic lymph node dissection; fT, Free testosterone; GS, Gleason score; HR, Hazard ratio; LNI, Lymph node invasion; OR, Odds ratio; PCa, Prostate cancer; PSA, Prostate-specific antigen; TT, Total testosterone

5.6.2. SHBG in human PCa tissue

SHBG is overexpressed at both protein and *mRNA* levels in many tumours, including breast carcinoma [67, 74, 142], ovarian carcinoma [449], and colon cancer [450]. Also, SHBG overexpression has been previously established in PCa explants and cell lines [22, 75, 83, 146].

Both *SHBG* mRNA and protein is overexpressed in human PCa tissue [22, 75, 86], PCa explants [74, 83] as well as in established PCa cell lines (human LNCaP, DU 145 and PC 3) [22, 75, 146, 434], suggesting that SHBG is locally synthesized. *SHBG* mRNA and protein are expressed in human PCa to a significantly greater extent than in benign prostate tissue [75, 83, 146], and the level of expression is positively associated with higher stage and grade of PCa, seminal vesicle invasion and lymph node metastasis [146]. Moreover, SHBG appears to have a role in inducing and maintaining stemness of LNCaP cells and PC3 cells upon DHT exposure *in vitro* [142, 146, 434].

Although established PCa cell lines have provided important molecular insights into PCa biology, the majority of research has been undertaken in only three cell lines, LNCaP, PC3, and DU145, which were all derived from metastases with loss or mutation of the AR and are, therefore, not representative of primary disease [451]. PC3 cells are typically AR-negative, which may suggest a T/DHT/SHBG mechanism independent of AR and highlights the potential importance of SHBG in mediating resistance to androgen deprivation. LNCaP cells are arguably more representative of true PCa than the other lines (e.g., they express the androgen receptor, PSA, prostate-specific membrane antigen, and PAP). However, these cell lines were all derived from metastases with loss or mutation of the AR and are, therefore, not representative of primary disease [451] that highlight the requisite in primary prostate tissues. Despite these lines of evidence linking prostate specific SHBG expression to PCa cancer progression, many facets of this relationship remain largely uncharacterized. For example, the SHBG gene is composed

of 13 different exons that generate at least six transcription units (TU's), only one of which (TU-1), expressed in hepatocytes, generates a secretory protein. Alternative SHBG transcripts in human prostate [86], are differentially downregulate SHBG translation [83, 85] but whether any of the SHBG TU's is specifically associated with PCa is unknown. Nakhla et al. reported that only the eight exons long SHBG transcript is generated in normal prostate tissue. However, normal prostate revealed a low abundance of transcripts derived from the two upstream promoters [22]. In striking comparison, the LNCaP cell lines exhibited a dramatic relative increase in both the number of alternatively spliced transcripts and transcripts from upstream promoters [22, 86]. This raises the possibility that the alternative SHBG transcripts identified in human PCa cells encode SHBG isoforms that function within the cells rather than having to be secreted to act, but this will only be resolved when these intracellular SHBG isoforms are isolated and characterised from human tissues. Taken together, the SHBG transcripts may be a valuable provider of diagnostic, prognostic, and predictive biomarkers for individuals with PCa.

Similarly, with one exception [75] attempts to correlate *SHBG* mRNA expression levels and protein corresponding to the same specimen, are not extensively studied. Details regarding the overexpression of SHBG in PCa cell lines exist, but whether the same overexpression of SHBG exist in human PCa biopsy sample compared to normal prostate or benign prostate epithelial cells is not known. Moreover, the role of SHBG in regulating androgen signalling remains largely uncharacterized.

While an improved understanding of the molecular basis of PCa tumorigenesis has generated increased prognostic and predictive measures, the early identification of aggressive primary PCa is still not resolved. Taken together, whether SHBG was locally expressed or was delivered to these cells through the plasma, whether SHBG has a distinct role in the pathophysiology of PCa and whether the increased expression of

SHBG is a marker of aggressive PCa and is involved in the pathogenesis of resistance to androgen deprivation, remained a question.

De novo lipogenesis in PCa

Dysregulated lipid metabolism, specifically increased lipid synthesis (lipogenesis) has been increasingly recognised as a hallmark for a wide variety of cancers including PCa [452, 453] where it is strongly androgen-regulated. Living cells acquire fatty acids for their metabolic demand from two major sources, exogenous dietary sources and by *de novo* endogenous synthesis. Although normal cells predominantly take up circulating fatty acids for lipogenesis, the primary source of fatty acids for lipid synthesis in cancer cells is *de novo* lipogenesis even in the presence of abundant exogenous fatty acids [452-454]. Continuous *de novo* lipogenesis provides cancer cells with membrane building blocks, signalling lipid molecules and key energy supply to support rapid cell proliferation [455, 456]. The expression and activity of many enzymes involved in fatty acid synthesis, i.e., ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN), are upregulated in many types of cancers [452, 457], and, of particular relevance to the current application, their expression is co-ordinately regulated by androgens. In PCa, increased expression and activity of key lipogenic enzymes [452, 453] and a higher rate of *de novo* fatty acid (FA) and cholesterol synthesis, are associated with tumour progression and a poorer prognosis [452]. Elevated expression of FASN has been found in the earliest stages of cancer development and becomes more pronounced during tumour progression [458, 459]. Fatty acid-binding protein 5 (FABP-5), a cancer promoter and metastasis inducer, is overexpressed in the majority of prostatic carcinomas. Investigation of molecular mechanisms involved in the tumour-promoting activity of FABP-5 has established that there is a fatty acid-initiated signalling pathway leading to malignant progression of PCa cells. The expression levels of FABP-5 in PCa cell lines and the cytoplasm and nuclei of carcinoma tissues is significantly higher

compared to benign cell lines and BPH tissues, and significantly correlated with the advanced grade and stage of carcinomas [452, 460].

SHBG and lipid metabolism in the prostate

SHBG expression by the liver is influenced by its metabolic status. Activation of *de novo* lipogenesis decreases hepatic SHBG production, whereas activation of β -oxidation will increase SHBG production [29, 57]. Thus, SHBG is a sensitive marker of *de novo* lipogenesis in the liver, where it is regulated by several transcription factors that are also involved in the regulation of *de novo* lipogenesis. HNF4 α is a transcription factor that constitutively binds fatty acids [452], and is a positive regulator of SHBG and inversely related to hepatic *de novo* lipogenesis[94, 452]. Normally HNF4 α mediates androgen-dependent transcription in the kidney and liver [457]. HNF4 α mutations [461] or increased expression in tissue sections has been reported from some PCa [462] raising the possibility that aberrant expression may contribute to androgen-independent AR activity. The transcription factor PPAR γ induces increased lipogenesis in the liver[463], is associated with FASN expression [452], and is a repressor of SHBG expression [33]. PPAR γ expression is increased in PCa tissue and is associated with disease aggressiveness [400, 463] and FASN expression [452, 459]. Ligand activation of PPAR γ inhibits growth and invasion of PCa [464, 465]. Taken together, these data suggest that there is an uncoupling of the regulation of SHBG from lipogenesis in PCa and alternate transcriptional regulation of SHBG in aggressive PCa. Alternatively, does SHBG expression increased because lipid turnover is increased in PCa, the regulatory mechanism seen for hepatic SHBG synthesis, needs to be established.

6. Regulation of plasma SHBG in men

Statement of Authorship

Title of Paper: Cross-sectional and longitudinal determinants of serum sex hormone

- binding globulin (SHBG) in a cohort of community-dwelling men

Statement of Authorship

Title of Paper	Cross-sectional and Longitudinal Determinants of Serum Sex Hormone Binding Globulin (SHBG) in a Cohort of Community-Dwelling Men
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6.1. Abstract

Despite its widespread clinical use, there is little data available from population-based studies on the determinants of serum sex hormone-binding globulin (SHBG). We aimed to examine multifactorial determinants of circulating SHBG levels in community-dwelling men. Study participants comprised randomly selected 35-80 y.o. Men (n=2563) prospectively-followed for five years (n=2038) in the Men Androgen Inflammation Lifestyle Environment and Stress (MAILES) study. After excluding men with illness or medications known to affect SHBG (n=172), data from 1786 men were available at baseline, and 1476 at follow-up. The relationship between baseline body composition (DXA), serum glucose, insulin, triglycerides, thyroxine (fT4), sex steroids (total testosterone (TT), oestradiol (E2)), and pro-inflammatory cytokines and serum SHBG level at both baseline & follow-up was determined by linear and penalized logistic regression models adjusting for age, lifestyle & demographics, body composition, metabolic, and hormonal factors. Restricted cubic spline analyses were also conducted to capture possible non-linear relationships. At baseline there were positive cross-sectional associations between age ($\beta=0.409$, $p<0.001$), TT ($\beta=0.560$, $p<0.001$), fT4 ($\beta=0.067$, $p=0.019$) and SHBG, and negative associations between triglycerides ($\beta=-0.112$, $p<0.001$), abdominal fat mass ($\beta=-0.068$, $p=0.032$) and E2 ($\beta=-0.058$, $p=0.050$) and SHBG. In the longitudinal analysis, the positive determinants of SHBG at 4.9 years were age ($\beta=0.406$, $p<0.001$), TT ($\beta=0.461$, $p<0.001$), and fT4 ($\beta=0.040$, $p=0.034$) and negative determinants were triglycerides ($\beta=-0.065$, $p=0.027$) and abdominal fat mass ($\beta=-0.078$, $p=0.032$). Taken together, these data suggest low SHBG is a marker of abdominal obesity and increased serum triglycerides, conditions which are known to have been associated with low testosterone and low T4.

6.2. Introduction

Sex hormone binding globulin (SHBG) is a circulating homodimeric glycoprotein, primarily synthesised in the liver that binds circulating sex steroids with high affinity [26, 97]. Variations in circulating SHBG levels are observed in several physiologic and pathological conditions. In men, low serum SHBG levels are associated with insulin resistance [138], obesity [194], non-alcoholic fatty liver disease (NAFLD) [237], type 2 diabetes (T2D) [29, 466], and cardiovascular disease [29]. Higher circulating SHBG is protective against the development of T2D in humans [173]. Overexpression of SHBG protects against T2D development in transgenic mice [87].

The synthesis and secretion of SHBG are subject to regulation by hormonal, metabolic, and nutritional factors [29, 57, 100, 247, 248]. Factors shown to be inversely associated with SHBG levels include growth hormone [208], oestrogen (oestradiol) [110, 217], insulin [217, 244], body composition [169, 175, 217], intrahepatic fat [173, 175, 205], triglycerides [188, 189], monosaccharides [94], C-reactive protein (CRP) [255], and moderate alcohol consumption [467]. SHBG has been positively associated with testosterone [217, 248], follicle stimulating hormone [248], serum thyroxine [217], adiponectin [93], olive oil [95], red wine (resveratrol) [37], increasing age [215], physical activity [467] and resistance training [205, 212]. However, these data are derived largely from cross-sectional studies with a variety of limitations including small sample size, non-representative samples, restricted age ranges, limited biochemistry, non-concurrent variables [175, 189, 217, 247, 468] generally leading to inconclusive and inconsistent findings. Recent longitudinal data from the Boston Area Community Health/Bone Survey examining changes in anthropometric measures and sex steroids demonstrated that SHBG at baseline was not associated with changes in any of the included measures of body composition [171]. Another recent study (n=1316) [469] undertook a secondary analysis of serum SHBG determinants. Their data suggested a possible effect of BMI,

glucose, and lipids on SHBG levels. However, the follow-up period was relatively short, men were all older, and adjustment for confounders was limited [469].

Accordingly, in a longitudinal cohort study, we examined cross-sectional and longitudinal relationships between lifestyle & demographic factors, body composition, metabolic, hormonal factors, and serum SHBG, simultaneously, in a large, community-dwelling, representative cohort, of middle-aged to elderly Australian men.

6.3. Subjects and methods

6.3.1. Study Design and Participants

Participants for the present study were from the Men Androgen Inflammation Lifestyle Environment and Stress (MAILES) study. The MAILES study is a cohort of men aged 35 years and older, pooled from two existing population-based studies using identical sampling methods in the Northern and Western suburbs of Adelaide, South Australia: The Florey Adelaide Male Ageing Study (FAMAS) and North West Adelaide Health Study (NWAHS). A more detailed description of the study design, procedures, and recruitment were published previously [470]. Briefly, the FAMAS includes 1195 randomly-selected men, aged 35–80 years at recruitment who attended baseline clinic visits in 2002–2005 and follow-up clinic visits in 2007–2010. The NWAHS includes men and women aged 18-years at recruitment in 1999–2000, who attended three clinic waves (n=2336). For the MAILES study, all FAMAS men and NWAHS age-matched (35–80 years at stage 2) men were included, yielding a final sample size of 2563 men at MAILES stage 1 (i.e., FAMAS baseline and NWAHS stage 2) and 2038 men at MAILES stage 2 (FAMAS 5-year follow-up and NWAHS stage 3). The mean follow-up period for the MAILES study is 4.9 years. The study was approved by the research ethics committees of the Royal Adelaide Hospital and the North Western Adelaide Health Service. A written informed consent form was given to the eligible participants who signed in-clinic.

For the current analysis, we excluded men with haemolysed samples (n=14), confounding health conditions ((thyrotoxicosis, hypothyroidism, epilepsy, alcoholism, hepatitis c, heart bypass surgery, orchiectomy, primary testicular disease, & prostate cancer) (n=56)) or on medications known to affect the hypothalamic pituitary gonadal (HPG) axis and hepatic SHBG synthesis (testosterone, antiandrogens, glucocorticoids, anti-epileptics, aromatase inhibitors, and thyroid hormone and anti-thyroid agents; n=102), leaving a total sample of 1786 at baseline and 1476 at follow-up for analysis (Figure 40).

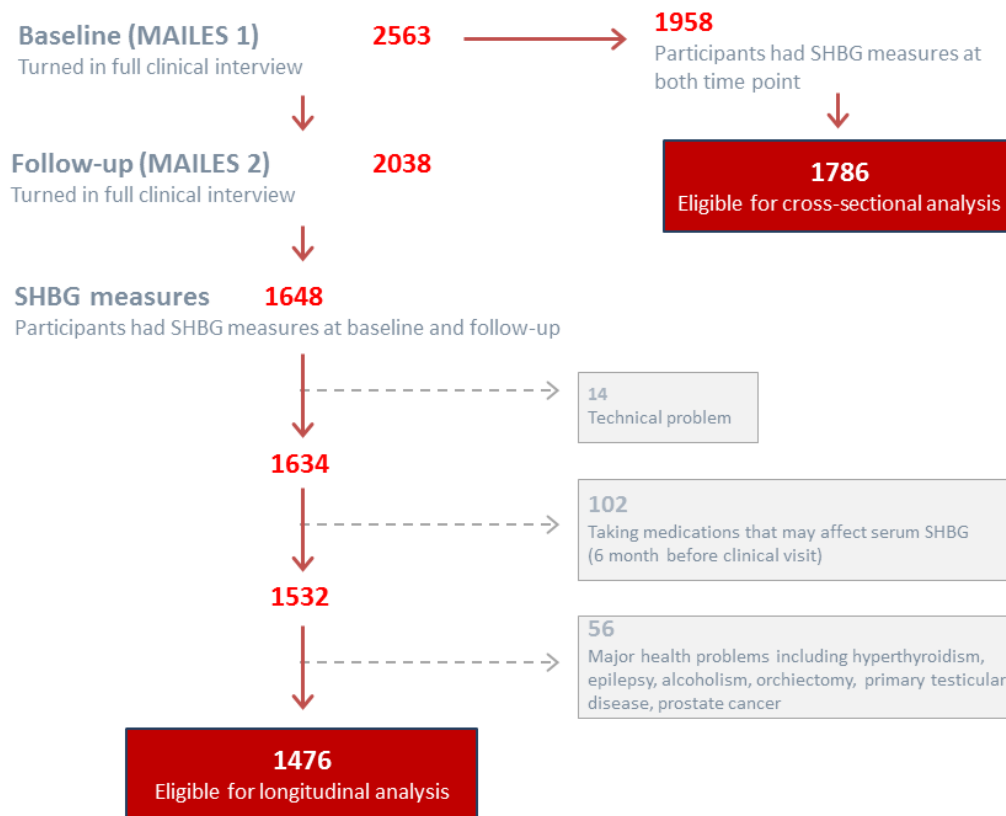


Figure 40. Description of MAILES sample enrolment.

Abbreviations: MAILES, Men androgen inflammation lifestyle environment and stress; SHBG, Sex hormone-binding globulin.

6.3.2. Measurements

Socio-demographic and behavioural characteristics

Information on sociodemographic and behavioural characteristics (age, gender, physical activity, smoking status, and alcohol consumption), as well as medical history, including information about medical procedures, and medication use was obtained by a validated, self-report questionnaire [470]. Body composition was assessed using Dual-energy X-ray Absorptiometry (DXA) using the Lunar DPX+ and Prodigy pencil beam densitometers (Lunar Radiation Corporation, Madison, USA). Both densitometers show excellent congruence, with <1% difference in measurement across a range of measured areas [471].

Biochemical and hormonal assays

Full details of the laboratory methods and quality control data have been reported previously [470]. Briefly, blood samples were drawn between 8:00 AM and 11:00 AM after a 12-hour overnight fast. Samples were immediately placed on ice and transported to a laboratory certified by the National Association of Testing Authorities (NATA) within 4-hours, then centrifuged, fractionated, and serum stored at -80°C until measurement (between 1 - 13 months after collection). Samples were randomly ordered for assay, and the laboratory technicians were blinded to participant characteristics. Serum SHBG levels were measured separately at baseline and follow-up by diluting serum to 1:21 through SHBG sample diluent, and then assayed using the Immulite Autoanalyser and a solid-phase, two-site chemiluminescent immunoassay (Siemens Medical Solutions, New York, USA; inter-assay CV: 4.0% at 32.3 nmol/L; lower detection limit: 0.17 nmol/L). Other plasma/serum measures included metabolic markers (glucose, triglycerides, insulin and alanine transaminase (ALT) activity), hormones (free thyroxine (fT4), total testosterone (TT) and oestradiol (E2)) and inflammatory markers (interleukin-6 (IL-6); tumour necrosis factor-alpha (TNF- α)); a marker of macrophage activation (myeloperoxidase activity (MPO)); and a marker of vascular endothelial inflammation (sE-

selectin (eSel)). The inter-assay coefficient of variation (CV) were <10.6 % for all measurements. Concentrations of all selected analytes are stable after multiple freeze-thaw cycles [166].

6.3.3. Statistical analysis

Descriptive analyses of selected independents and outcome measures were conducted using chi-square- (categorical) and t-tests (continuous). To estimate cross-sectional and longitudinal determinants of SHBG variability, we implemented unadjusted, then age-adjusted and multi-adjusted linear regression and penalised logistic regression (using the least absolute shrinkage and selection operator (LASSO)) models. The cross-sectional models utilised serum SHBG levels and selected independents variables from baseline clinic visits. The longitudinal models fitted serum SHBG at follow-up (median 4.94 years follow-up, interquartile range 4.34-5.00) against selected determinants from baseline clinic visits. In separate models, we also assessed the effect of absolute changes between visits for SHBG determinants against the change in serum SHBG between visits. Normality and linearity assumptions were examined for all independents. Non-normal independents were log-transformed for analysis with results back-transformed for data presentation. Interaction effects were also examined for selected independents with resultant terms included in multi-adjusted models, where appropriate. All analyses were performed with the IBM SPSS statistical package (version 23.0 Armonk, NY, USA). A p-value < 0.05 was considered statistically significant. Explained variance (R^2) of the models are also presented.

To access possible non-linear relationships, restricted cubic splines were performed using STATA (version 15.0, STATA Corporation, Texas, USA) with 95% Confidence Intervals. Analyses were multi-adjusted with 3 knots at the 10th, 50th, and 90th percentiles of the distribution. To further explore these non-linear associations, tests for nonlinearity comparing a model with only the linear term to a model with the linear and

restricted cubic spline terms were conducted using likelihood ratio tests (alternate middle knot locations). If a test for nonlinearity was not significant, a test for linearity was conducted comparing a model with the linear term to a model with only the covariates of interest (Appendix 1).

6.5. Results

There were 1786 men at baseline, with a mean age of 55 years. At follow-up, complete data were available for 1476 men with a mean age 59 years. The characteristics of the MAILES stage 1 (baseline) and MAILES stage 2 (follow-up over 4.9 years) study populations are summarised in Table 22. No significant difference exists between baseline characteristics of those subjects who missed the follow-ups (n=310) and those who did not (data have not been shown).

Table 22. Characteristics of the study participant at baseline and 5- year follow-up.

Parameters	Baseline (n=1786)	5-Year follow-up (n=1476)	P-value
Age, Years	55.0±12.2	59.0±11.5	<0.001
BMI (Kg/m ²)	28.5±4.5	28.8±4.5	<0.001
Abdominal total fat mass (%)	34.8±8.2	36.3±8.0	<0.001
Triglycerides (mmol/L)*	1.79±1.42	1.73±1.33	0.157
Glucose (mmol/L)	5.20±1.41	5.49±1.42	<0.001
Insulin (µIU/mL) ^a	10.9±9.3	9.43±8.81	<0.001
ALT activity (U/L) ^b	33.8±19.1	32.0±18.4	0.005
fT4 (pmol/L)	14.4±2.3	16.2±2.81	<0.001
TT (nmol/L)	17.0±5.8	16.4±5.7	<0.001
E2 (pmol/L) ^c	96.3±42.8	94.6±35.2	<0.001
SHBG (nmol/L)	33.0±13.5	33.5±13.6	<0.001
IL-6 (pg/mL)	2.03±1.76	2.07±1.98	0.331
TNF-α (pg/mL)	1.94±2.88	2.07±2.67	0.064
MPO activity (µg/L)	208.4±295.6	181.7±199.8	0.002
eSel (ng/mL)	36.8±16.9	36.4±17.2	0.461

Values are mean ± standard deviation unless stated otherwise. Statistically significant associations (P <0.05) are shown in bold.

Abbreviations: BMI, body mass index; ALT, alanine transaminases; f T4, free thyroxine; TT, total testosterone; E2, oestradiol; SHBG, sex hormone binding globulin; IL-6, interleukin 6; TNF-α, tumour necrosis factor alpha; MPO, myeloperoxidase; eSel, sE-selectin.

* geometric mean was 1.467 & median was 1.433 at baseline and mean 1.493 & median was 1.455 at five-year follow-up respectively.

^a n = 1099, ^b n=1096, ^c n=1136

The cross-sectional analysis at MAILES stage 1 is shown in Table 23. In unadjusted analysis, SHBG was positively associated with age, fT4, TT and E2, and inversely associated with alcohol consumption, smoking, abdominal total fat mass, triglycerides, glucose, insulin, ALT activity, and eSel levels. After adjustment for age, SHBG remained positively associated with fT4, TT and E2 and inversely associated with smoking, abdominal total fat mass, triglycerides, glucose, insulin, ALT activity, IL-6, MPO activity and cell levels. After multi-adjustment SHBG was positively associated with age, fT4 and TT and inversely associated with abdominal fat mass, triglycerides and E2. LASSO procedure selected TT, age, and fT4, respectively for having the highest positive regression coefficient while triglycerides and abdominal fat mass, respectively for having the highest negative regression coefficient.

Table 23. Unadjusted, age-adjusted and multi-adjusted generalized linear regression and lasso regression model to estimate cross-sectional determinants of SHBG in community-dwelling men (n=1786).

Determinants/factors	Unadjusted model			Age-adjusted			Multi-adjusted (Full model)		LASSO regression	
	Standardized β	P-value	R ²	Standardized β	P-value	R ²	Standardized β	P-value	Standardized β	P-value
Demographic, behavioural & anthropometric factors										
Age, Years	0.355	<0.001	0.126	-	-	-	0.409	<0.001	0.418	<0.001
Physical activity	0.022	0.375	0.000	0.008	0.724	0.132	-0.012	0.667		
Alcohol consumption	-0.088	<0.001	0.008	-0.044	0.055	0.127	-0.041	0.145		
Smoking status	-0.078	0.001	0.006	-0.149	<0.001	0.148	0.005	0.873		
Abdominal total fat mass (%)	-0.233	<0.001	0.054	-0.302	<0.001	0.202	-0.068	0.032	-0.081	0.006
Blood chemistry & hormones										
Triglycerides (mmol/L)	-0.251	<0.001	0.063	-0.227	<0.001	0.177	-0.112	<0.001	-0.121	<0.001
Glucose (mmol/L)	-0.061	0.010	0.004	-0.126	<0.001	0.141	0.033	0.269		
Insulin (μIU/mL) ^a	-0.171	<0.001	0.029	-0.201	<0.001	0.182	0.002	0.945		
ALT activity (U/L) ^b	-0.187	<0.001	0.035	-0.108	<0.001	0.152	-0.029	0.350		
fT4 (pmol/L)	0.105	<0.001	0.011	0.120	<0.001	0.139	0.067	0.019	0.086	0.002
TT (nmol/L)	0.535	<0.001	0.286	0.581	<0.001	0.461	0.560	<0.001	0.539	<0.001
E2 (pmol/L) ^c	0.110	<0.001	0.012	0.084	0.002	0.141	-0.058	0.050		
Inflammatory markers										
IL-6 (pg/mL)	-0.017	0.514	0.000	-0.065	0.008	0.123	-0.007	0.795		
TNF-α (pg/mL)	-0.003	0.896	0.000	-0.023	0.356	0.117	-0.019	0.504		
MPO activity (μg/L)	-0.042	0.111	0.002	-0.024	0.001	0.117	0.011	0.705		
eSel (ng/mL)	-0.178	<0.001	0.032	-0.160	<0.001	0.143	-0.014	0.651		

Statistically significant associations (P <0.05) are shown in bold. Multi-adjusted generalised linear model R² was 0.537 and lasso regression model R² was 0.530.

Abbreviations: ALT,alanine transaminases; fT4,free thyroxine; TT,total testosterone; E2,oestradiol; SHBG, sex hormone binding globulin; IL-6,interleukin 6; TNF- α , tumour necrosis factor alpha; MPO, myeloperoxidase; eSel, sE-selectin.

^a n = 1097, ^b n = 1095,^c n= 1134

The regression estimates of longitudinal analysis are shown in Table 24. Unadjusted analysis of the longitudinal data showed significant a positive association between absolute SHBG at 4.9 years and age, physical activity, fT4, TT, E2, IL-6, and TNF- α at baseline and inverse association with alcohol consumption, abdominal total fat mass, triglycerides, glucose, insulin, ALT activity, MPO activity, and eSel levels at baseline with absolute SHBG levels at 4.9 years. After adjustment for age, SHBG remained positively associated with fT4, TT and TNF- α and inversely with alcohol consumption, abdominal total fat mass, triglycerides, glucose, insulin, ALT activity, MPO activity and eSel levels. After multi-adjustment SHBG was positively associated with age, fT4 and TT and inversely associated with abdominal total fat mass and triglycerides. LASSO procedure selected TT, age, and fT4, respectively for having the highest positive regression coefficient while triglycerides and abdominal fat mass for having the highest negative regression coefficient.

Table 24. Unadjusted, age-adjusted and multi-adjusted generalised linear regression and lasso regression model to estimate longitudinal determinants of SHBG in community-dwelling men (n=1476).

Determinants/factors	Unadjusted model			Age-adjusted			Multi-adjusted (Full model)			LASSO regression	
	Standardized β	P-value	R ²	Standardized β	P-value	R ²	Standardized β	P-value	Standardized β	P-value	
Demographic, behavioural & anthropometric factors											
Age, Years	0.320	<0.001	0.103	-	-	-	0.406	<0.001	0.419	<0.001	
Physical activity	0.060	0.025	0.004	0.045	0.075	0.114	0.005	0.874			
Alcohol consumption	-0.106	<0.001	0.011	-0.070	0.006	0.110	-0.056	0.083			
Smoking status	0.006	0.827	0.000	-0.059	0.018	0.107	0.050	0.135			
Abdominal total fat mass (%)	-0.243	<0.001	0.059	-0.292	<0.001	0.143	-0.078	0.032	-0.081	0.014	
Blood chemistry & hormones											
Triglycerides (mmol/L)	-0.224	<0.001	0.050	-0.208	<0.001	0.146	-0.065	0.027	-0.088	0.006	
Glucose (mmol/L)	-0.094	<0.001	0.009	-0.162	<0.001	0.129	-0.049	0.145			
Insulin (μ U/mL) ^a	-0.157	<0.001	0.025	-0.178	<0.001	0.132	-0.009	0.806			
ALT activity (U/L) ^b	-0.239	<0.001	0.057	-0.168	<0.001	0.158	-0.054	0.118			
fT4 (pmol/L)	0.057	0.030	0.003	0.079	0.001	0.109	0.040	0.034	0.038	0.011	
TT (nmol/L)	0.450	<0.001	0.202	0.489	<0.001	0.339	0.461	<0.001	0.448	<0.001	
E2 (pmol/L) ^c	0.098	0.004	0.010	0.047	0.150	0.129	-0.023	0.490			
Inflammatory markers											
IL-6 (pg/mL)	0.062	0.016	0.004	0.018	0.470	0.104	0.047	0.150			
TNF- α (pg/mL)	0.074	0.005	0.006	0.056	0.025	0.109	0.037	0.243			
MPO activity (μ g/L)	-0.093	<0.001	0.009	-0.076	0.002	0.110	0.037	0.253			
eSel (ng/mL)	-0.169	<0.001	0.029	-0.151	<0.001	0.128	0.045	0.208			

Statistically significant associations (P < 0.05) are shown in bold. Multi-adjusted generalised linear model R² was 0.420 and lasso regression model R² was 0.404.

Abbreviations: ALT, alanine transaminases; fT4, free thyroxine; TT, total, testosterone; E2, oestradiol; SHBG, sex hormone binding globulin; IL-6, interleukin 6; TNF- α , tumour necrosis factor alpha; MPO activity, myeloperoxidase; eSel, sE-selectin.

^a n = 748, ^b n = 744, ^c n = 789

To account for the effect of changes in selected independent variables on the outcome measure, we modelled the absolute difference for independents between clinic visits against the change in SHBG at follow-up (Appendix 1). Furthermore, possible non-linear relationships between SHBG levels and independents have been assessed with multi-adjusted restricted cubic splines with 3 knots at the 10th, 50th, and 90th percentiles of the distribution. We found evidence of non-linear associations between SHBG levels and triglycerides ($P_{\text{non-linearity}} < 0.001$), glucose ($P_{\text{non-linearity}} < 0.001$), insulin ($P_{\text{non-linearity}} < 0.001$), alanine transaminases ($P_{\text{non-linearity}} < 0.001$), oestradiol ($P_{\text{non-linearity}} < 0.0001$), IL-6 ($P_{\text{non-linearity}} < 0.001$), TNF- α ($P_{\text{non-linearity}} < 0.001$) and eSel ($P_{\text{non-linearity}} < 0.001$) (Appendix 2 A-H). To further explore these non-linear associations, we applied generalised models, with alternate middle knot locations specified by visual inspection and likelihood ratio tests of the corresponding regression curves (Appendix 2). Except for E2 and ALT activity, all other independent variables within and above the cut-off were similar to the original linear regression model with continuous variables (Appendix 3).

6.6. Discussion

This study showed a positive relationship of serum SHBG levels with age, thyroxine and total testosterone, and an inverse relationship with abdominal total fat mass, triglycerides and oestradiol at baseline. Longitudinally, there was a positive relationship of SHBG levels with baseline age, thyroxine, and total testosterone and an inverse relationship with abdominal total fat mass, and triglycerides.

Our finding of a positive association between age and SHBG is consistent with most previous studies [195, 198, 215, 472]. The only study to find no association between SHBG levels and age included participants younger than 45 years [217]. We also did not find any significant association of serum SHBG levels and age among men younger than 45 years (n=458) (data not shown).

An independent inverse association between SHBG and obesity as defined by body mass index (BMI) and waist circumference (WC) has been reported cross-sectionally [170, 215, 473] and longitudinally [169, 189]. We have shown an even stronger inverse association using a DXA based estimate of visceral adiposity (abdominal total fat mass) consistent with prior cross-sectional data [172, 205, 217]. Further, we now demonstrate an inverse, longitudinal association between visceral adiposity and SHBG.

Although not directly addressed by the current study, our data are consistent with a considerable body of basic science data that links *de novo* lipogenesis and SHBG production [29, 33, 34, 57]. Prior studies have also suggested that circulating SHBG decreases when fat accumulates in the liver as a result of *de novo* lipogenesis [161, 169, 173]. Taken together, these data suggest a coupling between the regulation of SHBG and *de novo* lipogenesis. The strong inverse association between serum triglycerides and SHBG levels that we observed accord with the results of previous cross-sectional [175, 188, 248, 468] and limited longitudinal studies [168, 180]. This study

is the largest study to date to examine the association of serum triglycerides and SHBG using comprehensive clinical, demographic, anthropometric and bio-psychosocial data simultaneously.

Although SHBG is inversely associated with insulin in some cross-sectional and longitudinal studies [7, 11, 13, 15], we found, no association between insulin and SHBG either cross-sectionally or longitudinally. We also found an inverse association between insulin and SHBG among men with normal glucose or those with prediabetes but did not find a significant association between insulin and SHBG among men with T2D (data not shown). Insulin has been reported to inhibit hepatic SHBG production [109, 241] directly. However, there is no direct mechanism by which insulin can regulate transcription of the SHBG gene [94]. Rather, the effect is likely mediated indirectly via inhibition of hepatic *de novo* lipogenesis [94]. This may explain why SHBG tends to be higher in lean individuals with type 1 diabetes, and lower in the presence of obesity with hepatic insulin resistance [138, 161].

Consumption of a diet high in monosaccharides, particularly fructose, can reduce serum SHBG levels by about 80% in people without diabetes, and by about 40-50% among those with diabetes [94]. Glucose and fructose reduce SHBG production in HepG2 hepatocarcinoma cells by inducing lipogenesis [33]. We did not examine monosaccharide consumption, but there was no significant association between serum SHBG and serum glucose, as has been shown previously [176, 318]. We did not find any significant association between glucose and SHBG among men categorised as normoglycemic, impaired glucose tolerance and T2D group cross-sectionally but was significant longitudinally. In these data, SHBG levels were significantly low among T2D group (data not are shown).

T4 stimulates the production of SHBG in HepG2 hepatocarcinoma cells, indirectly by increasing HNF4- α gene expression and by reducing cellular palmitate levels [96,

109]. T4 has previously been shown to be positively associated with SHBG levels in men with hyperthyroidism and inversely associated with SHBG levels in men who are hypothyroid [279]. Our findings are consistent with these data despite two earlier observations reporting no relationship between T4 and SHBG [217, 280]. As far as we can determine the longitudinal association between T4 and SHBG, that we report, has not previously been demonstrated. Our data also accord with clinical observations of the effect of thyroid hormones on SHBG [100, 248].

SHBG production in HepG2 hepatocarcinoma cells is inhibited by testosterone, but the mechanism by which this occurs has not been elucidated [28, 29, 100]. As boys progress through puberty serum SHBG levels decrease as T levels increase [195]. Exogenously administered androgens, even at low doses when taken orally, suppress SHBG [264]. These findings notwithstanding, we found that serum T levels are positively correlated with SHBG cross-sectionally consistent with prior reports [175, 176, 217, 220, 248, 274]. As far as we can determine, our data are the first to show a strong positive association between T and SHBG levels longitudinally. Taken together, it seems that this reflects the expected steady-state relationship between T and SHBG as reflected by the measurement of total T.

The observed inverse association between E2 and SHBG in the cross-sectional data is consistent with the findings of others [110, 205, 217, 247] but stands in contrast to the effect of E2 to increase SHBG in HepG2 hepatocarcinoma cells, an effect most likely dependent on estrogen receptor α (ER- α) mediated upregulation of HNF4- α gene expression [24, 28, 261]. Furthermore, our longitudinal analyses revealed no association between E2 and SHBG. These observations were also confirmed by additional results obtained with permitted flexible nonlinear associations by restricted cubic spline analyses. The most likely explanation for the difference in cross-sectional vs. longitudinal results and findings within the model is that circulating E2 levels are

primarily dependent on T, but not age or percentage total fat mass and total T (measured in our study), which in general will be lower when SHBG is lower. The effect of E2 to stimulate SHBG is dependent on a first pass effect through the liver [24]. This applies to both men and women. Oral E2 in women is well described to increase SHBG, whereas topical delivery has a minimal effect [474]. The same is true in men [475].

Moreover, there is a general conclusion from longitudinal studies that serum E₂ declines when serum T declines in men [476], therefore any effects are likely obscured by the strong positive association between SHBG and total T levels. Support for this explanation comes from a study in which it was shown that with increasing obesity, both T and E2 decrease but T does so to a greater extent [477]. Furthermore, we assessed the expression of adipose tissue aromatase in male volunteers to determine the effect of 28 days overfeeding a high-fat energy dense diet (weight gain) and observed no increase in aromatase per unit of adipose tissue. Accordingly, we presume that expanded adipose tissue mass increases biotransformation of T to E accounting for the observed relative preservation of E2 compared with T.

TNF- α at both physiological and supraphysiological concentrations reduces SHBG production in HepG2 cells by downregulating HNF4- α gene expression, an effect mediated via hepatocyte nuclear factor kappa B (NF- κ B) [92]. Prior epidemiological studies have reported an inverse association of serum SHBG levels with WBC count and serum fibrinogen but not CRP [253, 256]. We did not find any statistically significant relationships between circulating pro-inflammatory cytokines (TNF- α and IL-6), a marker of macrophage activation (MPO activity), or a marker of vascular endothelial function (eSel) and SHBG. We also did not find any significant association of WBC count with serum SHBG in a subset of the cohort (n=792) for whom data were available (data not are shown).

Taken together these and other data suggest that circulating levels of SHBG are a marker of the presence and severity of hepatic insulin resistance [138], *de novo* lipogenesis [100], and associated conditions including NAFLD [237], type 2 diabetes [100], and cardiovascular disease [29]. From a clinical standpoint, the measurement of serum SHBG facilitates the identification of this component of metabolic dysfunction and monitoring of the response to treatment [29, 54]. Interventions that reduce weight, ameliorate insulin resistance, fatty liver and lower serum triglycerides lead to increases in serum SHBG [100]. Conversely worsening of these conditions leads to a decrease in serum SHBG [29, 57]. In patients with type I diabetes, SHBG levels should be normal or raised [325]; a decrease in SHBG level reflects the emergence of hepatic insulin resistance and therefore is a possible indicator of type II diabetes [29, 57]. For instance, SHBG levels were shown to be increased when patients with T2D are treated with rosiglitazone, which reduces insulin resistance by ~30% [161]. A further example is in evaluating the significance of the low testosterone in patients with metabolic syndrome [57].

The strengths of our study include the use of a well-characterised, large population-based and broadly representative sample of men, with ages ranging from 35 to 80 years, and the simultaneous measurement of numerous potential determinants and confounders. Further, sex steroids were measured, concurrently at each time point, using validated tandem mass spectrometry assays, and body composition were assessed by DXA. Study limitations include the predominant Caucasian population, which limits generalisability given known ethnic variations in SHBG [268].

6.7. Conclusions

In conclusion, our study has confirmed an age-related rise in circulating SHBG in men, beginning from middle-age. Beyond this gradual increase, reduced SHBG levels largely reflect obesity, particularly men with a substantial or predominant visceral component with associated metabolic consequences. We suggest that variation in SHBG reflects de novo lipogenesis within the liver that occurs in the context of insulin resistance. The observed positive relationship between thyroid hormones and SHBG levels suggests that thyroid hormone may modulate circulating SHBG, either directly or indirectly. High circulating sex steroids are associated with higher SHBG levels reflecting the direct effect of sex steroids.

7. Role of SHBG as a marker of T2D

Statement of Authorship

Title of Paper: The role of sex hormone-binding globulin (SHBG), testosterone, and other sex steroids, on the development of type 2 diabetes in a cohort of community-dwelling middle-aged to elderly men

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Signature		Date	23/10/18

7.1. Abstract

Aims: Contrasting findings exist regarding the association between circulating sex hormone-binding globulin (SHBG) and testosterone levels and type 2 diabetes (T2D) in men. We examined prospective associations of SHBG and sex steroids with incident T2D in a cohort of community-dwelling men. **Design and Methods:** Participants were from a cohort study of community-dwelling (n=2563), middle-aged to elderly men (35-80 years) from Adelaide, Australia (the Men Androgen Inflammation Lifestyle Environment and Stress (MAILES) study). The current study included men who were followed for five years and with complete SHBG and sex steroid levels (total testosterone (TT), dihydrotestosterone (DHT) and oestradiol (E2)), but without T2D at baseline (n=1597). T2D was identified by either self-report, fasting glucose (≥ 7.0 mmol/L), HbA1c ($\geq 6.5\%$ / 48.0 mmol/mol), and/or prescriptions for diabetes medications. Logistic binomial regression was used to assess associations between SHBG, sex steroids and incident T2D, adjusting for confounders including age, smoking status, physical activity, adiposity, glucose, triglycerides, symptomatic depression, SHBG and sex steroid levels. **Results:** During an average follow-up of 4.95 years, 14.5 % (n=232) of men developed new T2D. Multi-adjusted models revealed an inverse association between baseline SHBG, TT, and DHT levels, and incident T2D (odds ratio (OR) = 0.77, 95%CI= [0.62, 0.95], $p=0.02$; OR=0.70 [0.57, 0.85], $p<0.001$ and OR=0.78 [0.63, 0.96], $p=0.02$), respectively. However, SHBG was no longer associated with incident T2D after additional adjustment for TT (OR=0.92 [0.71, 1.17], $p=0.48$; TT in incident T2D: OR=0.73 [0.57, 0.92], $p=0.01$) and after separate adjustment for DHT (OR=0.83 [0.64, 1.08], $p=0.16$; DHT in incident T2D: OR=0.83 [0.65, 1.05], $p=0.13$). There was no observed effect of E2 in all models of incident T2D. **Conclusions:** In men, low TT but not SHBG and other sex steroids, best predicts the development of T2D after adjustment for confounders.

7.2. Introduction

Sex hormone-binding globulin (SHBG) is a circulating homodimeric glycoprotein synthesised in the liver and extrahepatic tissues, although the majority of circulating SHBG is of hepatic origin [26]. SHBG was originally thought to exclusively act as a carrier protein that binds circulating sex steroids with high affinity, thereby regulating their transport, distribution, biological activity, and metabolic clearance [26, 97]. However, emerging data have suggested that SHBG may also play a direct role in the intracellular signalling mediated by SHBG-receptors (R_{SHBG}) located on the target cell membranes that serve various metabolic functions [84, 131]. In men, 20% of circulating SHBG is unbound without known biological function(s) [28]. Recent studies have demonstrated that the synthesis and secretion of SHBG by hepatocytes is regulated by hormonal, metabolic, and nutritional factors that influence the metabolic state of the liver, with these effects reflected by alterations in circulating SHBG levels [29, 100]. This has raised the possibility of whether circulating SHBG may be reflective of broader metabolic changes, such as the development of type 2 diabetes mellitus (T2D).

The available epidemiological evidence is ambiguous, with some studies showing that SHBG is inversely associated with T2D risk in men [184, 186, 344], while another report no association [177, 185, 304]. Most likely these differences are attributable to differences in methodological design, principally not accounting for the role of testosterone in men or sub-optimal measurement of testosterone (e.g. absence of liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays [184, 186, 344, 349, 353-355], or the lack of standardized blood sampling time [177, 185] or the use of samples with extended periods of storage [184, 354]). SHBG and testosterone (T) concentrations co-vary with one other, and it remains unclear whether the association with T2D in men for SHBG is independent of T or is simply explained by T. However, a greater amount of evidence has shown an inverse association between T and incident T2D development [343, 351, 478-480], suggesting that testosterone may have a

protective role in the development of T2D. Other design issues from prospective studies that may have limited an understanding of the association between SHBG and T2D in men include differences in the definition [175, 184, 186, 347, 350, 353, 354], and/or low number of cases [184, 350], of incident diabetes; age ranges restricted to younger or older men [344, 354, 355]; failure to adjust for other confounders visceral obesity [186, 353, 354] and other diabetes risk factors [184, 349] .

Thus, we aim to comprehensively evaluate the associations between SHBG, sex steroids and incident T2D using data from a large, representative and well-characterised cohort of middle-aged to elderly men.

7.3. Materials and Methods

7.3.1. Study Design and Population

Participants for the present study were selected from the Men Androgen Inflammation Lifestyle Environment and Stress (MAILES) study. The MAILES study is comprised of suburban, community-dwelling men aged 35-80 years randomly selected from the Northern and Western Statistical Local Areas of Adelaide, Australia. A more detailed description of the study design, procedures, and recruitment were published previously [26]. Briefly, a total of 2563 age-matched men from two existing prospective cohorts (the Florey Adelaide Male Ageing Study (FAMAS) [27] and the North West Adelaide Health Study (NWAHS) [28] were harmonized into a dataset incorporating detailed information on sociodemographic, clinical, behavioural, chronic disease, and medication data. The present study involved participants from MAILES 1 (2002-2006) and MAILES 2 (2007-2010) (median follow-up period of 4.94 years, interquartile range 4.34-5.00 years). Comparisons between the sampled population and MAILES participants using 2006 and 2010 Australian Census data for MAILES 1 and 2, respectively showed that MAILES participants matched the target population for most key demographics, although younger groups and never-married men were underrepresented and older participants were overrepresented [27]. Among the 2563 participants enrolled at baseline (MAILES 1), 525 of them (20.5%) either died between waves (n=138), were lost-to-follow-up (n=77) or refused to participate (n= 310), providing an eligible sample of (n= 2038) during follow-up (MAILES 2). The analytic cohort for the present study consisted of those men with complete T2D data, SHBG and sex steroid levels (total testosterone (TT), dihydrotestosterone (DHT) and oestradiol (E2)), and without T2D at baseline (Figure 41). Among the 2563 participants enrolled at baseline, 562 of them either had missing data on T2D (n=73) or had prevalent T2D at baseline (n=489), providing an eligible sample of 2001. After further excluding 369 individuals without baseline SHBG measurements and eight men receiving either testosterone supplementation or anti-androgen

(Leuprorelin, Goserelin, Bicalutamide) therapy, the analytic cohort consisted of n=1597. A separate analysis was conducted to compare baseline demographics between the most recent MAILES survey (MAILES 2) and the analytic cohort to ensure a reasonable representation of the entire participants (Appendix 4). This study was approved by the research ethics committees of the Royal Adelaide Hospital and the North Western Adelaide Health Service. A written informed consent form was given to the eligible participants who signed in-clinic.

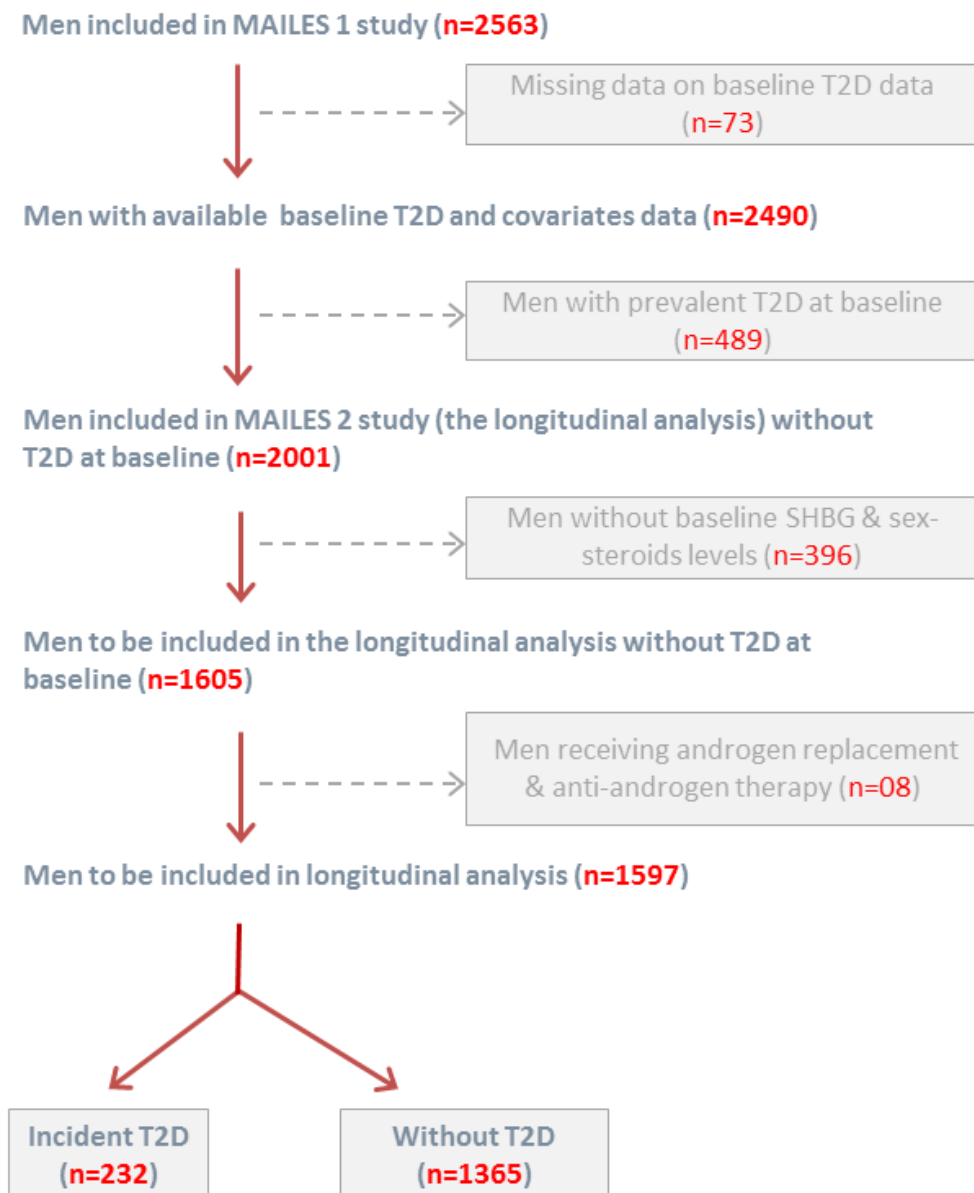


Figure 41. Consort diagram for the final analytical samples for the current analyses.

Abbreviations: MAILES, Men Androgen Inflammation Lifestyle Environment and Stress; T2D, Type 2 diabetes; SHBG, Sex hormone-binding globulin.

The antiandrogen therapy included were Leuprorelin, Goserelin and Bicalutamide.

7.3.2. Outcome measures

Our primary outcome was the occurrence of an incident case of T2D. At baseline and during follow-up, T2D was identified according to International Diabetes Federation (IDF) criteria by either self-reported doctor-diagnosed diabetes, fasting glucose (≥ 7.0 mmol/L / ≥ 126.0 mg/dL), glycated haemoglobin ((HbA1c) $\geq 6.5\%$ / ≥ 48.0 mmol/mol) and/or prescriptions for antidiabetic medications. Information regarding the use of antidiabetic medications was derived from both structured home interviews and data linkage with the national medication registry.

7.3.3. Sex hormone-binding globulin (SHBG) and other biochemical analyses

Full details of the laboratory methods and quality control data have been reported previously [223, 470, 481]. Briefly, venous blood samples were drawn between 8:00 AM and 11:00 AM after a 12-hour overnight fast and 20 min in a sitting position. Samples were immediately placed on ice and transported to a laboratory certified by the National Association of Testing Authorities (NATA) within 4-hours, then centrifuged, fractionated, and serum stored at -80°C until measurement (between 1 - 13 months after collection). Samples were randomly ordered for assay, and the laboratory technicians were blinded to participant characteristics. Blood samples from the two cohorts were analysed in the same laboratory, and samples from the two-time points were analysed concurrently. Serum SHBG levels were measured by diluting serum to 1:21 through SHBG sample diluent, and then assayed using the Immulite Autoanalyser and a solid-phase, two-site chemiluminescent immunoassay (Siemens Medical Solutions, New York, USA; inter-assay CV: 4.0% at 32.3 nmol/L; lower detection limit: 0.17 nmol/L). Serum sex steroids [total testosterone (TT), dihydrotestosterone (DHT) and oestradiol (E2)] were measured by a validated stable-isotope dilution liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (API-5000, Applied Biosystems/MDS SCIEX, Ontario, Canada). The inter-assay coefficients of variation (CV) were as follows: 10.1% at 0.43 nmol/L, 11.1% at 1.66 nmol/L, and 4% at 8.17 nmol/L for TT; 13.2% at 0.43 nmol/L, 8.0% at

1.68 nmol/L, and 7.4% at 8.37 nmol/L for DHT and 9.2% at 22pmol/L, 2.2% at 83 pmol/L, and 6.1% at 411 pmol/L for E2. HbA1c was measured by high-pressure liquid chromatography using a spherical cation exchange gel (Bio-Rad Laboratories, California, USA). Plasma glucose and serum triglycerides were measured using an automated chemistry analyser (Olympus AU5400; Olympus Corp, Tokyo, Japan). The inter-assay coefficient of variation (CV) were <10.6 % for all measurements. Concentrations of all selected analytes are stable after multiple freeze-thaw cycles [166].

7.3.4. Other covariates

Socio-demographic, behavioural and lifestyle characteristics

Information on socio-demographic characteristics (age, gender, education and marital status), behavioural & lifestyle factors (smoking status, physical activity, and alcohol consumption), as well as medical history, including information about medical procedures, and medication use was obtained by a validated, self-report questionnaire and data linkage with the national medication registry as described previously [470]. Smoking behaviour was recorded as smokers (current and former smoker) and non-smokers. In this analysis, smoking categories were a current smoker and non-current smoker. The physical activity questions from the Australian National Health Surveys were used to classify participants as sedentary or having low, moderate, or high levels of physical activity [223, 470]. Alcohol use was determined at clinical interview and recorded in standard drinks consumed per day; none, low ≤ 2 , and high ≥ 3 .

Anthropometry

Waist circumference (WC) was measured to the nearest 0.1 cm using an inelastic tape maintained in a horizontal plane at the level of the narrowest part of the waist, and read from the mid-axillary line, with the subject standing comfortably with weight distributed evenly on both feet. Low, moderate and high risk of abdominal adiposity were defined as WC (cm) ≤ 94.99 , 95.0-99.9 & ≥ 100.0 , respectively. Body composition was assessed

using Dual-energy X-ray Absorptiometry (DXA) using the Lunar DPX+ and Prodigy pencil beam densitometers (Lunar Radiation Corporation, Madison, USA). Both densitometers show excellent congruence, with <1% difference in measurement across a range of measured areas [471]. Body composition was measured at baseline in a subset of the cohort (n =1735): NWAHS participants aged ≥50 years (n=666), and FAMAS participants (n=1069).

Chronic conditions

Medication use was obtained from Medicare Australia by confidential unit record linkage, classified according to the Anatomical Therapeutic Chemical (ATC) Classification. The number of distinct medication classes (at the ATC third level) 6 months before baseline or follow-up clinical examination were treated as covariates. Family history of diabetes was obtained by a validated, self-report questionnaire. Depressive symptoms were assessed using the Centre for Epidemiological Studies-Depression Scale (CES-D) (score ≥16) in NWAHS and the Beck Depression Inventory (BDI) (score≥10) in FAMAS at both time points. The CES-D and BDI are comparable in terms of the validity and details of these have been published previously [223].

7.3.5. Statistical Analysis

Initial descriptive analyses of selected independents and outcome measures were conducted using χ^2 (categorical) and *t*-tests (continuous) and are reported as means ± standard deviation (SD) and percentages, as appropriate. Normality and linearity assumptions were examined for all covariates. To assess the association between baseline circulating SHBG (and sex steroids) levels and incident T2D, we implemented unadjusted, then age-adjusted and multi-adjusted logistic binomial regression models. For multi-adjusted regression models, selected covariates with an age-adjusted association with the outcome variable of $p \leq 0.20$, were entered into a final multi-adjusted model of incident T2D. To *evaluate* the individual and combined

association between SHBG, sex steroids and incident T2D, we included a multi-adjusted model with SHBG, TT and DHT, separately, as well as in combination with SHBG. The goodness of fit for competing regression models was evaluated by Akaike's Information Criterion (AIC) [482] and Nagelkerke R^2 values. Effects were presented as odds ratios (ORs) together with their 95% confidence interval (95% CI) per standard deviation (SD) increase in the SHBG and sex steroids levels (TT, DHT and E2). Since DXA based abdominal fat mass is a better measure of visceral adiposity, we performed the additional analysis substituting WC with DXA based abdominal total fat mass (%).

Multi-adjusted quartile regression analyses were additionally performed to examine non-linear associations between SHBG (and sex steroids) and T2D. Sub-group regression analyses by age group were also performed to examine for differential associations in younger (<65 years) versus older (≥ 65 years) men in multi-adjusted logistic binomial regression models. Finally, we conducted sensitivity analyses to assess the effect of medication usage on the observed regression coefficients for incident T2D men in multi-adjusted logistic binomial regression models. Also, inverse probability weighting (IPW) was conducted with weights estimated from a logistic binomial regression model to correct for possible selection bias due to loss to follow up.

Statistical analyses were performed with the IBM SPSS statistical package (version 23.0 New York, USA). All statistical tests were two-sided, and $p < 0.05$ was considered statistically significant. Akaike's Information Criterion (AIC) and explained variance (R squared) values of the models are also presented.

7.4. Results

Table 25 details the selected demographic, clinical and laboratory variables of the analytic sample at baseline and by incident T2D at follow-up. Of the 1597 eligible men without prevalent diabetes at baseline, 232 (14.5%) men developed T2D over a median follow-up of 4.95 years. Among these new cases of diabetes, 43 individuals (18.5%) were identified by self-reported doctor-diagnosed diabetes, 16 individuals (6.9%) through data linkage with the national medication registry, 23 individuals (9.9%) by IDF fasting glucose criteria and 195 (84.1%) were identified by IDF HbA1c criteria.

Table 25. Baseline characteristics of the overall sample and by incident type 2 diabetes.

Predictors	Overall sample (n=1597)	Without incident T2D (n=1365)	With incident T2D (n=232)	p-value [#]
Questionnaire & clinical evaluations				
Age(Years)	53.9±11.3	53.3±11.3	57.5±11.0	<0.001
Marital status				
Married / Partnered	1254(78.1)	941(78.2)	168(79.6)	0.953
Separated / Divorced	208(13.0)	152(12.6)	25(11.8)	
Widowed	40(2.5)	30(2.5)	6(2.8)	
Never married	98(6.1)	76(6.3)	11(5.2)	
Education Level				
Higher school or lower	459(28.6)	329(27.3)	71(33.6)	0.005
Trade / Apprenticeship	508(31.7)	390(32.4)	54(25.6)	
Diploma / Certificate	404(25.2)	292(24.3)	63(29.9)	
Bachelor or higher	210(13.1)	176(14.6)	19(9.0)	
Smoking status (current), n (%)				
Non-smoker	1258(78.4)	242(20.1)	36(17.1)	0.345
Smoker	343(21.4)	958(79.6)	174(82.5)	
Leisure time physical activity, n (%)				
Sedentary	393(24.5)	284(23.6)	65(30.8)	0.015
Low, moderate, high	1132(70.5)	855(71.1)	134(63.5)	
Alcohol consumption				
None/day	14(0.9)	11(0.9)	3(1.4)	0.479

1-2 drinks/day	840(52.3)	605(50.3)	115(54.5)	
>3 drinks/day	684(42.6)	529(44.0)	86(40.8)	
Cholesterol-lowering medication, <i>n</i> (%)				
Yes	46(2.9)	155(12.9)	55(26.1)	<0.001
No	1557(97.0)	1043(86.7)	156(73.9)	
Antihypertensive medication,%				
Yes	322(20.1)	217(18.0)	69(32.7)	<0.001
No	1278(79.6)	981(81.5)	142(67.3)	
Family history of diabetes, <i>n</i> (%)				
Yes	445(27.7)	321(26.7)	69(32.7)	0.018
No	1157(72.1)	881(73.2)	140(66.4)	
Depression, <i>n</i> (%)				
Yes	222(13.8)	136(11.3)	37(17.5)	0.015
No	1350(84.1)	1045(86.9)	168(79.6)	
Abdominal total fat mass (%) ^a	33.9±8.3	33.5±8.2	37.0±7.9	<0.001
WC (cm)	99.5±11.4	98.5±11.4	103.7±12.5	<0.001
BMI (kg/m ²)	28.1±4.2	27.8±4.0	29.6±4.6	<0.001
Systolic blood pressure (mmHg)	133.7±17.9	131.6±16.9	139.5±19.4	<0.001
Diastolic blood pressure (mmHg)	85.1±9.1	84.5±9.0	86.7±9.5	<0.001
Hypertension, <i>n</i> (%)				
Yes	717(44.7)	494(41.1)	89(42.2)	<0.001
No	885(55.1)	706(58.7)	122(57.8)	

Blood biochemistry & hormones				
Glucose (mmol/L)	4.8±1.35	4.8±0.58	5.1±0.76	<0.001
Glycosylated haemoglobin,% (mmol/mol))	5.5±0.35 (36.5±3.8)	5.4±0.34 (39.7±2.7)	5.8±0.25 (36.0±3.7)	<0.001
Insulin (µIU/mL) ^b	9.8±7.2	9.1±6.4	12.7±9.6	<0.001
Total cholesterol (mmom/L)	5.5±1.06	5.5±1.0	5.5±1.1	0.961
LDL-C(mmol/L)	3.5±0.882	3.5±0.88	3.5±0.91	0.671
HDL-C(mmol/L)	1.26±0.31	1.3±0.31	1.2±0.32	0.008
Triglycerides (mmol/L)	1.7±1.4	1.7±1.3	2.0±1.6	0.003
TT (nmol/L)	17.6±6.1	17.8±5.8	15.6±5.8	<0.001
DHT (nmol/L) ^c	1.8±0.80	1.8±0.76	1.6±0.67	0.001
E2 (pmol/L) ^d	96.0±42.0	98.9±43.3	100.8±38.1	0.630
SHBG (nmol/L)	34.0±15.0	33.8±14.5	31.5±13.4	0.033
Prevalence & incidence of the outcome				
Prevalent T2D, <i>n</i> (%)	489 (19.1)	-	-	-
Incident T2D, <i>n</i> (%)	-	232 (11.6)	-	-

Statistically significant associations ($p < 0.05$) are shown in bold.

Abbreviations: T2D, Type 2 diabetes; WC, Waist circumference; BMI, Body mass index; LDL, Low-density lipoprotein; HDL, High-density lipoprotein; TT, Total testosterone; DHT, Dihydrotestosterone; E2, Oestradiol; SHBG, Sex hormone-binding globulin.

^a n=1174 ^b n=772 ^c n=1359 ^d n=841.

‡ Indicates the *p*-value for the overall difference between men with and without T2D at follow-up based on χ^2 for categorical variables and *t*-test for continuous variables.

7.4.1. Baseline SHBG and prediction of incident T2D

In our data set, a multi-adjusted model with TT in combination with SHBG was the most favourable model followed by a multi-adjusted model with TT, multi-adjusted model with DHT in combination with SHBG, multi-adjusted with SHBG and multi-adjusted with DHT, respectively (AIC values of 739.2, 743.0, 744.4, 744.4 and 749.8, respectively).

Table 26 summarises the regression estimates for incident T2D for SHBG and selected predictors at baseline. In unadjusted regression models, increasing TT, DHT, and SHBG levels (per SD increase) at baseline were associated with a decreased risk of incident T2D (OR 0.644 [CI 0.541 - 0.767], OR 0.739 [CI 0.614 - 0.890], and OR 0.828 [CI 0.697 - 0.985]), respectively. This inverse association persisted after adjustment for age, (OR 0.653 [CI 0.548 - 0.779]) for TT, (OR 0.656 [CI 0.538 - 0.801]) for SHBG, and (OR 0.724 [CI 0.600 - 0.873]) for DHT. In separate multi-adjusted models, increasing TT (OR 0.696 [CI 0.567 - 0.853]), increasing DHT (OR 0.780 [CI 0.632 - 0.861]), and increasing SHBG (OR 0.765 [CI 0.616 - 0.951]) were independently associated with decreased risk of incident T2D. Following additional adjustment for TT, no significant association was observed between SHBG and T2D (OR 0.915 [CI 0.714 - 1.172]). There was, however, a strong, inverse association between TT and incident T2D (OR 0.725 [CI 0.573 - 0.919]) in this model. After substituting DHT for TT, the association between SHBG and T2D was also non-significant (OR 0.830 [CI 0.640 - 1.077]), while a null effect of DHT was observed with incident T2D (OR 0.828 [CI 0.650 - 1.054]). Other covariates that demonstrated an independent association with incident T2D were age (OR 1.564 [CI 1.264 - 1.934]), glucose (OR 1.739 [CI 1.459 - 2.072]), and symptomatic depression (OR 2.189 [CI 1.404 - 3.411]). Total E2, in contrast, was not associated with risk of developing T2D in all models of incident T2D. No association was observed between free testosterone (FT, as per Vermeulen equation [68] and incident T2D (data not shown). In a separate logistic binomial regression models, we substituted, WC with abdominal total fat mass (%), in a subset of the cohort (n=1067) for whom data were available,

none of the observed regression coefficients for associations between SHBG, TT, DHT and E2 with incident T2D were affected (Appendix 5).

Table 26. Unadjusted, age-adjusted, and multi-adjusted logistic regression models for baseline SHBG, sex steroids and other selected predictors on incident type 2 diabetes in community-dwelling men.

Predictors	Unadjusted		Adjusted											
	OR (95%CI)	p-value	+Age		Multi-adjusted model with SHBG		Multi-adjusted model with TT		Multi-adjusted model with DHT‡		Multi-adjusted model with SHBG & TT		Multi-adjusted model with SHBG & DHT‡	
			OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value
SHBG (nmol/L)	0.828(0.697-0.985)	0.033	0.656(0.538-0.801)	<0.001	0.765(0.616-0.951)	0.016	-	-	-	-	0.915(0.714-1.172)	0.481	0.830(0.640-1.077)	0.160
Testosterone (nmol/L)	0.644(0.541-0.767)	<0.001	0.653(0.548-0.779)	<0.001	-	-	0.734(0.601-0.896)	0.002	-	-	0.725(0.573-0.919)	0.008	-	-
DHT (nmol/L)‡	0.739(0.614-0.890)	0.001	0.724(0.600-0.873)	<0.001	-	-	-	-	0.780(0.632-0.961)	0.020	-	-	0.828(0.650-1.054)	0.125
E2 (pmol/L)	1.044(0.877-1.242)	0.629	-	-	-	-	-	-	-	-	-	-	-	-
Age , Years	1.487(1.281-1.726)	<0.001	-	-	1.660(1.351-2.041)	<0.001	1.514(1.255-1.826)	<0.001	1.661(1.365-2.021)	<0.001	1.564(1.264-1.934)	<0.001	1.776(1.426-2.212)	<0.001

Marital status (Married or partnered/Never married)	0.900(0.504-1.609)	0.723	-	-	-	-	-	-	-	-	-	-	-	-
Education level (Low or above high school)	0.940(0.781-1.132)	0.516	-	-	-	-	-	-	-	-	-	-	-	-
Glucose (mmol/L)	4.256(3.007-6.023)	<0.001	3.902(2.748-5.540)	<0.001	1.739(1.461-2.069)	<0.001	1.729(1.455-2.056)	<0.001	1.795(1.496-2.154)	<0.001	1.739(1.459-2.072)	<0.001	1.786(1.484-2.150)	<0.001
WC (cm) (<95, 95-99.9 & ≥100)	1.473(1.249-1.737)	<0.001	1.425(1.206-1.683)	<0.001	1.241(1.017-1.514)	0.034	1.194(0.977-1.459)	0.083	1.187(0.966-1.459)	0.103	1.173(0.957-1.438)	0.125	1.168(0.947-1.439)	0.147
Triglycerides (mmol/L)	1.196(1.056-1.355)	0.005	1.246(1.097-1.416)	0.001	1.062(0.913-1.236)	0.435	1.072(0.923-1.245)	0.360	1.111(0.945-1.305)	0.201	1.056(0.907-1.230)	0.483	1.084(0.918-1.279)	0.343
Parental history of diabetes	1.427(1.061-1.920)	0.019	1.584(1.171-2.144)	0.003	1.302(0.914-1.855)	0.143	1.337(0.941-1.899)	0.105	1.310(0.905-1.898)	0.153	1.319(0.924-1.882)	0.127	1.292(0.887-1.883)	0.182
Smoking status (current; Yes/No)	1.156(0.879-1.519)	0.199	1.337(0.949-1.882)	0.097	1.167(0.814-1.673)	0.401	1.142(0.800-1.631)	0.463	1.104(0.767-1.591)	0.594	1.149(0.802-1.648)	0.449	1.100(0.760-1.592)	0.613
Alcohol consumption (None, 1-2/day & ≥3/day)	0.844(0.642-1.110)	0.225	-	-	-	-	-	-	-	-	-	-	-	-

Depression(Sym ptomatic)	1.623(1.096-2.403)	0.016	1.749(1.174-2.604)	0.006	2.167(1.393-3.370)	0.001	2.181(1.404-3.389)	0.001	2.007(1.256-3.207)	0.004	2.189(1.404-3.411)	0.001	2.017(1.257-3.236)	0.004
Physical activity (Sedentary, low-,moderate- & high-exercise level)	0.862(0.741-1.004)	0.056	0.846(0.725-0.988)	0.035	0.941(0.789-1.121)	0.493	0.929(0.780-1.106)	0.407	0.929(0.775-1.114)	0.426	0.939(0.787-1.121)	0.486	0.937(0.779-1.126)	0.486

Statistically significant associations ($P < 0.05$) are shown in bold. Nagelkerke R Square (multi-adjusted + SHBG): 0.149, Nagelkerke R Square (multi-adjusted + TT): 0.152, Nagelkerke R Square (multi-adjusted + DHT): 0.154, Nagelkerke R Square (multi-adjusted + SHBG & TT): 0.159 and Nagelkerke R Square (multi-adjusted + SHBG & DHT): 0.161. AIC value for multi-adjusted + SHBG & TT: 739.2, AIC value for multi-adjusted + TT: 743.0, AIC value for multi-adjusted + SHBG & DHT: 744.4, AIC value for multi-adjusted + SHBG: 744.4 and AIC value for multi-adjusted + DHT: 749.8.

Abbreviations: SHBG, sex hormone-binding globulin; Odds Ratio, OR; DHT, dihydrotestosterone; E2, oestradiol; WC, waist circumference; AIC, Akaike's Information Criterion

‡ Includes 1129 men without T2D and 191 men with incident T2D

Quartile Analysis

Higher quartiles of TT, compared with the lowest quartile, was associated with a significantly reduced risk of T2D in multi-adjusted models in combination with SHBG [OR 0.778 [95% CI 0.632 - 0.958]; $P_{\text{trend}} = 0.02$). However, there was no significant change across SHBG levels [OR 0.930 [95% CI 0.748 - 1.155]; $P_{\text{trend}} = 0.51$). Likewise, there was no relationship observed across DHT and E2 quartiles [OR 0.850 (CI 0.693 - 1.042)]; $P_{\text{trend}} = 0.12$) and [OR 1.050 (CI 0.865 - 1.275)]; $P_{\text{trend}} = 0.62$), respectively in the multi-adjusted model in combination with SHBG (Figure 42).

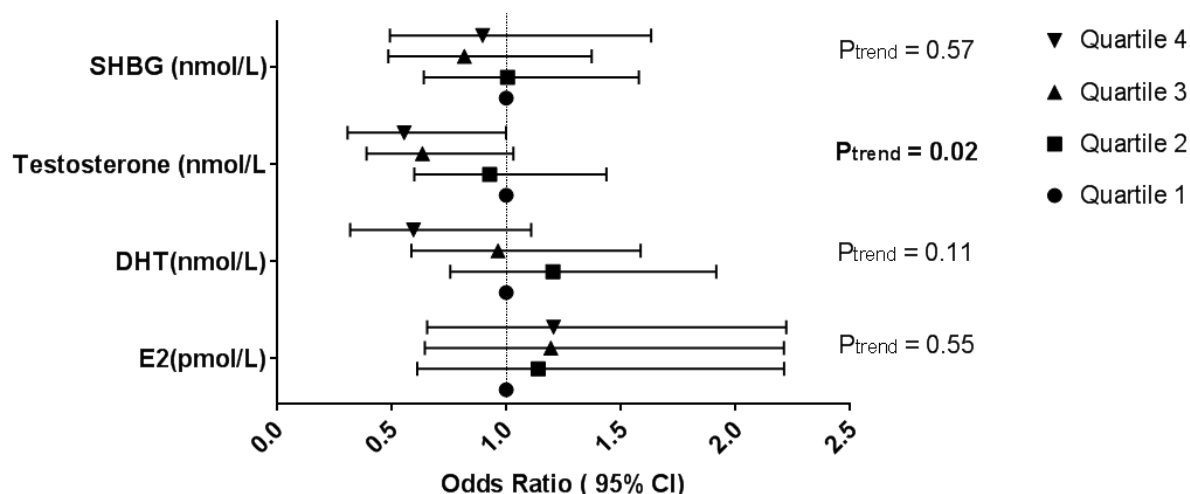


Figure 42. The odds ratio for incident T2D according to the quartiles of SHBG and sex steroids (TT, DHT & E2) in the multi-adjusted model.

Abbreviations: SHBG, Sex Hormone-Binding Globulin; TT, total testosterone; DHT, dihydrotestosterone; E2, oestradiol. The multi-adjusted model consisted of baseline age, glucose, waist circumference, triglycerides, parental history of diabetes, smoking status, physical activity and symptomatic depression with SHBG in combination with TT, DHT and E2, separately.

The dashed line represents the reference line, where OR = 1.00.

Subgroup analysis

When stratified according to middle-aged to younger men (< 65 years) and elderly men (≥ 65 years), the overall observed effect of TT on incident T2D risk in multi-adjusted models, was strengthened in the younger group (OR 0.648 [0.468 - 0.863]) and attenuated in the latter group (OR 1.035 [CI 0.684 - 1.568]) (Figure 43).

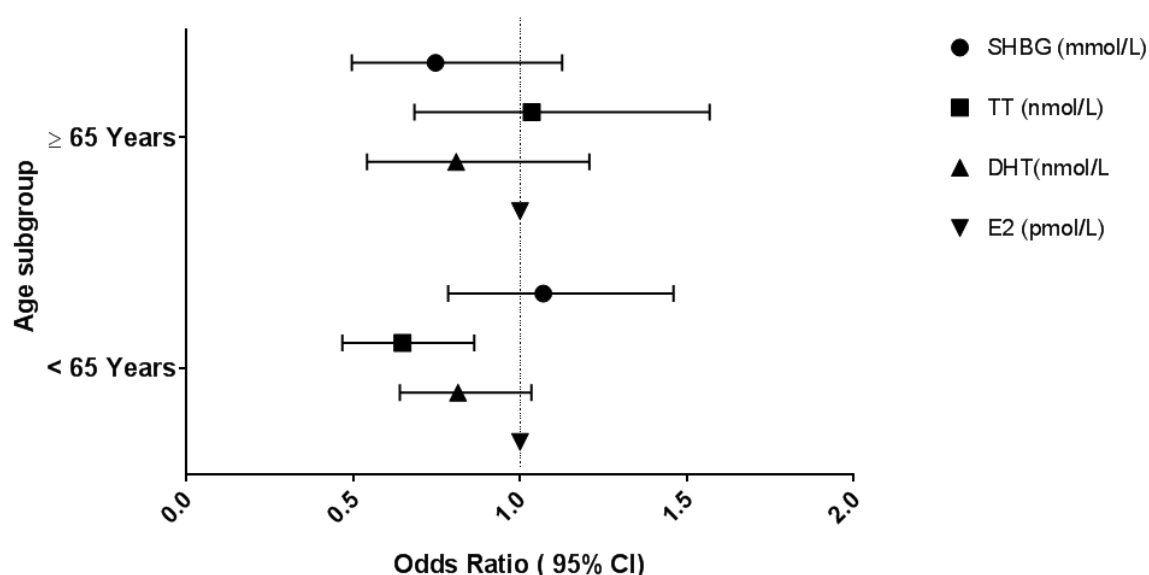


Figure 43. The odds ratio for an association between SHBG (and sex steroids) levels and T2D by age subgroup (<65 years and ≥65 years) in the multi-adjusted model.

Abbreviations: SHBG, sex hormone-binding globulin; TT, total testosterone; DHT, dihydrotestosterone; E2, oestradiol. SHBG, Sex Hormone-Binding Globulin; TT, total testosterone; DHT, dihydrotestosterone; E2, oestradiol. The multi-adjusted model consisted of baseline age, glucose, waist circumference, triglycerides, parental history of diabetes, smoking status, physical activity and symptomatic depression with SHBG in combination with TT and DHT, separately.

The dashed line represents the reference line, where OR = 1.00.

Sensitivity analysis

In a sensitivity analysis, none of the observed regression coefficients for associations between SHBG (and sex steroids) and T2D in multi-adjusted logistic binomial regression models were affected by further adjustment for pharmacotherapy, including lipid-lowering, hypoglycaemic, anti-hypertensive and anti-depressants (Table 27). Also, the IPW estimation indicated that none of the observed regression coefficients for SHBG (and sex steroids) and T2D in multi-adjusted logistic binomial regression models were affected by selection bias due to loss to follow up (data not shown).

Table 27. Effect of medication usage on the observed regression coefficients for associations between SHBG (and sex steroids) and T2D in multi-adjusted logistic binomial regression models.

Medications		Incident T2D				
		%(n)	OR	95% CI		R ² change
Lipid lowering (including Statin)	Baseline	1.8(29)	0.905	0.706	1.161	
	+/-	1.1(19)	0.927	0.724	1.188	-0.001
	+/+	0.6(10)	0.924	0.720	1.185	-0.002
	-/+	15.2(244)	0.916	0.698	1.203	+0.003
	-/-	72.5(1158)	Ref			
Hypoglycaemic	Baseline	6.7(44)	0.913	0.713	1.170	
	+/-	1.8(46)	0.865	0.664	1.127	+0.069
	+/+	1.0(16)	0.879	0.679	1.138	+0.039
	-/+	1.2(31)	0.913	0.713	1.170	0.000
	-/-	79.8(1274)	Ref			
Anti-hypertensive	Baseline	20.8(315)	0.908	0.707	1.165	
	+/-	11.9(181)	0.937	0.731	1.201	-0.007
	+/+	31.6(478)	0.937	0.731	1.201	-0.003
	-/+	11.9(181)	0.953	0.741	1.225	+0.002
	-/-	81.5(1231)	Ref			
Anti-depressants	Baseline	5.25(84)	0.920	0.719	1.178	
	+/-	1.56(25)	0.915	0.715	1.171	+0.003
	+/+	1.62(26)	0.918	0.717	1.176	0.000
	-/+	3.63(58)	0.914	0.714	1.170	+0.002
	-/-	96.9(1488)	Ref			

Data presented are OR (95% CI) from binomial regression of incident T2D. Medication usage assessed through the Pharmaceutical Benefits Scheme linkage. Data include those men who were found to take selected medications up to 6 months prior to initial visit (baseline), had ceased taking selected medications between baseline and follow-up (+/-), those who had commenced taking selected medications after baseline visit and up to 6 months prior to follow-up visit (-/+), those who had been taking selected medications after baseline visit and up to 6 months prior to follow-up visit (+/+) and those who were found to have not used the selected medications (-/-) exposure referent category. Statistically significant associations ($p < 0.05$) are shown in bold.

7.5. Discussion

In this prospective cohort study of community-dwelling, middle-aged to elderly men, serum levels of SHBG, TT and DHT were inversely associated with incident T2D, independent of established diabetes risk factors including age, WC, fasting plasma glucose, serum triglycerides, parental history of diabetes, current smoking status, symptomatic depression and physical inactivity. However, after mutual adjustment for SHBG and TT and, separately, SHBG and DHT, there was no significant association between SHBG, DHT, and incident T2D, whereas TT remained inversely associated with risk of incident T2D.

In contrast to the consistently demonstrated effect of low TT on T2D development in men [478, 480, 483], studies relating to the effect of SHBG have produced more variable outcomes.

Of the comparatively few studies that have examined the influence of SHBG on T2D development [177, 184-186, 304, 317, 344, 348, 349, 353, 356], most of them have not accounted for the concurrent effects of visceral adiposity [177, 184-186, 304, 344, 348, 349, 353] and TT [185, 304, 317, 349, 356]. Our data that demonstrates the association between SHBG and T2D is attenuated after adjustment for TT, by most of the available prospective [177, 185, 304, 317, 346, 348, 349, 356], cross-sectional [351, 484], and case-control [176] studies. However, these previous studies had some acknowledged design limitations. For instance, neither the San Antonio Heart [356] or the Hawaii-Los Angeles-Hiroshima [317] studies accounted for the effect serum testosterone, although this would be expected to bias results towards a positive, not a negative association. Similarly, the Finnish (Lieto) longitudinal study [348] had sex hormones, and SHBG levels quantified by immunoassay and had a very small number of incident T2D cases (n=30). To our knowledge, the present study has the highest number of incident T2D in examining the association between SHBG and T2D, in addition to a

comprehensive panel of sex hormones measured by the use of the gold standard LC-MS/MS. Recently available data from the DPP (3 years of follow-up), which included the use of LC-MS/MS to measure sex steroids, showed no association between SHBG and incident T2D [304]. However, entry criteria for this study included men known to be at high risk of diabetes. Hence the relationships observed with SHBG may have been confounded. Likewise, the CHS also utilised LC-MS/MS assays and reported that SHBG was not associated with an increased risk of diabetes development [177]. However, they included the elderly (≥ 65 years) men only, lacked a standardised blood sampling time; again likely limiting the interpretation of the observed association between SHBG and T2D. The observed associations in the present study appeared linear across the observed range of SHBG quartile levels. A minority of prospective studies have demonstrated an independent association between SHBG with T2D development in men [184, 186, 344, 349, 353]. The discrepancies between the majority of findings from other longitudinal studies, including our own, is likely related to differences in the definition [175, 184, 186, 347, 350, 353, 354], and/or low number of cases [184, 350], of incident diabetes; the populations studied [344, 354, 355]; lack of standardized blood sampling time [177, 185]; long-term storage of samples [184, 354]; failure to adjust for confounders such as sex steroids levels [185, 304, 317, 349, 356]; visceral adiposity [177, 184-186, 304, 344, 348, 349, 353] and serum TT not measured by LC-MS/MS [184, 186, 344, 349, 353-355].

Our data are supported by two Mendelian randomisation analyses where there was no evidence of a causal role of SHBG on the risk for T2D [283, 304] after accounting for the effect of testosterone. Two other Mendelian randomisation studies that did find an apparent direct effect of SHBG on incident diabetes had not accounted for the effect of testosterone [168, 238].

Our results are consistent with recent data from the DPP study, which showed no association between DHT (measured by LC-MS/MS) and incident T2D [304]. Although the CHS [177] found that higher levels of DHT (measured by LC-MS/MS) are associated with incident T2D, only the elderly (≥ 65 years) men were included.

Our data confirm the null association between E2 and incident T2D observed in the previous prospective [184, 485] and cross-sectional [351] studies. The CHS lacked standard data required for the identification of cases of incident T2D [184] while LC-MS/MS was not used to measure E2 in the Rancho Bernardo Study [485]. In contrast, earlier prospective [185, 304, 346] and cross-sectional [320, 350] studies showed a positive association between E2 and incident T2D in men, though the DPP study population was already at risk of T2D [304] while serum E2 was not measured by LC-MS/MS in both the Tromsø study [185] and the Environment, Inflammation and Metabolic Diseases Study (EIMDS) [346].

Our data demonstrate that the most likely mechanism by which changes in serum SHBG are linked to T2D development is via incidental changes to serum testosterone, and there are plausible mechanisms by which testosterone may favourably modify glucose metabolism [288]. Biochemical evidence indicates that testosterone controls the expression of key regulatory enzymes involved in glucose and lipid metabolism in major insulin-responsive target tissues, such as liver, adipose tissue and skeletal muscle [288]. There are no such data for SHBG, where the weight of evidence is that SHBG is a marker of the metabolic abnormalities associated with insulin resistance [28, 100, 138].

The strengths of the current study is the use of data from a large and well-characterized cohort of middle-aged to elderly men, adequately powered through a high number of incident cases of T2D, standardised time of sampling, accurate measurement of visceral adiposity (DXA), the use of LC-MS/MS where the samples from both time points were concurrently measured, and adequate adjustment for confounding variables

including testosterone and visceral adiposity. However, one of the key limitations of our study include the 20.5 % loss to follow-up, and a few baseline characteristics were found different comparing those lost to follow-up with all eligible participants though unlikely to influence our findings as these were mostly due to inevitable high mortality rate of an older male population, which accounted for nearly 27% of the loss in our cohort. Nevertheless, IPW was conducted with weights estimated from a logistic binomial regression model to correct possible selection bias due to loss to follow up whereas the follow-up rate was comparable as is commonly seen in prospective cohort studies of elderly participants [177, 185, 186]. The generalisability of our findings is limited due to the use of a predominantly Caucasian study population, representative of urban men only, and the use of a relatively shorter follow-up period when compared with comparable longitudinal studies [177, 184, 344, 353].

7.6. Conclusions

In summary, our data concord with the most recent epidemiological data demonstrating that low TT, not SHBG and other sex steroids, is independently associated with an increased risk of T2D development. There is, however, at this stage, no evidence from large-scale, randomised control studies that T-replacement therapy is beneficial for T2D in otherwise healthy men. This suggests that patients presenting with traditional T2D risk factors, in addition to low TT, should continue to be conservatively managed.

CHAPTER 8

8. Role of SHBG as a marker of CVD

Statement of Authorship

Title of Paper: Higher serum sex hormone-binding globulin (SHBG) levels are associated with incident cardiovascular disease (CVD) but not CVD related mortality in a cohort of community-dwelling middle-aged to elderly men

Statement of Authorship

Title of Paper	Higher serum sex hormone-binding globulin (SHBG) levels are associated with incident cardiovascular disease (CVD) but not CVD related mortality in a cohort of community-dwelling middle-aged to elderly men	
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style	
Publication Details		

Principal Author

Name of Principal Author (Candidate)	Prabin Gyawali		
Contribution to the Paper	Conceptualization, project design, analysis & interpretation of data, writing, review, and/or revision of the manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	06/11/2018

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Contribution to the Paper	Conceptualization, project design, data collection, acquisition of data, analysis and interpretation of data, writing, review, and/or revision of the manuscript, and final approval of manuscript.		
Signature		Date	19/12/2018

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Contribution to the Paper	Analysis and interpretation of data, writing, review, and final approval of the manuscript.		
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Name of Co-Author	Alicia J. Jenkins		
Contribution to the Paper	Project design, analysis and interpretation of data, assays/ laboratory analysis, writing, review, and final approval of the manuscript.		
Signature		Date	11/12/18

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Contribution to the Paper	Analysis and interpretation of data, assays/ laboratory analysis, writing, review, and final approval of the manuscript.		
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Signature		Date	24/10/2018

8.1. Abstract

Background: Sex hormone-binding globulin (SHBG) levels are associated with cardiovascular disease (CVD) risk factors. However, prospective data on the association between SHBG levels and CVD events are sparse, with conflicting results. We examined the associations between serum SHBG, sex steroids, and incident CVD and CVD-related mortality in middle-aged to elderly men. **Design and Methods:** Data on 2563 community-dwelling men (35-80 years) were obtained from participants in the Men Androgen Inflammation Lifestyle Environment and Stress (MAILES) cohort. The analytic sample included 1492 men without baseline (2002-2007) CVD and with fasted morning serum SHBG and total testosterone (TT) available at both baseline and follow-up (2007-2010), and without medications affecting TT or SHBG. Incident CVD cases were identified by self-report, occurrence of a CVD-related hospitalization, while CVD - related mortality was obtained by data linkage through June 2014. Associations of baseline SHBG and TT, with incident CVD and CVD - mortality, were analysed using logistic regression for incident CVD and Cox's proportional hazard regression for CVD mortality, adjusting for established CVD risk factors. Sensitivity analyses assessed the mediation effects of anxiety, sleep duration, shift work ≥ 3 years, and obstructive sleep apnoea (OSA). **Results:** Over a 4.95 year median follow-up period, 101 (6.7%) men developed CVD. There were 56 (3.2%) CVD - related deaths recorded by the census date. In multivariable models, elevated baseline SHBG and lower baseline TT were independently associated with incident CVD (OR=1.54 [1.15, 2.06] per SD increase in SHBG, $p=0.003$) and (OR =0.71 [0.52, 0.97] per SD decrease in TT, $p=0.03$), respectively. There was no association between E2 and incident CVD. A decrease in TT between time points was associated with incident CVD (OR=0.72 [0.56, 0.92], $P=0.02$). There was no association between change in SHBG and incident CVD. In age-subgroup analyses, incident CVD was higher in older men (≥ 65 years) with elevated SHBG levels (OR=1.71, [1.07, 2.76], $p=0.03$) or lower TT levels (OR=0.58, [0.35, 0.95], $p=0.03$). Neither SHBG nor TT were significantly associated with

all-age CVD mortality (HR=0.69 [0.29, 1.63], p=0.40 & HR=0.60 [0.28, 1.26], p=0.18, respectively). However, in age- subgroup analyses, CVD related mortality was higher in older men (≥ 65 years) with elevated SHBG levels (OR=1.72, [1.08, 2.71], p=0.02) or lower TT levels (OR=0.60, [0.37, 0.97], p=0.04). In the sensitivity analyses there was evidence for mediation effects of shift-work on the association of TT, but not SHBG with incident CVD (both baseline & change models). **Conclusions:** Among all men and men aged over 65, both elevated SHBG and lower TT were independently associated with both a greater risk of CVD and an increased CVD mortality risk. Shift-work mediates associations of TT with incident CVD.

8.2. Introduction

Sex hormone-binding globulin (SHBG) is a circulating homodimeric glycoprotein predominantly synthesized and secreted from the liver [14, 26]. The principle known role of SHBG is to bind and transport sex steroids in the circulation, which regulates their biological activity and metabolic clearance rate [26, 97]. Experimental and clinical studies have provided evidence that the synthesis and secretion of SHBG from the liver are regulated by hormonal, metabolic, and nutritional factors that are in turn reflected by alterations in circulating SHBG levels [29, 57, 168, 486, 487]. An emerging understanding of the complexity of SHBG synthesis and physiology has raised the possibility of its potential role in a suite of disorders, including cardiovascular disease (CVD) [57, 100].

Previous population-based studies have consistently demonstrated that low serum SHBG is associated with CVD risk factors (e.g. hypertension [187-189], dyslipidaemia [188-190, 323, 379], obesity [187, 189, 190, 323], inflammation (CRP) [188, 323], insulin resistance [168, 323] and dysglycemia [168, 187, 189, 190]). However, data on the relationship between SHBG and CVD events are still lacking. To date, there have only been four longitudinal studies that have examined the relationship between CVD events and SHBG, while accounting for the influence of other related sex steroids [191, 305, 381, 382]. A more recent examination of over 5000 elderly men followed for 6.2 years, as part of the Outcome Reduction with an Initial Glargine Intervention trial (ORIGIN) reported that SHBG, but not TT, is an independent predictor of CVD in men with impaired glucose metabolism at increased risk of CVD and without accounting for the role of total testosterone (TT) [382]. In the Osteoporotic Fractures in Men (MrOS) study (n=2416), using a composite endpoint of unstable angina, MI, revascularization, stroke, transient ischemic attack and LC-MS/MS assays for sex steroids, baseline SHBG levels were not found to be associated with incident CVD, in multi-adjusted models that included both SHBG and TT [305]. A recent analysis of data

from 1804 Australian men aged 17-97 years followed for 14.9 years men as part of the Busselton Health Study (BHS) was also unable to detect associations between SHBG levels and incident CVD without accounting for the role of testosterone [191]. Likewise, the Cardiovascular Health Study (CHS) study (n=1032) which examined men over an average of 9.8 years of follow-up, reported no association between SHBG levels and incident CVD [381]. Both MrOS and CHS studies, however, included older men (>65 years), with unstandardized blood sampling time. Furthermore, it is unclear whether there are any mediating factors for the associations between SHBG and TT with incident CVD.

Low SHBG levels have also been suggested as a marker for increased CVD mortality risk. Again, however conflicting results are available; of the seven longitudinal studies to date, two have indicated an inverse association [190, 392], while five were unable to detect associations [385, 392-395] and one indicated a positive association [396], between SHBG levels and CVD mortality. Moreover, to what extent serum SHBG translate into real hard endpoint remains controversial.

Therefore, we aim to comprehensively evaluate the prospective associations between SHBG, TT, and incident CVD and risk of CVD mortality using data from a large, representative and well-characterized cohort of middle-aged to elderly men and using gold standard methods for measurements of sex steroids.

8.3. Research Design and Methods

8.3.1. Study Design and Participants

The study population is based on the Men Androgen Inflammation Lifestyle Environment and Stress (MAILES) study, a prospective cohort of men aged 35 years and older, pooled from two existing population-based studies using identical sampling methods in the Northern and Western suburbs of Adelaide, Australia: The Florey Adelaide Male Ageing Study (FAMAS) and North West Adelaide Health Study (NWAHS). The description of the study design and recruitment processes have been described in detail previously [470, 481]. Briefly, the FAMAS includes 1195 randomly-selected men, aged 35-80 years at recruitment who attended baseline clinic visits in 2002-2005 and follow-up clinic visits in 2007-2010. The NWAHS includes men and women aged 18-years at recruitment in 1999-2000, who attended three clinic waves (n=2336). For the MAILES study, all FAMAS men and NWAHS age-matched (35-80 years at stage 2) men were included, yielding a final sample size of 2563 men at MAILES stage 1 (i.e., FAMAS baseline and NWAHS stage 2) and 2038 men at MAILES stage 2 (FAMAS 5-year follow-up and NWAHS stage 3). The median follow-up period for the MAILES study is 4.94 years (interquartile range 4.34-5.00 years).

8.3.2. Population under analysis

The present study used data from the second wave (MAILES 2) and the baseline examinations of the first wave (MAILES 1). For the current analyses, participants with a missing data on CVD (n=10), a prevalent history of CVD (n=299), receiving either testosterone supplementation or anti-androgen (Leuprorelin, Goserelin, Bicalutamide) therapy (n=5) were excluded and the final cohort comprised of 1492 subjects with non-missing information on serum SHBG and sex steroid levels (total testosterone (TT), dihydrotestosterone (DHT) and oestradiol (E2), several CVD risk markers, and cardiovascular outcomes. A flow chart of participants eligible for the analysis is provided in Figure 44. The research ethics committees of the Royal Adelaide Hospital and the

North Western Adelaide Health Service approved the MAILES study and was conducted by the Declaration of Helsinki. A written informed consent form was provided by each eligible participants who signed in-clinic at each study Visit.

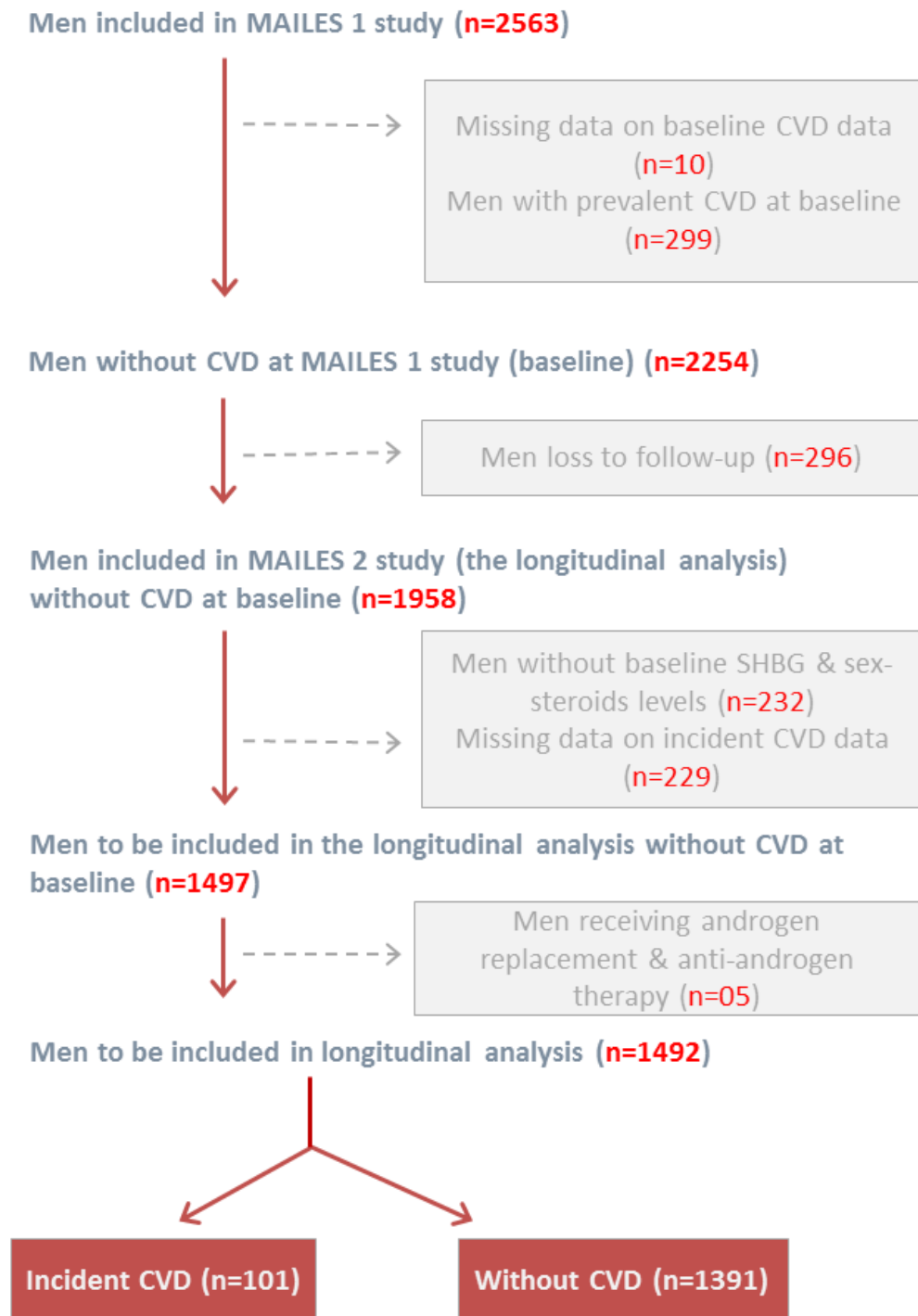


Figure 44. Schematic representation final analytical samples for the current analyses.

Abbreviations: MAILES, Men Androgen Inflammation Lifestyle Environment and Stress; CVD, Cardiovascular disease; SHBG, Sex hormone-binding globulin.

The antiandrogen therapy included were Leuprorelin, Goserelin and Bicalutamide.

8.3.3. Sex hormone-binding globulin (SHBG) and other biochemical analyses

Full details of the laboratory methods and quality control data have been reported previously [470, 481]. Briefly, venous blood samples were drawn between 8:00 AM and 11:00 AM after a 12-hour overnight fast and 20 min in a sitting position. Samples were immediately placed on ice and transported to a laboratory certified by the National Association of Testing Authorities (NATA) within 4-hours, then centrifuged, fractionated, and serum stored at -80 °C until measurement (between 1 - 13 months after collection). Samples were randomly ordered for assay, and the laboratory technicians were blinded to participant characteristics. Blood samples from the two cohorts were analysed in the same laboratory, and samples from the two-time points were analysed concurrently. Serum SHBG levels were measured by diluting serum to 1:21 through SHBG sample diluent, and then assayed using the Immulite Autoanalyser and a solid-phase, two-site chemiluminescent immunoassay (Siemens Medical Solutions, New York, USA; inter-assay CV: 4.0% at 32.3 nmol/L; lower detection limit: 0.17 nmol/L). Serum sex steroids [TT, DHT and E2] were measured by a validated stable-isotope dilution liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (API-5000, Applied Biosystems/MDS SCIEX, Ontario, Canada). The inter-assay coefficients of variation (CV) were as follows: 10.1% at 0.43 nmol/L, 11.1% at 1.66 nmol/L, and 4% at 8.17 nmol/L for TT; 13.2% at 0.43 nmol/L, 8.0% at 1.68 nmol/L, and 7.4% at 8.37 nmol/L for DHT and 9.2% at 22 pmol/L, 2.2% at 83 pmol/L, and 6.1% at 411 pmol/L for E2. HbA1c was measured by high-pressure liquid chromatography using a spherical cation exchange gel (Bio-Rad Laboratories, California, USA). Plasma glucose and serum lipids were measured using an automated chemistry analyser (Olympus AU5400; Olympus Corp, Tokyo, Japan). Plasma high-sensitivity C-reactive protein (hs-CRP) was quantitated with an enzyme-linked immunosorbent assay (ELISA) using Cobas autoanalyser (Roche Diagnostics, New Jersey, US). The inter-assay coefficient of variation (CV) were <10.6 % for all

measurements. Concentrations of all selected analytes are stable after multiple freeze-thaw cycles [166].

8.3.4. Incident cardiovascular disease and CVD-related mortality outcomes

The primary outcome of this study was incident CVD, a composite endpoint of incident non-fatal CVD events, which was obtained via self-report of physician-diagnosed CVD (NWAHS heart attack, stroke, angina, or transient ischaemic attack; FAMAS angina, or other heart conditions) or the first episode of hospitalization by any cardiovascular cause (International Classification of Diseases, 10th Revision (ICD-10) codes I00-I78). Information (on hospitalization) for incident CVD events was obtained from Integrated South Australian Activity Collection (SA ISSAC) or ED Data, registry of the Australian Institute of Health and Welfare (AIHW). The prevalent and incident CVD events were classified as angina pectoris (ICD-10 code 120), acute myocardial infarction (ICD-10 code 121), acute and subacute ischemic heart disease (ICD-10 code 125), myocarditis (ICD-10 code 140), cardiomyopathy (ICD-10 code 142), cardiac arrest (ICD-10 code 146), cardiac arrhythmias (ICD-10 code 147,148 &149), congestive heart failure (ICD-10 code 150) and other heart disease (ICD-10 code 151 & 152). Information on dates and cause of death was obtained from the National Death Index (NDI) by linkage using each patient's unique identification number. Participants were followed until death or through 30 June 2014. The secondary outcome was CVD-related mortality.

8.3.5. Baseline covariates for multi-adjusted analyses

Socio-demographic, behavioural and lifestyle characteristics

Information on socio-demographic characteristics (age, gender, education and marital status), behavioural & lifestyle factors (smoking status, leisure-time physical activity, and alcohol consumption), as well as medical history, including information about medical procedures, and medication use was obtained by a validated, self-report questionnaire and data linkage with the national medication registry as described previously [470].

Smoking behaviour was recorded as smokers (current and former smoker) and non-smokers. In this analysis, smoking categories were a current smoker and non-current smoker. The physical activity questions from the Australian National Health Surveys were used to classify participants as sedentary or having low, moderate, or high levels of physical activity [223, 470]. Alcohol use was determined at clinical interview and recorded in standard drinks consumed per day; none, low ≤ 2 , and high ≥ 3 .

Anthropometry

Waist circumference (WC) was measured to the nearest 0.1 cm using an inelastic tape maintained in a horizontal plane at the level of the narrowest part of the waist, and read from the mid-axillary line, with the subject standing comfortably with weight distributed evenly on both feet. Low, moderate and high risk of abdominal adiposity were defined as WC (cm) ≤ 94.99 , 95.0-99.9 & ≥ 100.0 , respectively. Height to the nearest 0.1 cm and weight to the nearest 0.1 kg were measured with subjects unshod and in light clothing. Body mass index (BMI) was calculated using weight (kg) divided by height (m) squared. Normal, overweight and obesity were defined as BMI 18.5-24.99, ≥ 25 or ≥ 30 kg/m², respectively.

Chronic conditions

Hypertension was defined as systolic blood pressure ≥ 140 mm Hg and diastolic blood pressure ≥ 90 mm Hg, or use of antihypertensive medication. Medication use was obtained from Medicare Australia by confidential unit record linkage, classified according to the Anatomical Therapeutic Chemical (ATC) Classification. The number of distinct medication classes (at the ATC third level) 6 months before baseline or follow-up clinical examination were treated as covariates. Family history of heart disease was obtained by a validated, self-report questionnaire. Depressive symptoms were assessed using the Centre for Epidemiological Studies-Depression Scale (CES-D) (score ≥ 16) in NWAHS and the Beck Depression Inventory (BDI) (score ≥ 10) in FAMAS at both time points. The CES-

D and BDI are comparable in terms of the validity and details of these have been published previously [223].

8.3.6. Statistical Analysis

Initial descriptive analyses of selected independents and outcome measures were conducted using χ^2 (categorical), and *t*-tests (continuous) and are reported as means $\pm$ standard deviation (SD) and percentages, as appropriate. Normality and linearity assumptions were examined for all covariates. To assess the associations between baseline circulating SHBG (and sex steroids) levels and incident CVD, we implemented unadjusted, then age-adjusted, age + TT + SHBG, and multi-adjusted logistic binomial regression models. For multi-adjusted regression models, selected covariates with an age + TT + SHBG, adjusted association with the outcome variable of $p \leq 0.20$, were entered into a final multi-adjusted model of incident CVD. Effect modification between covariates and study factors were also examined in base multivariable models, and both main and interaction terms were included in final models where appropriate. Sub-group analyses by age group were also performed to examine for differential associations in younger (<65 years) versus older (≥ 65 years) men. Additional logistic regression models were used to assess the association between SHBG and sex steroids and coronary heart disease (CHD) events (defined as the first occurrence of angina pectoris, non-fatal myocardial infarction and non-fatal ischemic heart disease) while rest of the events fall to non-CHD events. Finally, we conducted additional sensitivity analyses to assess the effect of medication usage on the observed regression coefficients for incident CVD men in multi-adjusted logistic binomial regression models and incorporated inverse probability weighting (IPW) to correct for possible selection bias due to loss to follow up.

Associations between serum SHBG, TT and CVD-related mortality were examined using Cox proportional hazards regression models using similar staging. Effects were

presented as odds ratios (ORs) and hazard ratios (HRs) together with their 95% confidence interval (95% CI).

Multi-adjusted quartile regression models were additionally performed to examine non-linear associations between SHBG (and sex steroids) and incident CVD. Statistical analyses were performed with the IBM SPSS statistical package (version 23.0 New York, USA). All statistical tests were two-sided, and $p < 0.05$ was considered statistically significant.

8.4. Results

8.4.1. Participant characteristics

Of 1492 men included in the analysis, a total of 101 (6.7%) men developed incident CVD over a median follow-up of 4.9 years. At baseline, men who developed incident CVD were older, have type 2 diabetes, hypertensive, have higher WC, LDL-Cholesterol, HbA1c, SHBG, hs-CRP levels, and have lower TT levels and physical activity (Table 28).

Table 28. Characteristics of men at the MAILES 1 (baseline) followed for incident CVD through MAILES 2.

Predictors	Overall sample (n=1492)	No CVD (n=1391)	CVD (n=101)	p-value†
Questionnaire				
Age(Years)	54.2±11.1	53.6±10.9	62.0±10.5	<0.001
Marital status				0.04
Married / Partnered	1180(78.8)	1100(79.1)	80(79.2)	
Separated / Divorced	184(12.3)	175(12.6)	09(8.9)	
Widowed	41(2.7)	34(2.4)	07(6.9)	
Never married	86(5.7)	81(5.8)	05(5.0)	
Education Level				0.48
Higher school or lower	426(28.5)	393(26.3)	33(32.7)	
Trade / Apprenticeship	469(31.3)	440(29.4)	29(28.7)	
Diploma / Certificate	374(25.0)	345(23.1)	29(28.7)	
Bachelor or higher	205(13.7)	196(13.1)	09(8.9)	
Smoking status (current), n (%)				0.92
Smoker	284(19.0)	265(19.0)	19(18.8)	
Non-smoker	1208(80.7)	1126(80.8)	82(81.2)	
Leisure time physical activity, n (%)^a				0.53
Sedentary	849(56.7)	789(59.7)	60(63.2)	
Low, moderate, high	415(27.7)	387(29.3)	28(29.5)	
Alcohol consumption				0.53
None/day	16(1.1)	15(1.1)	01(1.1)	
1-2 drinks/day	781(52.2)	724(54.7)	57(60.6)	
>3 drinks/day	621(41.5)	585(44.2)	36(38.3)	
Cholesterol-lowering medication, n (%)				0.002
Yes	36(2.4)	29(2.1)	07(6.9)	
No	1459(97.5)	1365(97.9)	94(93.1)	
Antihypertensive medication,%				<0.001
Yes	321(21.4)	285(20.5)	36(35.6)	
No	1171(78.2)	1106(79.5)	65(64.4)	
Hypertension, n (%)				<0.001
Yes	682(45.6)	619(44.4)	63(62.4)	

No	812(54.2)	774(55.6)	38(37.6)	
Family history of heart disease, n (%)				0.37
Yes	699(46.7)	734(52.8)	58(57.4)	
No	792(52.9)	656(47.2)	43(42.6)	
Diabetes, n (%)				0.002
Yes	159(10.6)	139(10.0)	20(19.8)	
No	1338(89.4)	1257(90.0)	81(80.2)	
Diabetic medication, n (%)				0.09
Yes	18(1.2)	15(1.1)	03(3.0)	
No	1475(98.5)	1377(98.9)	98(97.0)	
Depression, n (%)				0.34
Yes	194(13.0)	178(13.0)	16(16.3)	
No	1275(85.2)	1193(87.0)	82(83.7)	
Physical measurements				
WC (cm)	100.2±11.8	99.9±11.7	104.9±12.0	<0.001
BMI (kg/m ²)	28.3±4.4	28.3±4.4	28.8±4.4	0.21
Change in weight (Δ Weight), Kg	0.68±5.95	0.82±5.56	-0.61±8.81	<0.001
Systolic blood pressure (mmHg)	133.7±17.6	133.1±17.2	142.9±19.8	<0.001
Diastolic blood pressure (mmHg)	85.1±9.2	85.0±9.1	86.4±10.4	0.13
Lipid markers				
Total cholesterol (mmol/L)	5.5±1.04	5.53±1.04	5.38±1.14	0.15
LDL-C(mmol/L)	3.5±0.87	3.52±0.87	3.33±0.92	0.04
HDL-C(mmol/L)	1.2±0.31	1.25±0.31	1.23±0.30	0.50
Triglycerides (mmol/L)	1.76±1.38	1.75±1.39	1.77±1.27	0.90
Metabolic and inflammatory markers				
Glucose (mmol/L)	5.0±1.19	5.01±1.18	5.21±1.33	0.10
Glycosylated haemoglobin (%)	5.7±0.75	5.7±0.74	6.0±0.88	<0.001
hS-CRP ^b	2.56±4.81	2.5±4.5	3.9±7.8	0.004
Sex steroids and SHBG				
TT (nmol/L)	17.2±6.0	17.3±6.0	15.9±5.9	0.02
DHT (nmol/L) ^c	1.73±0.75	1.73±0.75	1.75±0.74	0.79
E2 (pmol/L) ^d	99.2±41.7	99.7±42.5	92.4±28.4	0.18
SHBG (nmol/L)	32.9±14.3	32.6±13.8	38.1±18.5	<0.001

Prevalence & incidence of of the outcome			
Prevalent Composite CVD, <i>n</i> (%)	299 (11.7)	-	-
Incident Composite CVD, <i>n</i> (%)	-	101 (6.7)	-
All-cause mortality (through June 2014)	115 (6.7)	-	-
CVD related mortality (through June 2014)	56 (3.2)	-	-

Values are reported as number (percentage) for categorical variables and are reported as means $\pm$ standard deviation (SD) for continuous variables.

Abbreviations: BMI, Body mass index; CVD, Cardiovascular disease; DHT, Dihydrotestosterone; E2, Oestradiol; HbA1c, Glycosylated haemoglobin; hS-CRP, High sensitivity C-reactive protein; LDL, Low density lipoprotein; HDL, High density lipoprotein; TT, Total testosterone; SHBG, Sex hormone-binding globulin; WC, Waist circumference

^a *n*=1264 ^b *n*=1442 ^c *n*=1388 ^d *n*=893.

† indicates the *p*-value for the overall difference between men with and without CVD at follow-up based on χ^2 for categorical variables and *t*-test for continuous variables.

8.4.2. Baseline SHBG (and Sex steroids) and incident CVD

Table 29 and Table 30 summarises the regression estimates for incident CVD predicted by baseline SHBG, TT, and other factors. In unadjusted regression models, each 1-SD increase in baseline SHBG levels was associated with a greater risk of incident CVD (OR 1.42 [CI 1.18,1.70]) whereas each 1-SD increase in baseline TT levels was associated with a decreased risk of incident CVD (OR 0.76 [CI 0.61, 0.96]). The positive association observed for SHBG was attenuated after adjustment for age (OR 1.14 [CI 0.92, 1.41]) while the association remained unaltered for TT (OR 0.79 [CI 0.63, 1.00]). In multi-adjusted analyses, increasing SHBG (OR 1.54 [CI 1.15, 2.06]) was independently associated with greater risk of incident CVD. In separate multi-adjusted analyses, there was a strong, inverse association between TT and incident CVD (OR 0.71 [CI 0.52, 0.97]) in this model. Other baseline covariates that demonstrated an independent association with incident CVD were age (OR 1.96 [CI 1.43, 2.68]), waist circumference (OR 1.45 [CI 1.05, 2.01]), and high sensitivity-CRP levels (OR 1.16 [CI 1.01, 1.34]).

Table 29. Logistic regression models for incident CVD predicted by baseline SHBG and other factors among community-dwelling men.

Predictors	Unadjusted		Adjusted					
	OR (95%CI)	p-value	+Age		+Age + SHBG +TT		Full multi-adjusted model	
			OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value
SHBG (nmol/L)	1.42(1.18,1.70)	<0.001	1.14(0.92,1.41)	0.22	-	-	1.54(1.15,2.06)	0.003
Age , Years	2.32(1.84,2.94)	<0.001	-	-	-	-	1.96(1.43,2.68)	<0.001
Marital status (Married or partnered/Never married)	1.04(0.81,1.33)	0.74	1.09(0.84,1.43)	0.50	1.06(0.81,1.39)	0.66	-	-
Education level (Low or above high school)	0.91(0.76,1.08)	0.28	0.98(0.82,1.17)	0.82	0.99(0.83,1.17)	0.88	-	-
Smoking status (current; Yes/No)	0.98(0.59,1.65)	0.95	1.60(0.93,2.77)	0.09	1.59(0.91,2.78)	0.10	1.39(0.73,2.62)	0.31
Alcohol consumption (None, 1-2/day & ≥3/day)	0.80(0.53,1.21)	0.22	0.92(0.60,1.41)	0.71	0.96(0.62,1.47)	0.84	-	-
Physical activity (Sedentary, low-,moderate- & high- exercise level)	0.82(0.66,1.02)	0.08	0.77(0.62,0.99)	0.04	0.79(0.63,1.00)	0.05	0.85(0.66,1.09)	0.21
Diabetes status (Yes/No)	2.23(1.33,3.75)	0.002	1.58(0.93,2.70)	0.09	1.52(0.88,2.63)	0.13	1.39(0.77,2.50)	0.27
WC (cm) (<95, 95-99.9 & ≥100)	1.64(1.27,2.12)	<0.001	1.50(1.16,1.94)	0.001	1.53(1.16,2.02)	0.002	1.45(1.05,2.01)	0.02
Δ Weight	0.96(0.93,0.99)	0.02	0.98(0.94,1.01)	0.21	0.98(0.94,1.01)	0.20	0.98(0.95,1.02)	0.30
HTN (≥140 systolic/≥90 diastolic mm Hg)	2.64(1.68,4.13)	<0.001	1.71(1.07,2.74)	0.03	1.64(1.02,2.64)	0.04	1.41(0.82,2.42)	0.21
Parental history of ischaemic heart disease	1.21(0.80,1.81)	0.37	1.28(0.84,1.94)	0.25	1.29(0.84,1.96)	0.24	-	-

Depression (Symptomatic)	1.31(0.75,2.29)	0.35	1.50(0.85,2.67)	0.16	1.51(0.85,2.69)	0.16	1.48(0.81,2.71)	0.21
Fasting Triglycerides (mmol/L)	1.01(0.83,1.24)	0.90	1.09(0.89,1.33)	0.23	1.09(0.88,1.35)	0.42	-	-
HDL- Cholesterol (mmol/L)	93(0.75,1.15)	0.50	0.85(0.69,1.05)	0.13	0.84(0.67,1.04)	0.11	0.92(0.72,1.17)	0.48
LDL- Cholesterol (mmol/L)	0.79(0.63,0.99)	0.04	0.88(0.69,1.11)	0.20	0.91(0.72,1.15)	0.44	-	-
hs-CRP (mg/mL)	1.17(1.03,1.33)	0.01	1.15(1.02,1.32)	0.03	1.14(0.99,1.30)	0.06	1.16(1.01,1.34)	0.04
TT (nmol/L)	-	-	-	-	-	-	0.71(0.52,0.97)	0.03

All values represent the standardised coefficients of regression analyses. Statistically significant associations ($P < 0.05$) are shown in bold. Nagelkerke

R^2 (Full multi-adjusted model for SHBG): 0.165.

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; Δ , Change between time points; hs-CRP, high sensitivity C-reactive protein; HDL, high density lipoprotein; HTN, hypertension; LDL, low density lipoprotein; OR, Odds Ratio; SHBG, sex hormone-binding globulin; TT, total testosterone; WC, waist circumference

Table 30. Logistic regression models for incident CVD predicted by baseline testosterone and other factors among community-dwelling men.

Predictors	Unadjusted		Adjusted					
	OR (95%CI)	p-value	+Age		+Age + SHBG +TT		Full multi-adjusted model	
			OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value
TT (nmol/L)	0.76(0.61,0.96)	0.02	0.79(0.63,1.00)	0.05	-	-	0.71(0.52,0.97)	0.03
Age, Years	2.32(1.84,2.94)	<0.001	-	-	-	-	1.96(1.43,2.68)	<0.001
Marital status (Married or partnered/Never married)	1.04(0.81,1.33)	0.74	1.09(0.84,1.43)	0.50	1.06(0.81,1.39)	0.66	-	-
Education level (Low or above high school)	0.91(0.76,1.08)	0.28	0.98(0.82,1.17)	0.82	0.99(0.83,1.17)	0.88	-	-
Smoking status (current; Yes/No)	0.98(0.59,1.65)	0.95	1.60(0.93,2.77)	0.09	1.59(0.91,2.78)	0.10	1.39(0.73,2.62)	0.31
Alcohol consumption (None, 1-2/day & ≥3/day)	0.80(0.53,1.21)	0.22	0.92(0.60,1.41)	0.71	0.96(0.62,1.47)	0.84	-	-
Physical activity (Sedentary, low-, moderate- & high- exercise level)	0.82(0.66,1.02)	0.08	0.77(0.62,0.99)	0.04	0.79(0.63,1.00)	0.05	0.85(0.66,1.09)	0.21
Diabetes (Yes/No)	2.23(1.33,3.75)	0.002	1.58(0.93,2.70)	0.09	1.52(0.88,2.63)	0.13	1.39(0.77,2.50)	0.27
WC (cm) (<95, 95-99.9 & ≥100)	1.64(1.27,2.12)	<0.001	1.50(1.16,1.94)	0.001	1.53(1.16,2.02)	0.002	1.45(1.05,2.01)	0.02
Δ Weight	0.96(0.93,0.99)	0.02	0.98(0.94,1.01)	0.21	0.98(0.94,1.01)	0.20	0.98(0.95,1.02)	0.30
HTN (≥140 systolic/≥90 diastolic mmHg)	2.64(1.68,4.13)	<0.001	1.71(1.07,2.74)	0.03	1.64(1.02,2.64)	0.04	1.41(0.82,2.42)	0.21
Parental history of ischaemic heart disease	1.21(0.80,1.81)	0.37	1.28(0.84,1.94)	0.25	1.29(0.84,1.96)	0.24	-	-

Depression(Symptomatic)	1.31(0.75,2.29)	0.35	1.50(0.85,2.67)	0.16	1.51(0.85,2.69)	0.16	1.48(0.81,2.71)	0.21
Fasting Triglycerides (mmol/L)	1.01(0.83,1.24)	0.90	1.09(0.89,1.33)	0.23	1.09(0.88,1.35)	0.42	-	-
HDL-C (mmol/L)	93(0.75,1.15)	0.50	0.85(0.69,1.05)	0.13	0.84(0.67,1.04)	0.11	0.92(0.72,1.17)	0.48
LDL-C (mmol/L)	0.79(0.63,0.99)	0.04	0.88(0.69,1.11)	0.20	0.91(0.72,1.15)	0.44	-	-
hs-CRP (mg/mL)	1.17(1.03,1.33)	0.01	1.15(1.02,1.32)	0.03	1.14(0.99,1.30)	0.06	1.16(1.01,1.34)	0.04
SHBG (nmol/L)	-	-	-	-	-	-	1.54(1.15,2.06)	0.003

All values represent the standardised coefficients of regression analyses. Statistically significant associations (P <0.05) are shown in bold. Nagelkerke

R² (Full multi-adjusted model for TT): 0.164.

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; Δ, Change between time points; hs-CRP, high sensitivity C-reactive protein; HDL, high density lipoprotein; HTN, hypertension; LDL, low density lipoprotein; OR, Odds Ratio; SHBG, sex hormone-binding globulin; TT, total testosterone; WC, waist circumference

8.4.3. Quartile Analysis

For SHBG, the odds ratio (OR) derived from the logistic regression models were similar for the three higher quartiles compared with the lower quartile (reference category), our findings demonstrate a linear relationship for the risk of future CVD. No such relationships were observed for TT, DHT or E2 (Figure 45).

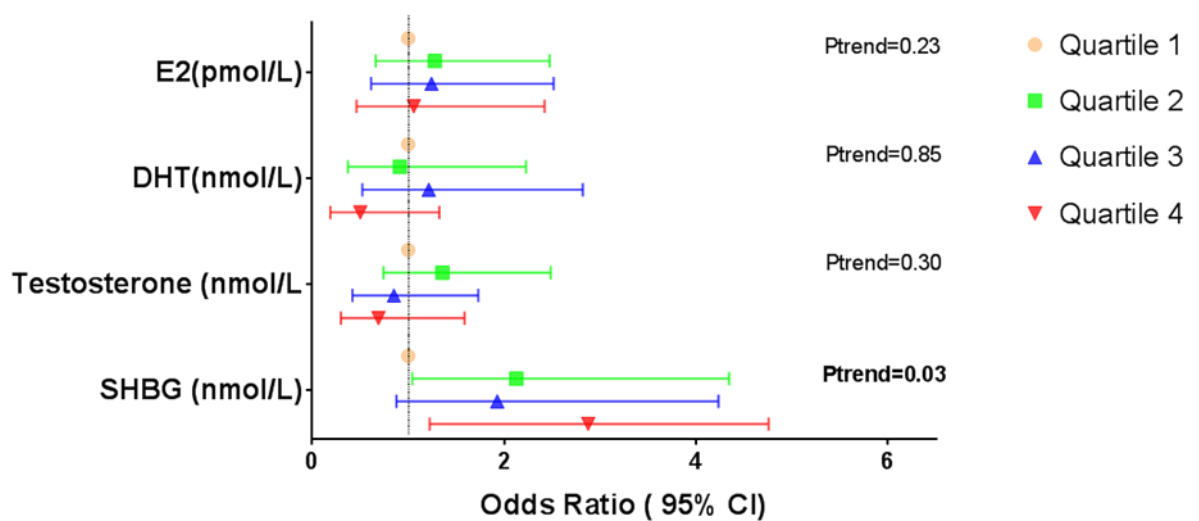


Figure 45. Multi-adjusted Odds ratio (95% CI) for incident CVD according to quartiles of SHBG and sex steroids (TT, DHT & E2) in 1492 middle-aged to elderly men in the MAILES study.

Abbreviations: CVD, cardiovascular disease; Δ , Change between time points; DHT, dihydrotestosterone; E2, oestradiol; hs-CRP, high sensitivity C-reactive protein; OR, Odds Ratio; SHBG, sex hormone-binding globulin; TT, total testosterone.

The Multi - adjusted model consisted of baseline age, current smoking status, physical activity, diabetes status, waist circumference, Δ weight, hypertension including current medication, symptomatic depression, HDL-cholesterol and high sensitivity-CRP with SHBG in combination with TT, DHT and E2, separately.

The dashed line represents the reference line, where OR = 1.00.

8.4.4. Associations between SHBG, TT and risk of CVD-related mortality

For CVD mortality, the median follow-up time was 12 years (2002 examinations through June 2014). There were 115 all-cause deaths (6.7%), 56 of which (48.6%) were attributed to CVD. When we adjusted for cardiovascular risk factors and other confounders, we found no significant associations between serum levels of SHBG and TT and CVD mortality across any models (Table 31).

Table 31. Hazard ratios and 95% CIs for the associations of SHBG and sex steroids with CVD related mortality in community-dwelling men.

Predictors	Unadjusted		Adjusted					
			+Age		+Age + SHBG + TT		Full multi-adjusted model*	
	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
SHBG (nmol/L)	0.83(0.62,1.11)	0.21	0.81(0.60,1.10)	0.16	0.84(0.60,1.17)	0.31	0.69(0.29,1.63)	0.40
Testosterone (nmol/L)	0.83(0.61,1.12)	0.22	0.85(0.62,1.15)	0.28	0.92(0.64,1.33)	0.65	0.60(0.28,1.26)	0.18

*Full multi-adjusted model included age, education level, parental history of ischemic heart disease, Δ weight, symptomatic depression, high sensitivity-CRP, SHBG and TT.

Abbreviation: CVD, cardiovascular disease; Δ , Change between time points; hs-CRP, high sensitivity C-reactive protein; HR, Hazard Ratio; SHBG, sex hormone-binding globulin; TT, total testosterone

8.4.5. Age-stratified analyses

There were significant interactions between age and SHBG levels on risk of incident CVD in our analytical sample therefore age specific subgroup analysis was done. In age subgroup analyses, the associations between SHBG (sex steroids) and incident CVD were generally consistent to our primary analysis in older men (≥ 65 years of age) whereas it was null in younger men (< 65 years of age) in multi-adjusted analyses (Figure 47).

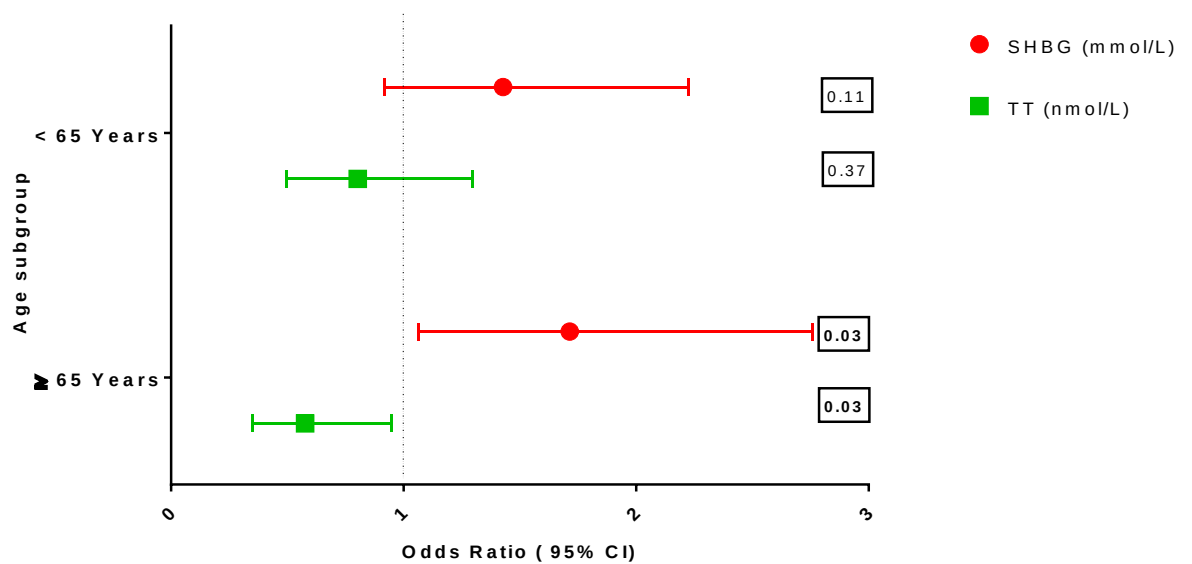


Figure 46. The odds ratio for an association between SHBG (and sex steroids) levels and incident CVD by age subcategory (<65 years and ≥65 years) in the multi-adjusted model.

Abbreviations: CVD, cardiovascular disease; Δ , Change between time points; HDL, high density lipoprotein; hs-CRP, high sensitivity C-reactive protein; OR, Odds Ratio; SHBG, sex hormone-binding globulin; TT, total testosterone

The Multi - adjusted model consisted of baseline age, current smoking status, physical activity, diabetes status, waist circumference, Δ weight, hypertension including current medication, symptomatic depression, HDL-cholesterol, high sensitivity-CRP, SHBG and TT. Dashed line represents the reference line, where OR = 1.00.

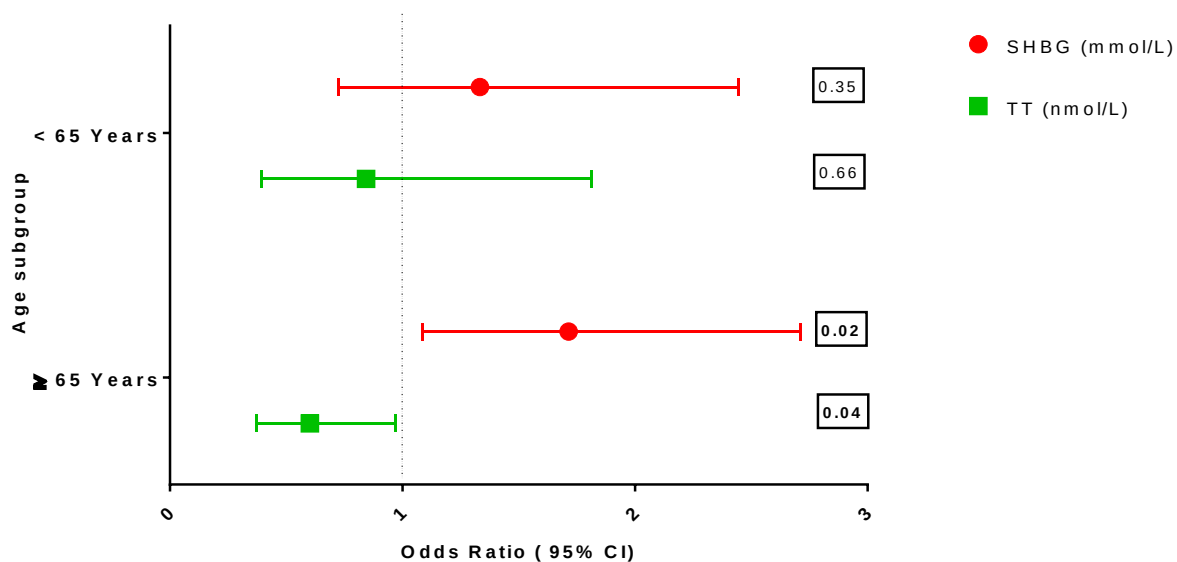


Figure 47. The hazard ratio for an association between SHBG (and sex steroids) levels and CVD related mortality by age subcategory (<65 years and ≥65 years) in the multi-adjusted model.

Abbreviations: CVD, cardiovascular disease; Δ , Change between time points; HDL, high density lipoprotein; hs-CRP, high sensitivity C-reactive protein; HR, Hazard Ratio; SHBG, sex hormone-binding globulin; TT, total testosterone

Multi - adjusted model consisted of baseline age, education level, parental history of ischemic heart disease, Δ weight, symptomatic depression, hs-CRP, SHBG and TT.

Dashed line represents the reference line, where HR = 1.00.

8.4.6. CHD and non-CHD category

Similarly, we found a stronger association of SHBG and TT levels for the outcome limited to CHD than for the overall CVD composite outcome in the comparison to non-CHD categories after multi-adjustment, the OR for incident CHD vs non-CHD was (OR =1.69 [1.20, 2.37] for SHBG and (OR =0.61 [0.43, 0.87]) for TT.

8.4.7. Sensitivity analysis

We performed several sensitivity analyses in our model for incident CVD. The significant association between increasing SHBG concentration expressed as increasing SD or as quartiles were not substantially modified by medications use (Appendix 6). We also repeated the analyses after substituting diabetes status with glycosylated haemoglobin (HbA1c). We found trends consistent with those in our main model, with a larger effect size for HbA1c (OR=1.19 [0.99, 1.42,] p=0.06). Finally, the significant association between increasing SHBG concentration expressed as increasing SD or as quartiles were not substantially modified by anxiety, sleep duration, OSA and shift work in our additional analysis, but trending in the same direction (Appendix 7).

All results were essentially similar when the inverse probability weighting analyses were performed in the regression model (Table 29 and Table 30).

8.5. Discussion

In this prospective cohort study of community-dwelling, middle-aged to elderly men without CVD at baseline, higher SHBG concentrations were associated with increased risk of incident CVD, after adjustment for established CVD risk factors and confounders. Lower TT was associated with increased risk of incident CVD. When we divided our sample into men aged below and above 65 years, the association between SHBG, TT and incident CVD held for men aged less than 65 years only. Neither SHBG nor TT was associated with risk of CVD mortality in all-age models of CVD mortality. In age-stratified analyses, the associations between SHBG, TT and CVD related mortality appeared to be significant in older men (≥ 65 years).

Similar to the present results, the Outcome Reduction with an Initial Glargine Intervention trial (ORIGIN) showed that SHBG, but not T, is an independent predictor of CVD in elderly men. The primary distinction between our study and ORIGIN study is the populations studied: men were aged 60 years, and 80% of men had previously established diabetes [382]. Our results differ from prior prospective studies that have evaluated the association between SHBG and incident CVD in elderly men [191, 305, 381, 382]. In the MrOS study (n=2416) using a composite endpoint of unstable angina, MI, revascularisation, stroke, transient ischemic attack and LC-MS/MS assays for sex steroids, SHBG was not found to be associated with incident CVD in multi-adjusted models that included both SHBG and TT [305]. In a longitudinal analysis of 1032 elderly men from the CHS, no association found between SHBG and incident CVD was reported [381]. The primary distinction between our study and other studies is the populations studied (elderly men only) [305, 381], and the lack of a standardised blood sampling time [305, 381]. However, a recent analysis of data from 1804 Australian men aged 17-97 years followed for 14.9 years men as part of the Busselton Health Study (BHS) also reported no association between SHBG and incident CVD [191]. However, the BHS didn't provide any age-stratified data, 68.9% of their population were aged <60 years, and

SHBG was not simultaneously adjusted for TT levels. Both CHS and BHS observed the same direction of an association as ours but non-significant. Therefore the finding of higher SHBG levels associated with greater risk of CVD, cannot be ruled out.

Moreover, in comparison with these studies, our study included several important confounders not previously included in any examination of SHBG and incident CVD (e.g. family history of CVD, visceral obesity, Δ weight, co-morbid conditions, inflammatory markers, and medications). Further, when our population was stratified by age, an inverse association was seen in men ≥ 65 years, but not in men < 65 years. This study highlights the necessity of age stratification in reporting the association between SHBG and the risk of developing CVD.

Consistent with the previous finding from the MrOS study (n=2416), we also observed an inverse association between TT and incident all-cause CVD [305]. However, we additionally show this association was partly confounded by SHBG. In contrast, the BHS [191], the CHS [381], the Framingham Heart Study [192, 488], the Atherosclerosis Risk in Communities study [489], and a meta-analysis of 19 prospective studies [490] found no association between serum TT levels and incident CVD. The discrepancies between our findings and other longitudinal studies could, in part, be related to differences in the study population [192, 381], LC-MS/MS not used to measure serum T levels [192], and failure to adjust for SHBG concentrations [192, 488, 489].

In contrast to the relationship between SHBG and incident CVD, there was no association between SHBG and risk of CVD mortality. This is consistent with the majority of prior prospective studies [190, 381, 392, 394, 398, 399]. However, these prior studies have design limitations, such as failure to adjust for TT [392, 394, 398], sex steroids not measured by LC-MS/MS [398, 399], and participants being older men. Two studies have observed an inverse association between SHBG and CVD mortality -Third National Health and Nutrition Examination Survey (NHANES III) Mortality Study [399] and the Health in Men Study (HIMS) [191]. Their findings are consistent with our age-

stratified analyses showing significant associations between SHBG, TT and CVD related mortality in older men (≥ 65 years). The NHANES III Mortality Study demonstrated that low SHBG is associated with an increased risk of CVD mortality at 18 years but not at nine years of follow-up [399], suggesting that SHBG may have some utility as a long-term prognostic marker of CVD in men. Whereas, HIMS [396] did not measure sex steroids by LC-MS/MS method and included only older men (70-88 years).

In addition to a consensus and documented inverse relationship between SHBG and CVD risk factors [187-190], it appears that increased concentrations of circulating SHBG are also independently associated with an increased risk of incident CVD among men. These findings suggest that SHBG has a putative harmful effect on cardiovascular health, which is insufficiently described and may have clinical relevance. As an observational study, a reasonable possibility is that our findings might largely result from residual confounding, reverse causation, or secondary to the presence of subclinical CVD. Another more relevant and promising explanation is that SHBG acts through modulation of factors associated with frailty, supported by findings of a favourable relationship with high serum SHBG levels and increased risk of frailty [491, 492]. Other mechanisms and pathways besides those related to CVD risk factors and confounders also cannot be ruled out. SHBG is an indirect measure of androgenicity [3], adding SHBG to T might more predictive information that was not captured by T alone and vice versa. Additionally, it has been shown that the serum SHBG levels are associated with single-nucleotide polymorphisms (SNPs) that SNPs retards the plasma clearance of SHBG. The resulting higher plasma SHBG levels in individuals who carry that SNPs have been positively associated with the risk of developing CVD [57].

Strengths of our study include the diagnosis of major cardiovascular events were defined as composite endpoint CVD from a well-characterised prospective cohort study of community-dwelling middle-aged to elderly men. The diagnosis of CVD outcome in the

current study was based on either self-report or a hospital data linkage system that strengthens the validity of our results, although biases cannot be ruled out due to non-rigorous evaluation. The use of LC-MS/MS method to measure sex steroids levels is an additional strength of our study. Also, the possibility of effect modifiers, such as medications used was possible as the use of several medications were systematically collected. In spite of the strengths of our study, a few limitations deserve mention. The major limitation of our study was the relatively small number of incident cases (n=101) and CVD related mortality (n=56), which may have limited power to detect a true association of our observed effects. However, our prospective outcomes thoroughly measure the number of CVD risk factors and reflect the general good health of our participants. Another limitation was, only 80% of participants were successfully followed-up, and further 10% accounted for a loss of a baseline SHBG and outcome variable data.

Nevertheless, the follow-up rate and participants in an analytical sample were comparable to other prospective studies conducted in a comparable population [191, 305, 382]. An additional limitation is that our study also had a relatively shorter follow-up time compared with other longitudinal studies that followed men over 9-15 years [191, 381] and as an observational study, our inability to fully discern the potential mechanisms. Finally, our participants were predominantly Caucasian, generalisation in another ethnic group should be made with caution.

8.6. Conclusions

In conclusion, increased serum SHBG and decreased TT concentrations at baseline are independently associated with an increased risk of incident CVD, but not with increased risk of CVD mortality, over a follow-up period in community-dwelling men initially free of CVD. More research is needed to replicate these findings with larger populations. The reason for the associations with SHBG and whether treatment with testosterone will abrogate the risk of CV needs to be determined.

9. Role of SHBG as a marker of Prostate cancer aggressiveness

Statement of Authorship

Title of Paper: Sex hormone-binding globulin (SHBG) as a marker of aggressive prostate cancer

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Title of Paper	Sex hormone-binding globulin (SHBG) as a marker of aggressive prostate cancer
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Principal Author

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Contribution to the Paper	Conceptualization, project design, assays/ laboratory analysis, analysis & interpretation of data, writing, review, and/or revision of the manuscript		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Signature		Date	21/01/2019

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Contribution to the Paper	Analysis and interpretation of data, writing, review, and/or revision of the manuscript, and final approval of manuscript.		
Signature		Date	21/1/19

Name of Co-Author	Gary A. Wittert		
Contribution to the Paper	Conceptualization, project design, acquisition of data, analysis and interpretation of data, writing, review, and/or revision & final approval of the manuscript.		
Signature		Date	21/1/19

9.1. Abstract

Background: Sex hormone-binding globulin (SHBG) is a dimeric glycoprotein synthesised in, and secreted from, hepatocytes. SHBG is also expressed in, but not secreted from, the prostate, where its role is unclear. The expression of SHBG is linked to lipid metabolism, and it also modulates transport and availability of androgen. Given its expression in the prostate and role in the androgen signalling axis, we postulated that expression of SHBG would increase in prostate cancer (PCa), distinguishing it from both normal prostate and benign prostate hyperplasia (BPH), and reflect the aggressiveness of the disease. **Methods:** We utilised tissues and data from existing South Australian PCa Registries. *SHBG* mRNA was measured by qRT-PCR (n=47: 7 BPH and 40 PCa of varying Gleason score (GS)). SHBG protein was measured by immunohistochemistry and quantified by Image J software (n=125: 8 normal, 32 BPH and 85 PCa of varying GS). The expression of *SHBG* transcript variants was analysed by qRT-PCR from an independent set of PCa samples of varying GS (n=12: 1 with GS ≤ 6 , 9 with GS ~ 7 and 2 with GS ≥ 8) that had been treated with the androgen receptor antagonist Enzalutamide (10 μ M MDV) or vehicle control. **Results:** *SHBG* mRNA and SHBG protein are barely detectable in the normal prostate epithelium or BPH but increase in PCa epithelial cells ($p < 0.001$; compared to both). SHBG protein concentration was highest in with GS ≥ 8 compared to GS ≤ 6 and ~ 7 ($p = 0.002$). PCa with GS ≤ 6 (n=1) and GS ≥ 8 (n=2) expressed unique coding transcripts of 1382bp and 1311bp respectively. However, within PCa of GS ~ 7 (n=9), there was marked heterogeneity of SHBG transcripts; 463 bp, 522 bp (non-coding), 526bp and 1146bp transcripts predominated, none expressed the 1311 bp and 1382 bp transcript. In response to 10 μ M MDV, PCa with GS ≤ 6 (n=1) did not express, 522 bp transcript predominated in GS ≥ 8 (n=2) while GS ≥ 7 (n=9) expressed unique coding transcripts of 1382bp and 973bp respectively, but 522 bp transcript predominated. **Conclusions:** SHBG is a marker of PCa and may be markers for the aggressive behaviour of PCa and resistance to androgen ablation.

9.2. Introduction

Prostate cancer (PCa) is the most commonly occurring non-cutaneous cancer worldwide and the fifth leading cause of cancer deaths in men [405]. Aggressive PCa, defined by the progression from localised disease to metastasis, is responsible for the majority of PCa-related death [493]. With widespread awareness and screening for PCa, many cancers are diagnosed at an early stage and prostatectomy affords a potential cure [494]. However, prostatectomy carries a substantial risk of permanent impotence and ongoing lower urinary tract symptoms [493, 495]. Over-treatment remains a major clinical problem because we do not have a reliable way, other than “watchful waiting”, to differentiate aggressive from indolent tumours of apparently low-grade PCa at diagnosis and other benign prostatic diseases. Although PCa progresses very slowly, differentiation of aggressive PCa from indolent disease improves personalised treatment [496], highlighting the urgent medical need for novel biomarkers that distinguishes aggressive PCa from indolent disease.

Sex hormone-binding globulin (SHBG) is a dimeric glycoprotein synthesised in and secreted from, hepatocytes [28, 29] that functions to transport testosterone and other sex steroids in the circulation. As such, SHBG is likely to be a critical determinant of androgen action in target tissues such as the prostate [3, 114]. SHBG is also expressed in, but not secreted from, the prostate [74, 75, 84], where its role is unclear. SHBG within the prostate epithelium is likely to modify local androgen action by acting as an androgen receptor (AR) coactivator, especially under androgen deprivation conditions [83, 114, 434]. SHBG is a sensitive marker of *de novo* lipogenesis in the liver [29, 89]. Because lipid turnover is increased in PCa and is associated with disease aggressiveness [452, 453], this raises the possibility that there is an uncoupling of the regulation of SHBG from lipogenesis in PCa and alternate transcriptional regulation of SHBG in aggressive PCa.

Expression of SHBG protein by immunohistochemistry and *SHBG* mRNA by *in situ* hybridisation and RT-PCR has been demonstrated in prostate glandular epithelial and stromal cells [74, 75, 435], where it has been shown to increase DHT-mediated transcriptional activity [83]. Prior studies on both *SHBG* mRNA and protein expression in PCa were examined in human prostate cancer cell lines (LNCaP, DU145, PC3, and BPH1) [22, 75, 146, 434], in prostate cancer explants [74, 83] and in cultured prostate stromal and epithelial cells derived from patients with BPH [75]. However, these cell lines are not representative of primary disease and are unlikely to evaluate clinicopathological associations of *SHBG* mRNA and protein in primary PCa specimens and its aggressiveness. Furthermore, a recent study provided further evidence that the SHBG protein content is positively associated with higher Gleason grade and highly infiltrating tumour (seminal vesicle invasion and lymph node metastasis) [146]. However, they did not analyse *SHBG* mRNA expression in their study and the SHBG protein content in PCa epithelial cells was compared only to another prostatic disease, BPH and not to normal. Moreover, SHBG appears to have a role in upregulating stemness of PCa cells upon DHT exposure *in vitro* [142, 146, 434]. Despite these lines of evidence linking prostate-specific SHBG expression to PCa cancer progression, many facets of this relationship remain largely uncharacterised. For example, the *SHBG* gene is composed of 13 different exons that generate at least six transcription units (TUs), only one of which (TU-1), expressed in hepatocytes, generates a secretory protein. Alternative *SHBG* transcripts in human prostate differentially downregulate SHBG translation [85, 86], but whether any of the SHBG TUs are specifically associated with PCa is unknown.

Given that previous work focus: only on SHBG protein content, chiefly on PCa cell lines and patient-derived explants, and clinicopathological association and aggressiveness relative to SHBG expression were not evaluated. We postulated that the expression of SHBG would increase in PCa, distinguishing it from normal and benign prostate and reflect the aggressiveness of disease using primary PCa specimens. Therefore, we aimed to determine the nature of the *SHBG* transcripts

expressed and mRNA expression, together with SHBG protein, in normal epithelium, benign and malignant prostate tissues.

9.3. Materials and Methods

9.3.1. Analysis of SHBG mRNA expression in published data sets

To obtain confirmatory data relating to SHBG mRNA expression in PCa, we downloaded publically available transcriptomic data from cBioPortal for Cancer Genomics on November 20, 2018 [497, 498]. The Memorial Sloan Kettering Cancer Center (MSKCC) dataset used in this study SHBG mRNA expression data from 177 primary PCa specimens. The Cancer Genome Atlas (TCGA) dataset used in this study comprised SHBG mRNA expression data from 499 radical prostatectomies (RP) tumour samples. Similarly, dataset generated by the Sun Lab 2017 with whole-genome and transcriptome sequencing of 65 prostate adenocarcinoma patients was also evaluated. Comprehensive profiling of 57 PCa samples generated by Levi Garraway's lab at the Broad Institute and Mark Rubin's lab at Cornell was also analysed.

9.3.2. Access to tissues and existing data

We utilised tissues and data from the South Australian Branch of the Australian Prostate Cancer BioResource (APCBio) and the South Australian Prostate Cancer Clinical Outcomes Collaboration (SA-PCCOC) Registry, Australia. Ethical approval for the use of human prostate tumours was obtained from the University of Adelaide Human Research Ethics Committee (HREC) (Adelaide, Australia). All experiments were performed by the guidelines of the National Health and Medical Research Council (Australia).

9.3.3. *Ex Vivo* culture of human prostate tumours

Fresh PCa specimens were obtained with written informed consent through the Australian Prostate Cancer BioResource from men undergoing robotic radical prostatectomy (RP) at the Royal Adelaide Hospital (Adelaide, Australia) and cultured for 48 h as previously described [499]. To assess the effect of AR signalling on SHBG, tissues were treated by adding the AR antagonist Enzalutamide (10 μ M MDV) to the media. Tissues cultured with vehicle (DMSO) was used as controls. Tissues were cultured at 37 °C for 48 h, then formalin fixed and paraffin-

embedded for histology, snap frozen in liquid nitrogen for proteomics analysis, or stored in RNAlater (Invitrogen) for transcriptomic analysis. For RNA sequencing, the data (n=24) belonged to 12 patients and was patient-matched. Each patient-derived explants (n=1 with GS $\leq$ 6, n=9 with GS $\sim$ 7 and n=2 with GS $\geq$ 8) with two treatment (Enzalutamide treated and DMSO) thus each generated 12 samples.

RNA Sequencing

The androgen receptor antagonist Enzalutamide or vehicle-treated patient-derived explants) preserved in RNAlater were homogenised with the Precellys®24 Tissue Homogenizer (Bertin Technologies, France) using 0.5 mL Precellys®24 tubes containing 1.4 mm ceramic beads and 500 μ L Tri Reagent (Sigma, USA). RNA was then extracted using a Qiagen RNeasy mini kit adapted for Tri Reagent using the manufacturer instructions. Library preparation and RNA sequencing were performed at the David Gunn Genomics Facility (SAHMRI, Adelaide, Australia). The library was prepared using TruSeq Stranded Total RNA Library Prep Kit (Bioo Scientific) as single reads, according to the manufacturer's instructions. TruSeq Stranded adaptors were trimmed from short reads. Mapping was carried out in two levels of genes and transcripts to have the expression based on genes and based on transcripts.

9.3.4. RNA extraction

Total RNA was extracted from tissue derived from TURPs using TRIzol reagent (Sigma), essentially as described previously [500], except that the RNA was precipitated with 2.5 volume of ethanol, 10 mM MgCl₂, 0.1 volume of 5 M NaCl, and 1 μ L (20 μ g) of Glyco-Blue (Invitrogen) overnight at -20°C . The purity of RNA was determined using the Nanodrop ND-2000 device.

SYBR Green Quantitative Real-Time PCR

Primary PCa specimens were obtained with written informed consent through the Australian Prostate Cancer BioResource from 47 men (n=07 BPH and n=40 PCa of varying Gleason score) who underwent robotic RP at the Royal Adelaide Hospital (Adelaide, Australia). Total RNA was

reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. CA, USA), following the manufacturer's protocol. Quantitative PCR (qRT-PCR) was carried out using iQ SYBR Green Supermix (Bio-Rad) and primer pairs on a CFX384 real-time PCR detection system (Bio-Rad Laboratories, Inc. CA, USA) for 40 cycles at 94 °C for 15 seconds, 61 °C for 30 seconds, and 72 °C for 30 seconds. Gene expression was normalised to Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA levels. The sequences of primers for *SHBG* were as follows:

Forward : 5'- ATCACAAAACCTCCTCCTCCTT -3' and

Reverse : 5'- CGTCTCGAAGTCCCAGCATAA -3'.

Each sample was analysed in triplicate. The relative mRNA expression of *SHBG* was calculated using the $2^{-\Delta\Delta C_t}$ methods and presented as fold change relative to normalisation against the expression of *GAPDH*.

9.3.5. Immunohistochemical analysis

Primary PCa specimens (n=125) included and analysed in this study consisted of eight normal prostates, 32 BPH and 85 PCa of varying GS tumour samples which underwent robotic RP at the Royal Adelaide Hospital (Adelaide, Australia). The classical Gleason scoring system in tumour tissues derived from transurethral prostatectomies (TURPs) was re-evaluated according to the 2014 International Society of Urological Pathology (ISUP) Consensus on Gleason grading of PCa [501]. Immunohistochemistry (IHC) assays were performed to detect the expression of *SHBG* in primary prostate tissue by indirect methods of antigen detection using standard biotin-streptavidin complex method and a polyclonal antibody against *SHBG*. Anti-*SHBG* antibody (cat. no. AF2656; R& D systems; MN, USA) was optimised with positive controls (ab4348 and ab5051, abcam).

Unstained, formalin fixed, and two µm paraffin-embedded tissue sections on ultra plus slides were deparaffinised through xylene and rehydrated by three successive baths of absolute

ethanol. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide solution (Peroxidized; Biocare) for 5 min followed by a wash in 10X PBS. Antigen retrieval was carried out by incubating the slides at 110 °C in Decloaking chamber (Biocare Medical, CA USA) in 10 mM citrate buffer (pH 6.5) for 15 min, followed by a 30 minutes cooldown period on the bench. Slides were then rinsed in 10X PBS. Slides were then blocked in 5% rabbit serum (Sigma-Aldrich) diluted in 10X PBS for 30 minutes at room temperature in a humid chamber. Blocking solution was removed, and slides were incubated with goat polyclonal anti-SHBG antibody (primary antibody) (diluted to 1/400; cat. no. AF2656; R& D systems; MN, USA) overnight in a humid chamber at 4°C. After overnight incubation, all slides were incubated at room temperature with secondary biotinylated antibody and streptavidin label (Dako, Australia) successively, each for 60 minutes in a humid chamber, and each followed by a 10X PBS wash. The reaction products were detected by using a 3, 3-diaminobenzidine (DAB) as chromogen (Sigma) for 6 minutes followed by washing in running tap water and were counterstained with 1:2 Lillie Mayer's hematoxylin for 1 minute. Finally, the slides were dehydrated by three successive baths of absolute ethanol followed by xylene and mounted in DPX mounting medium (Sigma).

SHBG expression in liver tissue (ab4348, abcam) and prostate tissue (ab 5051 and ab5052, abcam) was always kept as a positive control in each running. Negative controls were sections immunostained as above, but the primary antibody was omitted.

Digital Scanning and immunohistochemical quantification

The slides were digitally scanned at 40 × magnification using Nanozoomer 2.0RS and analysed with the NDP view software (Hamamatsu, Herrsching am Ammersee, Germany). All immunostained sections were assessed blindly. High-power fields (n=8) for each slide were randomly selected to assess SHBG expression. The intensity of SHBG staining has been quantified by computer through the use of Fiji ImageJ software, with epithelial cells staining separated using deconvolution plug-in. As a first step, we used a colour deconvolution technique to separate the

pure DAB and hematoxylin stained areas leaving a complimentary image. The pixel intensities of separated DAB or hematoxylin images range from 0 to 255. Value 0 represents the lightest shade of the colour while 255 represent the darkest shade of the colour in the image. To assign an automated score by judging the pure DAB staining pattern, a histogram profile of every image, that is, the number of pixels of a specific intensity value versus their respective intensity was raised using ImageJ standard programme feature. The percentage of epithelium positively stained for SHBG was calculated by the following formula: $\text{Epithelium area} / (\text{Epithelium area} + \text{Stroma area}) \times 100\%$.

9.3.6. Statistical Analysis

Statistical comparisons of mRNA and protein levels of genes/transcripts/proteins between two groups amongst normal, benign and PCa epithelial cells was made using a Students t-test, and between groups was made using one-way analysis of variance (ANOVA). Spearman rank correlations were used for correlation analyses. GraphPad Prism (version 7.02) was used for data analysis, and the significance level was set to 0.05 (two-sided).

9.4. Results

9.4.1. Increased *SHBG* mRNA expression in PCa

In silico analyses of mRNA expression data from four PCa cohort indicated that *SHBG* mRNA levels are higher in primary PCa. However, *SHBG* mRNA was not associated with either tumour grade or differentiation according to Gleason's grade in both TCGA and MSKCC cohort and an independent dataset from Sun Lab and Levi Garraway's lab (Figure 48).

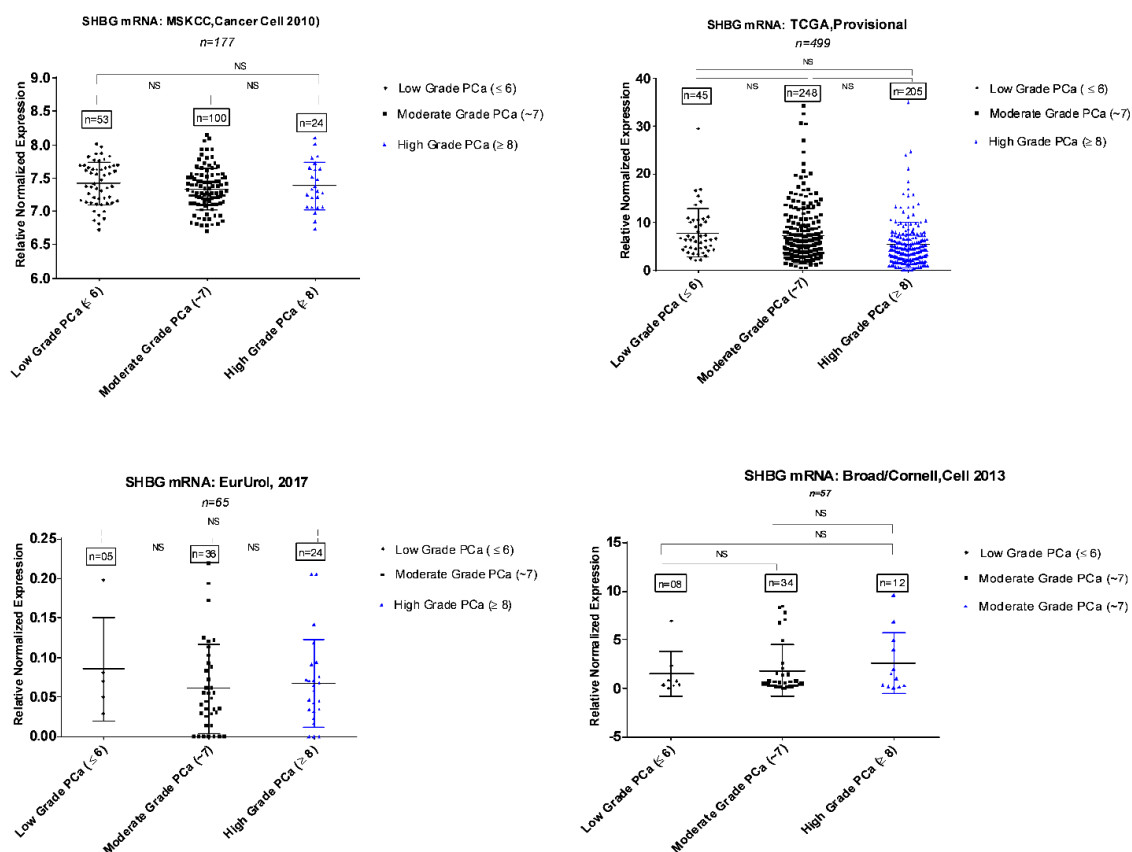
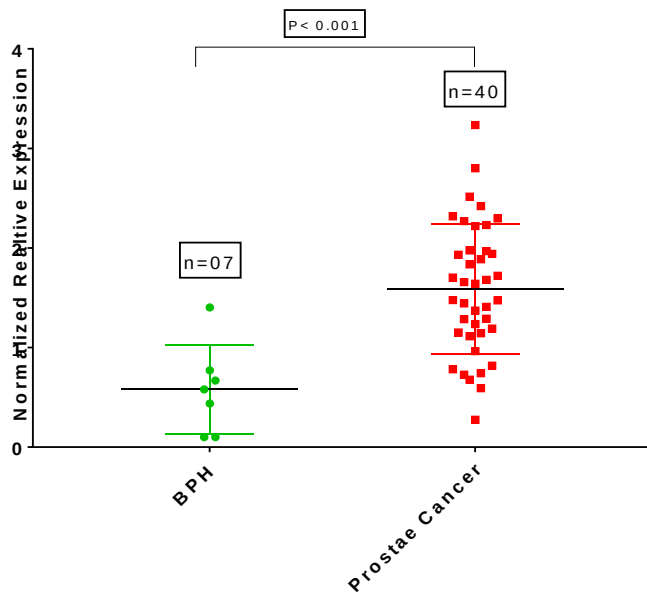
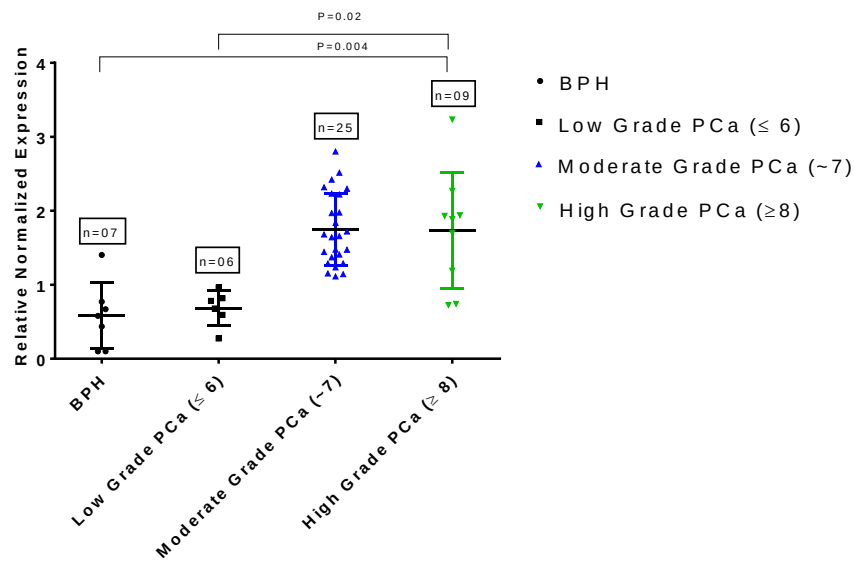


Figure 48. SHBG mRNA concentrations in localised PCa (obtained during RP) according to increasing Gleason score in published data sets.

SHBG mRNA concentrations were significantly increased in PCa compared with BPH ($P < 0.001$) (Figure 49A). When our primary tumour samples were stratified by Gleason grade, only high grade ($GS \geq 8$) tumours displayed significantly increased *SHBG* mRNA concentrations than BPH ($P = 0.004$) (Figure 2B). There was a significant difference in *SHBG* mRNA expression between high grade ($GS \geq 8$) vs low grade ($GS \leq 6$) ($P = 0.02$) (Figure 49B), but no significant difference in *SHBG* mRNA expression between high ($GS \geq 8$) vs moderate grade ($GS \sim 7$) ($P = 0.07$) and moderate ($GS \sim 7$) vs low grade ($GS \leq 6$) ($P = 0.12$) (Figure 49B). There was also no significant differences in the *SHBG* mRNA expression in moderate grade ($GS \sim 7$) with $GS\ 3+4$ compared to $4+3$ scored PCa ($P = 0.18$) (data not are shown).



[A]



[B]

Figure 49. SHBG mRNA expression in non-malignant prostate and PCa tissue samples.

[A] Mean SHBG mRNA expression in PCa is higher in comparison to BPH. [B] SHBG mRNA expression status in BPH and PCa according to histological grade (GS).

Error bars indicate the standard deviation. P-value set to <0.05.

9.4.2. SHBG protein content in PCa

SHBG protein was predominately detected in the cytoplasm of luminal epithelial cells of the prostate tissues and not in the stromal cells (Figure 50 A-C). We detected weakly positive or negative staining in normal prostatic epithelial cells, while a weak/moderate staining was observed in epithelial cells from BPH. We observed statistically significant increased SHBG protein concentrations in BPH compared to normal prostate epithelial cells ($P < 0.001$) (Figure 50A). In contrast, intense cytoplasmic staining was exhibited in PCa epithelial cells, and none of the tumours was negative for SHBG. SHBG was significantly higher in prostate tumours compared to both normal prostate epithelial cells & BPH ($p < 0.001$) (Figure 50A-C).

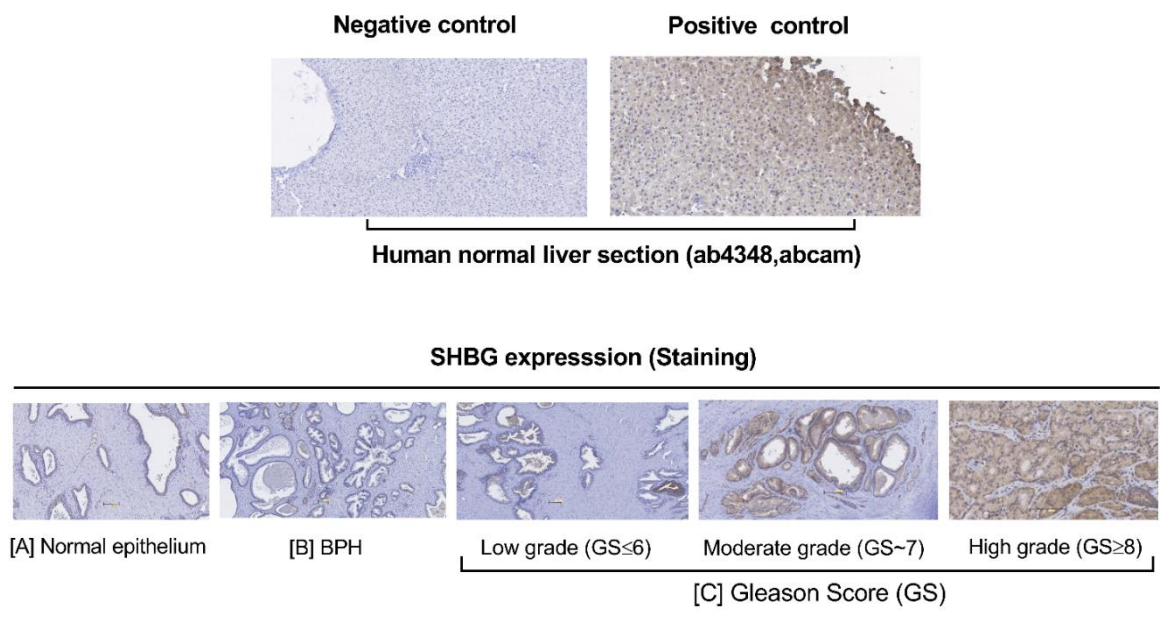


Figure 50. Immunohistochemical expression of SHBG.

Representative immunohistochemical staining in [A] Normal, [B] BPH, and [C] Prostate cancer epithelial cells.

SHBG increased with an advanced clinical stage in PCa ($P < 0.001$). A significant increase existed in SHBG protein concentrations between high grade (GS ≥ 8) and tumours that were a moderate grade (GS ~ 7) ($P = 0.002$). No difference was found between moderate grade (GS ~ 7) and low grade (GS ≤ 6) ($P = 0.34$) and high grade (GS ≥ 8) and low grade (GS ≤ 6) ($P = 0.15$) (Figure 51). There was also no significant differences in the proportion of SHBG protein concentrations in moderate grade (GS ~ 7) with GS 3+4 compared to 4+3 scored PCa ($P = 0.94$) (Figure 52).

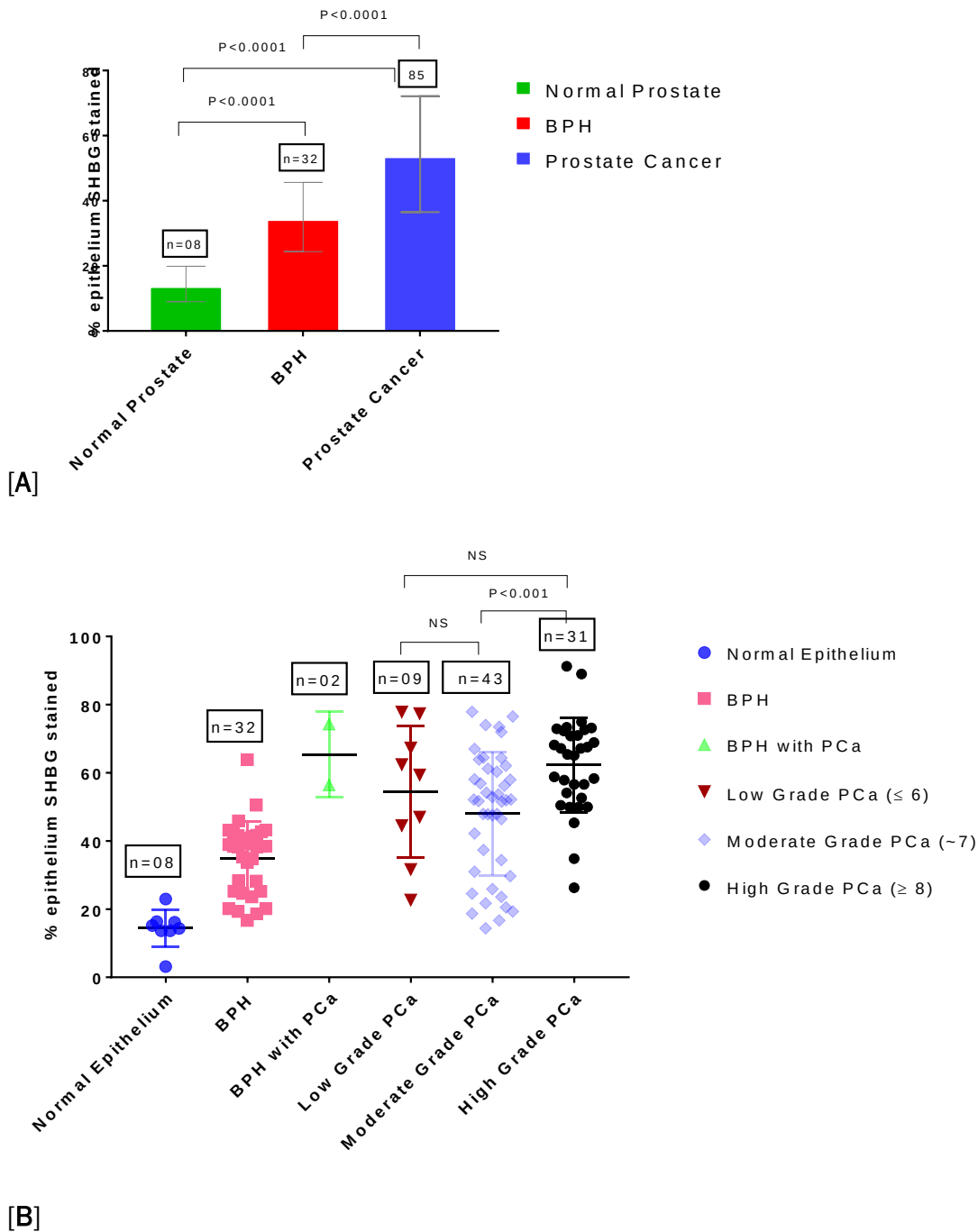


Figure 51. Immunohistochemical analysis of SHBG protein content in non-malignant prostate and PCa tissue samples.

[A] SHBG protein content is barely detectable in the normal prostate epithelium or weakly in BPH but increase in PCa epithelial cells. **[B]** SHBG expression in a normal, BPH and PCa with histological grade (Gleason score).

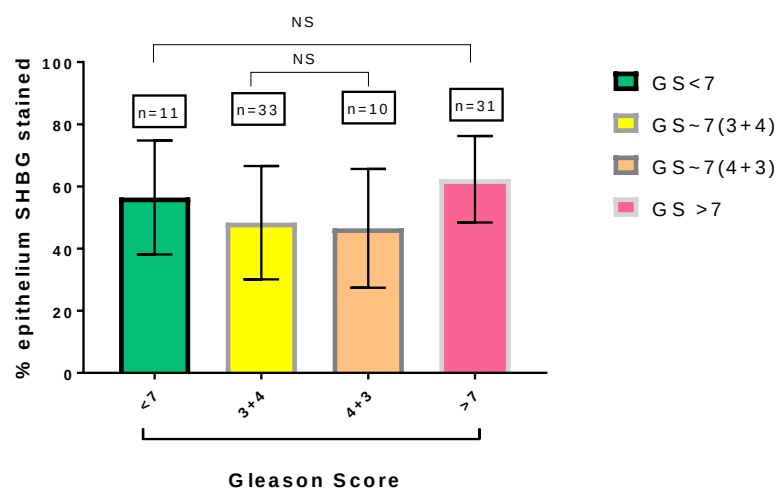


Figure 52. SHBG protein concentrations in PCa and its relationship with moderate grade (GS ~7) with GS 3+4 compared to 4+3 scored PCa.

9.4.3. SHBG mRNA and Protein in paired sample

There was no significant correlation between SHBG mRNA and SHBG protein concentrations (Spearman rho = 0.16, $P=0.33$) (Figure 53).

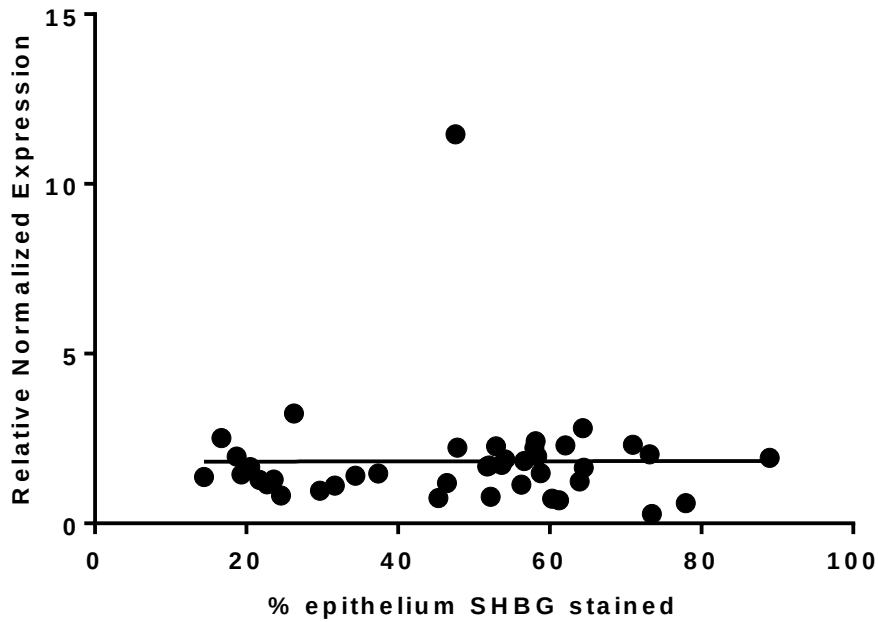


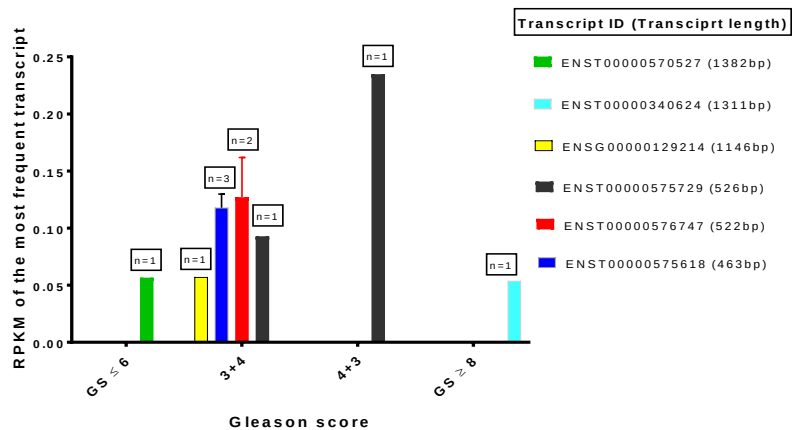
Figure 53. Correlation between SHBG mRNA and Protein content in paired samples ($n=41$).

9.4.4. *SHBG* Transcripts

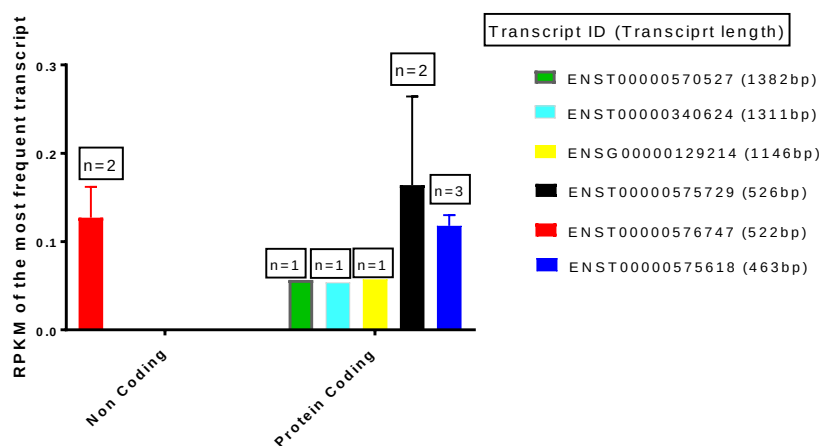
To evaluate which *SHBG* transcripts are expressed in PCa, we generated RNA-seq data from 12 tumour specimens. *SHBG* has 20 different annotated transcript isoforms (splice variants) of variable length (463bp to 1382bp). Our data showed a relative increase in both the number of alternatively spliced transcripts and transcripts from upstream promoters in both treatments (enzalutamide treated and DMSO).

Very low expression of *SHBG* was observed in patient-derived PCa explants cultured with vehicle (DMSO). There were six most frequently expressed transcript and were differentially in most but not all tumour specimens examined (10 of 12) (Figure 54 A & B). PCa with $GS \leq 6$ and $GS \geq 8$ expressed unique coding transcripts of 1382bp and 1311bp, respectively. Within PCa of $GS \sim 7$, there was marked heterogeneity of *SHBG* transcripts; 463 bp, 522 bp (non-coding), 526bp and 1146bp transcripts predominated, none expressed the 1311 bp and 1382 bp transcript.

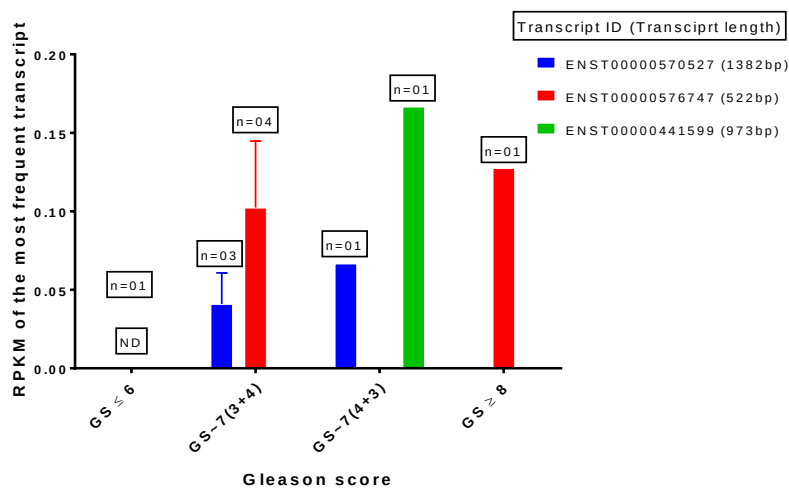
To determine whether *SHBG* transcript levels are regulated by AR, we evaluated RNA-seq data from matched tumours treated with the AR antagonist 10 μ M Enzalutamide. *SHBG* gene expression did not change in response to MDV. However, the non-coding transcripts of 522 bp, aberrantly expressed in PCa with $GS \geq 7$ along with coding transcripts of 1382 bp and 973 bp while none was detected in PCa with $GS \leq 6$ in response to 10 μ M MDV. Moreover, non-coding transcripts 522 bp was significantly up-regulated ($P < 0.05$) by 1.875 fold change while coding transcripts 463 bp and 526 bp both were significantly down-regulated ($P < 0.05$) in response to 10 μ M MDV (Figure 54 C).



[A]



[B]



[C]

Figure 54. Transcript isoforms of SHBG in PCa.

[A] *SHBG* transcript isoforms detected in PCa patient-derived explants cultured with vehicle (DMSO). [B] *SHBG* transcript isoforms based on protein-coding and non-coding. [C] *SHBG* transcript isoforms detected in patient-derived PCa explants in response to 10 uM MDV.

9.5. Discussion

We found *SHBG* mRNA is increased in PCa epithelial cells as compared to normal prostate and BPH. These data are consistent with those of others showing an increased expression of *SHBG* mRNA in primary PCa epithelial cells [74, 75, 86], in PCa explants [74, 83], and established PCa cell lines (human LNCaP, DU 145 and PC 3) [22, 75, 146, 434]. No previous studies have examined the relationship of *SHBG* mRNA expression to stage/grade of PCa. In the current study, there was no consistent relationship between *SHBG* mRNA and Gleason's grade either in the human primary PCa samples or in the *in silico* analyses.

As with the mRNA, we found SHBG protein content is increased in PCa epithelial cells. It has previously been demonstrated that in normal prostate, SHBG protein cannot be detected by immunohistochemistry [22, 74, 75, 83, 502], whereas it is weakly positive in BPH [75, 146, 502], and intensely positive in primary PCa epithelial cells [83, 146], in PCa explants [74, 83] and established PCa cell lines (human LNCaP, DU145, and PC3) [22, 75, 146, 434]. The data from the current study are in accordance with this. Further, we detected a substantial increase in SHBG protein content from, low grade, to high-grade PCa. Using the same antibody Ma and colleagues reported increased SHBG protein content is associated with high Gleason grade and increased exclusively in the high Gleason grade tumours with highly infiltrating tumour cells (seminal vesicle invasion and lymph node metastasis) [146].

As with the current study, two prior studies have reported that SHBG protein is high despite low *SHBG* mRNA content in PCa epithelial cells [74, 75]. One potential explanation has been the diffusion of SHBG from the plasma into the prostatic tissue [65, 83, 434]. While it is possible this may account for a proportion of the SHBG, there is clearly synthesis of SHBG in the PCa cells. Further, we identified at least six *SHBG* transcripts of variable length (463bp to 1382bp), of which the five longer coded for a

functional protein. Although not assessed in the current study, the glycosylation of SHBG is required for optimal protein stability and secretion [39]. SHBG synthesised in the prostate is not secreted and is only partially glycosylated [65]; this is possibly enough to increase protein stability but insufficient for secretion leading to intracellular accumulation.

Although multiple transcripts of SHBG mRNA have previously been reported in PCa cell lines [85, 86], this is the first study to show the presence of transcript heterogeneity in primary PCa tissue. Although the number of samples may be too few to enable definitive conclusions to be drawn, we noted that within the low ($\leq$ GS 6) and high grade ($\geq$ GS 8) PCa, there was a single unique coding transcript; 1382bp and 1311bp in the low and high grade respectively. Multiple SHBG transcripts were present within GS grade 7 PCa tissue. The predominant transcripts were 463 bp, 522 bp (non-coding), 526bp and 1146bp transcripts. The longer 1311 bp and 1382 bp transcripts were absent.

Further work is required to determine the relationship between SHBG transcript size and tumour behaviour. If the pattern of SHBG transcripts in PCa epithelial cells can predict the subsequent behaviour of the tumour, this may permit risk stratification, and refinement of management protocols, for men with PCa. An understanding of the biological roles of intra-prostatic SHBG may provide novel approaches to the management as well as risk stratification of PCa.

This study has a number of strengths: (1) the first documented analysis that examined pattern of differential expression of SHBG in PCa epithelial cells compared to both normal prostate and BPH epithelial cells; (2) tumour tissue was obtained sections of sent for pathology following prostatectomy; (3) all samples were re-evaluated by a single pathologist to apply the latest International Society of Urological Pathology (ISUP) Consensus on Gleason grading of PCa [501]. The limitations of this study include: (1)

only 33% of tissue samples was accessed for the pairwise comparison between *SHBG* mRNA and SHBG protein content. Therefore, any comparison is limited by the homogeneity of the specimens being compared but merits further investigations; (2) we could not include clinicopathological characteristics of PCa (for example tumour stage or biochemical recurrence) and patient's clinical outcomes; (3) there was lack of complete and resilient data regarding the primary treatment modality and androgen deprivation therapy (ADT), since ADT could potentially affect patient outcomes and thus our interpretations.

9.6. Conclusions

Taken together with the findings from the current study and those previously published we propose that SHBG protein abundance and transcript size are markers of advanced disease and may be a novel marker for aggressive tumour behaviour in early-stage disease. Given the androgen-dependent enzyme fatty acid synthase (FAS), required for *de novo* lipogenesis, is overexpressed in PCa and has been associated with aggressive disease. The synthesis of SHBG is linked to *de novo* lipogenesis. Therefore, the linkage between increased SHBG expression in PCa and *de novo* lipogenesis requires further investigations.

10. General discussion and future directions

10.1. Introduction

This thesis aimed to study the factors regulating serum SHBG concentration, and the role of SHBG as a marker of the risk of three common non-communicable chronic diseases in men, i.e. T2D, CVD and PCa.

10.2. Determinants of plasma SHBG

Data from the Men Androgen Inflammation Lifestyle Environment and Stress (MAILES) study, showed a positive relationship of serum SHBG concentrations with ageing, thyroxine and total testosterone, and an inverse relationship with abdominal total fat mass, triglycerides and oestradiol in community-based men (Chapter 6.4). We also demonstrated no association between insulin and SHBG when using a population-based sample with sufficient numbers of hypo-, eu-, and hyper-glycaemic men (Chapter 6.4). Taken together with the available literature, the data from this thesis strongly suggests that decreased SHBG in men is predominantly associated with elevated triglycerides within the liver that occurs in the context of insulin resistance.

10.2.1. Clinical implications

The accepted gold standard assay for T is total T (TT) measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Measurement of SHBG has an inherent value in the clinic in that changes refer to specific clinical conditions. An understanding of the factors affecting SHBG facilitates interpretation, and the clinical significance, of a TT level. The new information I have obtained supports the most recent Australian position statement on male hypogonadism [160] that, rather than relying on FT measured by suboptimal methodology, at high and low ends of the reference interval, serum TT concentrations should be interpreted about SHBG concentrations. Further, SHBG concentrations provide information relating to specific clinical conditions (e.g. insulin resistance, thyroid hormone resistance).

10.2.2. Future research

There are several areas of investigation that are warranted, given the results of my thesis:

- 1) The utility of SHBG to guide management of hypertriglyceridemia and insulin resistance;
- 2) What is the implication of genetic SHBG variants for the physiology of SHBG? Are there sex-related differences and implications?
- 3) How do variations in SHBG affect the physiology of SHBG?

10.3. Role of SHBG as a marker of T2D

In the MAILES population, I found that low serum SHBG is a marker of overall metabolic risk, i.e. T2D, dyslipidaemia (Chapter 7.0). SHBG, however, unlike T, is not an independent predictor of T2D development (Chapter 7.0). It is likely that the reported association between T2D and a low concentration of SHBG is due in part to an inverse correlation between SHBG and insulin resistance (Chapter 4.1) and dyslipidaemia (Chapter 3.3). The results of this thesis concur with recent Mendelian randomisation studies [283, 304] arguing against a causal role of low SHBG and T2D.

10.3.1. Clinical implications

Data from my thesis suggests that SHBG is a marker of T2D risk and can be used to assess the impact of interventions to abrogate the effect of risk factors for T2D that also impact in SHBG. The relevance of T as a marker of risk, and whether this allows clinical intervention, will be determined by ongoing studies such as T4DM [503].

10.3.2. Future research

There are several areas of investigation that are warranted, given the results of my thesis:

- 1) The clinical utility of SHBG as a marker of metabolic risk in people with T1D, or as a marker of risk for T2D in patients with T1D, requires further investigation;
- 2) Does the role of SHBG as a marker of risk apply equally to both sexes, and does it function as an integrated marker of risk?
- 3) Further studies are also needed to establish hormone thresholds at which T2D risk is significantly increased, thereby aiding in the identification of high-risk men in the clinical setting.

10.4. Role of SHBG as a marker of CVD

The new information I have obtained from the MAILES study indicate that community-based men with high circulating concentrations of SHBG are predisposed to a greater risk of incident CVD, but not CVD-related mortality (Chapter 8.3). I also demonstrated that higher concentrations of SHBG and lower concentrations of TT are independently associated with the risk of CVD in men. It remains unclear what are the major drivers of this elevated SHBG in the natural history of CVD in men. Our data also suggest that the observed association between serum SHBG and CVD risk are specific to SHBG, thereby leaving issues such as reverse causation or residual confounding to be addressed. Nevertheless, my data and others [345, 382, 386], have demonstrated a relatively strong association between increased SHBG and the risk for the development of CVD in men, and propose SHBG being utilised as a risk marker, rather than a causal risk factor.

10.4.1. Clinical implications

Data from my thesis adds strength to recent studies advocating serum SHBG measurement in the standard assessment of men for CVD [11, 29, 504]. I would caution however, that these not take the place of other established markers of CVD development (e.g. TT measured by LC-MS/MS) as per the Task Force of the Endocrine Society [129].

10.4.2. Future research

There are several areas of investigation that are warranted, given the results of my thesis:

- 1) Further work is required to precisely estimate the sensitivity and specificity of SHBG as a biomarker of incident CVD events in men, especially the demonstration of thresholds for SHBG at which CVD risk is significantly increased;
- 2) Research is still required to identify the precise mechanisms leading to higher SHBG in men at risk for CVD, to identify potential points of intervention where appropriate;

- 3) Larger studies utilising harmonised datasets (particularly in healthy, community-based men) are required to differentiate the association between SHBG and specific types of CVD.

10.5. Role of SHBG as a marker of prostate cancer aggressiveness

My thesis has presented novel data demonstrating increased *SHBG* mRNA and protein occurs almost exclusively in PCa epithelial cells, distinguishing it from both the normal prostate and benign prostate hyperplasia (BPH). The data demonstrate that the highest levels of expression occur in poorly differentiated and metastatic PCa epithelial cells (GS ≥ 7). Taken together with findings from previous studies, we propose that SHBG protein abundance and transcript size are markers of advanced disease and may be a novel marker for aggressive tumour behaviour in early-stage disease. The nature of the *SHBG* transcripts expressed in PCa epithelial cells indicates that there is clearly synthesis of SHBG in PCa cells and that this is unlikely as a result of the diffusion of SHBG from the plasma into the prostatic tissue. Therefore, as recently suggested in other conditions [3, 54], SHBG might have biological functions beyond transport and modulation of androgens. In the case of prostatic disease, SHBG may serve as a reservoir for locally produced androgens that can be accessed by the AR; thus, cancer progresses despite the limited supply of androgens and becomes more aggressive.

10.5.1. Clinical translation

My research has broad and promising avenues for clinical utilisation. My findings suggest that absolute quantification of SHBG in PCa biopsies may be helpful in early diagnoses and prognostic judgment of PCa. Moreover, an understanding of the biological roles of intra-prostatic SHBG may provide novel approaches to the management, as well as risk stratification, of PCa in the future.

10.5.2. Future research

There are several areas of investigation that are warranted, given the results of my thesis:

- 1) Further work is required to determine the relationship between *SHBG* transcript size and tumour behaviour. If the pattern of SHBG transcripts in PCa epithelial

cells can predict the subsequent behaviour of the tumour, this may permit risk stratification, and refinement of management protocols, for men with PCa;

2) It remains to be determined:

- a. What is the function of SHBG within PCa cells?
- b. Whether glycosylation plays an important role in the intracellular accumulation and function of intraprostatic SHBG;
- c. The possibility of an uncoupling of the regulation of SHBG from lipogenesis in PCa and/or alternate transcriptional regulation of SHBG in aggressive PCa given: a) SHBG is a sensitive marker of *de novo* lipogenesis in the liver [89]; b) lipid turnover is increased in PCa, and c) is associated with PCa aggressiveness [452].

3) Dynamics between *SHBG* mRNA translation and protein, and their stability within the prostate, requires further investigation;

4) Future investigations also are required to examine whether the intraprostatic SHBG expression contributes to the castration-resistant phenotype or biochemical recurrence, by accentuating the actions of small amounts of locally-produced intratumoral androgens.

10.6. Overall Conclusion

In conclusion, variation in serum SHBG reflects *de novo* lipogenesis and sex steroids metabolism within the liver that occurs in the context of insulin resistance. While the inverse relationship between insulin resistance and reduced plasma SHBG concentrations is incontrovertible, SHBG is a marker, rather than a mediator of incident CVD but not T2D. PCa epithelial cells contain higher concentrations of SHBG transcripts and immunoreactive SHBG. SHBG expression and transcript size is a novel marker for aggressive tumour behaviour in early-stage disease, is highly expressed in high grade and metastatic PCa. Moreover, SHBG might be utilised as a biomarker for metabolic and sex steroid-dependent diseases in men.

APPENDICES

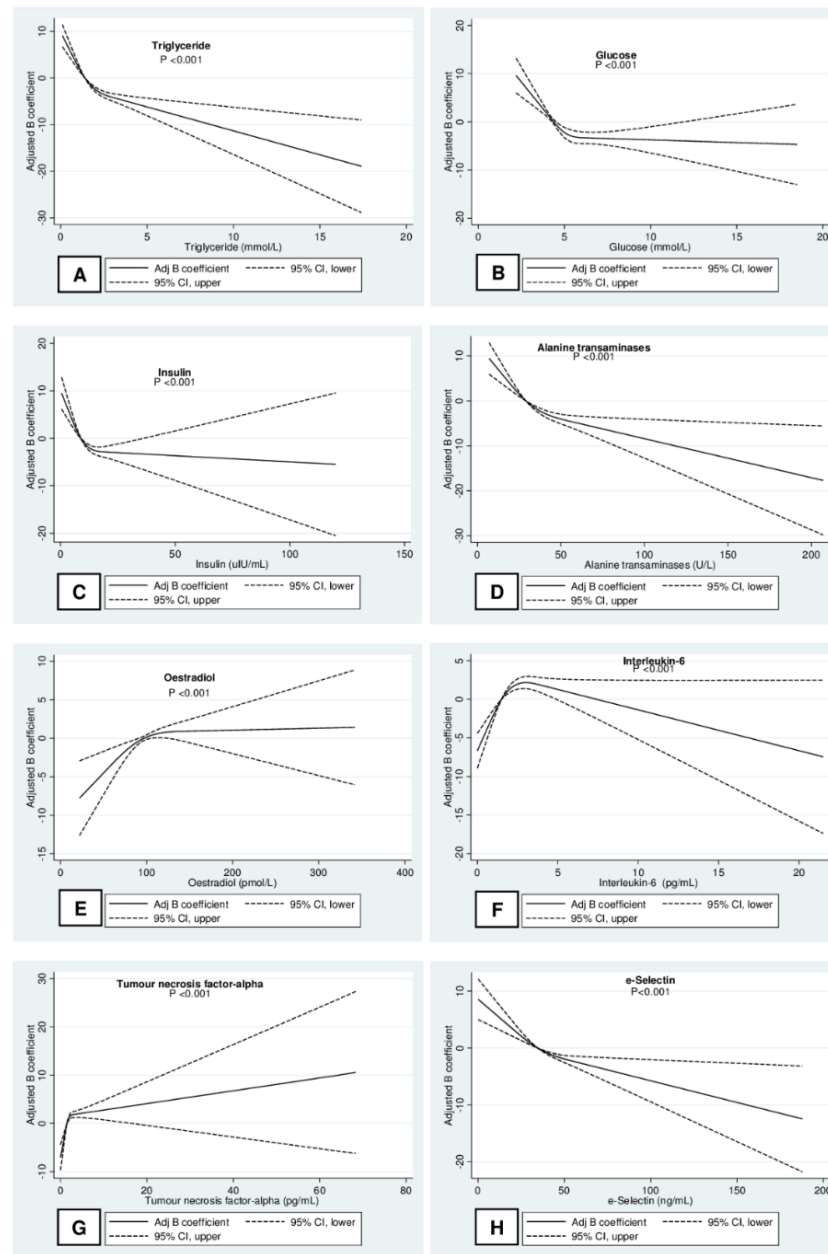
Appendix 1. Unadjusted, age-adjusted, and multi-adjusted generalised linear regression and lasso regression model to estimate associations between changes in potential determinants (Δ) against change in SHBG (Δ) between visits in community-dwelling men (n=1476).

Determinants/factors	Δ SHBG=1.38 $\pm$ 9.3								
	Unadjusted model			Age-adjusted		Multi-adjusted (Full model)		LASSO regression	
	Mean difference $\pm$ SD	Standardized β	P-value	Standardized β	P-value	Standardized β	P-value	Standardized β	P-value
Demographic, behavioural & anthropometric factors									
Δ Age , Years	4.6 $\pm$ 0.6	0.218	<0.001	-	-	0.000	0.999	-	-
Δ Physical activity	148.7 $\pm$ 2346.9	0.035	0.450	0.035	0.457	0.032	0.524	-	-
Δ Abdominal total fat mass (%)	-2401.8 $\pm$ 894.6	-0.063	0.100	-0.063	0.098	-0.045	0.369	-	-
Blood chemistry & hormones									
Δ Triglycerides (mmol/L)	-0.053 $\pm$ 1.53	-0.028	0.275	-0.030	0.242	-0.157	0.002	-0.165	0.001
Δ Glucose (mmol/L)	0.328 $\pm$ 1.257	0.020	0.440	-0.002	0.944	-0.007	0.888	-	-
Δ Insulin (μ U/mL) ^a	-1.2 $\pm$ 7.8	-0.058	0.101	-0.060	0.095	0.008	0.876	-	-
Δ ALT activity (U/L) ^b	-2.0 $\pm$ 19.7	0.002	0.950	0.003	0.929	0.083	0.106	-	-
Δ fT4 (pmol/L)	1.8 $\pm$ 3.3	-0.003	0.919	0.061	0.026	0.159	0.002	0.173	0.001
Δ TT (nmol/L)	-0.631 $\pm$ 4.3	0.333	<0.001	0.348	<0.001	0.406	<0.001	0.403	<0.001

Δ E2 (pmol/L) ^c	-9.3 ± 39.9	0.074	0.033	0.057	0.107	-0.114	0.035	-0.110	0.038
Inflammatory markers									
Δ IL-6 (pg/mL)	0.047 ± 1.9	-0.063	0.017	-0.074	0.004	-0.119	0.019	-0.109	0.028
Δ TNF- α (pg/mL)	0.137 ± 2.9	-0.079	0.003	-0.072	0.005	0.041	0.423	-	-
Δ MPO activity (μ g/L)	-26.7 ± 329.1	0.004	0.893	-0.005	0.849	0.060	0.249	-	-
Δ eSel (ng/mL)	-0.218 ± 11.4	-0.032	0.277	-0.036	0.164	-0.022	0.676	-	-

Statistically significant associations ($P < 0.05$) are shown in bold. Multi-adjusted generalised linear model R^2 was 0.240 and lasso regression model R^2 was 0.258. Δ = the change in value between baseline and on follow-up over 4.9 years, ALT, alanine transaminases; f T4, free thyroxine; TT, total testosterone; E2, oestradiol; SHBG, sex hormone binding globulin; IL-6, interleukin 6; TNF- α , tumour necrosis factor alpha; MPO, myeloperoxidase; eSel, sE-selectin.

^a n=748, ^b n=744, ^c n=789



Appendix 2. Non-linear regression curves of associations between SHBG and potential determinants in community-dwelling men.

[A] Triglycerides; [B] Glucose; [C] Insulin; [D] ALT; [E] E2; [F] IL-6; [G] TNF- α ; [H] eSel , among community-dwelling, middle-aged to elderly men. All analyses were adjusted for age, physical activity, smoking status, alcohol consumption, abdominal total fat mass(%), triglycerides, glucose, insulin, alanine transaminases (ALT); free thyroxine (fT4), total testosterone (TT), oestradiol(E2), interleukin 6 (IL-6), tumour necrosis factor alpha(TNF- α), myeloperoxidases (MPO) and e-Selectin (eSel).

Appendix 3. Multi-adjusted regression model (via splines) to estimate longitudinal determinants of SHBG in community-dwelling men (n=1476).

Determinants/factors	Multi-adjusted (Full model)					
	The test below likelihood ratio cut-off		The test above likelihood ratio cut-off		Test for overall association (continuous)	
	Standardized β	P-value	Standardized β	P-value	Standardized β	P-value
Blood chemistry & hormones						
Triglycerides (mmol/L) ^a	-0.171	<0.001	-0.143	<0.001	-0.065	0.027
Glucose (mmol/L) ^b	-0.003	0.925	0.003	0.930	-0.049	0.145
Insulin (μ U/mL) ^c	0.017	0.651	-0.030	0.423	-0.009	0.806
ALT activity (U/L) ^d	0.152	<0.001	-0.166	<0.001	-0.054	0.118
E2 (pmol/L) ^e	-0.138	<0.001	0.134	<0.001	-0.023	0.490
Inflammatory markers						
IL-6 (pg/mL) ^f	-0.007	0.845	0.006	0.871	0.047	0.150
TNF- α (pg/mL) ^g	0.009	0.807	-0.015	0.681	0.037	0.243
eSel (ng/mL) ^h	0.055	0.141	-0.052	0.168	0.045	0.208

Data presented are standardised regression coefficients (β) taken from generalised additive models, with cut points determined from corresponding cubic spline analyses and likelihood ratio (S1 Fig). Statistically significant associations ($P < 0.05$) are shown in bold. ALT, alanine transaminases; E2, oestradiol; IL-6, interleukin 6; TNF- α , tumour necrosis factor alpha; eSel, sE-selectin.

^a Triglycerides cut-off value was 2 mmol/L; ^b Glucose cut-off value was 6.0 mmol/L; ^c Insulin cut-off value was 20.0 μ IU/mL; ^d ALT cut-off value was 45.0 U/L; ^e E2 cut-off value was 110.0 pmol/L, ^f IL-6 cut-off value was 3.5 pg/mL; ^g TNF- α cut-off value was 4.0 pg/mL; ^h eSel cut-off value was 45.0 ng/mL.

Appendix 4. Baseline characteristics of participants with available and missing data on covariates in an analytical sample

Parameters	Participants in the analytical sample (n=1597)	Participants not in analytical sample (n=886)
Questionnaire & clinical evaluations		
Age (Years)	53.9±11.3	59.5±12.8
Marital status		
Married / Partner	1254(78.1)	604(68.2)
Separated / Divorced	208(13.0)	125(14.1)
Widowed	40(2.5)	53(6.0)
Never married	98(6.1)	55(6.2)
Education		
Higher school or lower	459(28.6)	335(37.8)
Trade / Apprenticeship	508(31.7)	246(27.8)
Diploma / Certificate	404(25.2)	180(20.3)
Bachelor or higher	210(13.1)	65(7.3)
Smoking status (current), n (%)		
Non-smoker	1258(78.4)	650(73.4)
Smoker	343(21.4)	190(21.4)
Leisure time physical activity, n (%)		
Low, moderate, high	1132(70.5)	544(61.4)
Sedentary	393(24.5)	228(25.7)
Alcohol consumption		
None/day	14(0.9)	Nil
1-2 drinks/day	840(52.3)	479(54.1)
≥3 drinks/day	684(42.6)	267(11.8)
Cholesterol-lowering medication, n (%)		
Yes	46(2.9)	266(30.0)
No	1557(97.0)	560(63.2)
Antihypertensive medication,%		
Yes	322(20.1)	328(37.0)
No	1278(79.6)	501(56.5)

Family history of diabetes, n (%)				
			445(27.7)	313(35.3)
Yes			1157(72.1)	526(59.4)
No				
Depression, n (%)				
Yes			222(13.8)	150(16.9)
No			1350(84.1)	712(80.4)
Abdominal total fat mass (%)			33.9±8.3	36.4±8.2
WC (cm)			99.5±11.4	104.1±13.4
BMI (kg/m ²)			28.1±4.2	29.3±5.1
Systolic	blood	pressure	133.7±17.9	137.9±19.5
(mmHg)				
Diastolic	blood	pressure	85.1±9.1	84.7±10.1
(mmHg)				
Hypertension, n (%)				
Yes			717(44.7)	447(50.5)
No			885(55.1)	380(42.9)
Blood biochemistry & hormones				
Glucose (mmol/L)			4.8±1.35	5.9±2.1
HbA1c,% (mmol/mol))			5.5±0.35 (36.5±3.8)	6.3±1.2 (45.3±13.1)
Insulin (µIU/mL)			9.8±7.2	13.7±12.4
Total cholesterol (mmom/L)			5.5±1.06	5.1±1.1
LDL-C (mmol/L)			3.5±0.882	3.1±0.98
HDL-C (mmol/L)			1.3±0.31	1.2±0.31
Triglycerides (mmol/L)			1.7±1.4	1.9±1.6
SHBG (nmol/L)			34.0±15.0	35.1±18.2
TT (nmol/L)			17.6±6.1	16.1±7.4
DHT (nmol/L)			1.8±0.80	1.7±0.89
E2 (pmol/L)			96.0±42.0	91.5±43.7

WC, waist circumference; BMI, body mass index; HbA1c, glycosylated haemoglobin; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; SHBG, sex hormone-binding globulin; TT, total testosterone; DHT, dihydrotestosterone; E2, oestradiol

Appendix 5. Unadjusted, age-adjusted, and multi-adjusted logistic regression models for baseline SHBG, sex steroids and other selected predictors on incident type 2 diabetes in community-dwelling men (n=1067)

Predictors	Unadjusted		Adjusted											
	OR (95%CI)	p- valu e	+Age		Multi-adjusted model with SHBG		Multi-adjusted model with TT		Multi-adjusted model with DHT‡		Multi-adjusted model with SHBG & TT		Multi-adjusted model with SHBG & DHT‡	
			OR (95%CI)	p- valu e	OR (95%CI)	p- valu e	OR (95%CI)	p- valu e	OR (95%CI)	p- valu e	OR (95%CI)	p- valu e	OR (95%CI)	p- valu e
SHBG (nmol/L)	0.828(0.697-0.985)	0.033	0.656(0.538-0.801)	<0.001	0.759(0.600-0.959)	0.021	-	-	-	-	0.908(0.694-1.188)	0.483	0.829(0.629-1.093)	0.183
Testosterone (nmol/L)	0.644(0.541-0.767)	<0.001	0.653(0.548-0.779)	<0.001	-	-	0.683(0.542-0.860)	0.001	-	-	0.716(0.546-0.938)	0.015	-	-
DHT (nmol/L)‡	0.739(0.614-0.890)	0.001	0.724(0.600-0.873)	<0.001	-	-	-	-	0.758(0.602-0.954)	0.018	-	-	0.836(0.645-1.083)	0.175
E2 (pmol/L)	1.044(0.877-1.242)	0.629	-	-	-	-	-	-	-	-	-	-	-	-
Age , Years	1.487(1.281-1.726)	<0.001	-	-	1.518(1.192-1.933)	0.001	1.390(1.109-1.742)	0.004	1.505(1.190-1.903)	0.001	1.439(1.121-1.847)	0.004	1.587(1.234-2.043)	<0.001

Marital status (Married or partnered/ Never married)	0.900(0.504-1.609)	0.723	-	-	-	-	-	-	-	-	-	-	-	-
Education level (Low or above high school)	0.940(0.781-1.132)	0.516	-	-	-	-	-	-	-	-	-	-	-	-
Glucose (mmol/L)	4.256(3.007-6.023)	<0.001	3.902(2.748-5.540)	<0.001	1.689(1.402-2.034)	<0.001	1.692(1.405-2.037)	<0.001	1.782(1.468-2.163)	<0.001	1.686(1.399-2.033)	<0.001	1.764(1.452-2.144)	<0.001
Abdominal total fat mass (%) #	1.571(1.316-1.876)	<0.001	1.501(1.256-1.793)	<0.001	1.306(1.054-1.618)	0.015	1.243(1.001-1.543)	0.049	1.275(1.021-1.592)	0.032	1.239(0.996-1.543)	0.055	1.268(1.012-1.589)	0.039
Triglycerides (mmol/L)	1.196(1.056-1.355)	0.005	1.246(1.097-1.416)	0.001	1.061(0.882-1.276)	0.529	1.077(0.897-1.294)	0.427	1.127(0.936-1.357)	0.206	1.055(0.876-1.270)	0.574	1.087(0.900-1.314)	0.386
Parental history of diabetes	1.427(1.061-1.920)	0.019	1.584(1.171-2.144)	0.003	1.263(0.853-1.870)	0.243	1.302(0.880-1.928)	0.187	1.324(0.880-1.992)	0.178	1.276(0.860-1.892)	0.226	1.290(0.855-1.946)	0.225
Smoking status (current; Yes/No)	1.156(0.879-1.519)	0.199	1.337(0.949-1.882)	0.097	0.888(0.534-1.479)	0.648	0.887(0.529-1.487)	0.649	0.879(0.516-1.498)	0.636	0.883(0.526-1.483)	0.638	0.864(0.506-1.474)	0.591

Alcohol consumption (None, 1-2/day & ≥3/day)	0.844(0.642-1.110)	0.225	-	-	-	-	-	-	-	-	-	-	-	-
Depression (Symptomatic)	1.623(1.096-2.403)	0.016	1.749(1.174-2.604)	0.006	1.853(1.130-3.039)	0.015	1.905(1.161-3.125)	0.011	1.868(1.115-3.132)	0.018	1.887(1.149-3.101)	0.012	1.852(1.103-3.112)	0.020
Physical activity (Sedentary, low-, moderate- & high-exercise level)	0.862(0.741-1.004)	0.056	0.846(0.725-0.988)	0.035	0.993(0.820-1.203)	0.944	0.982(0.810-1.191)	0.855	0.977(0.803-1.189)	0.817	0.990(0.816-1.201)	0.917	0.981(0.805-1.196)	0.852

Statistically significant associations ($P < 0.05$) are shown in bold. Nagelkerke R^2 (multi-adjusted + SHBG): 0.142, Nagelkerke R^2 (multi-adjusted + TT):

0.152, Nagelkerke R^2 (multi-adjusted + DHT): 0.161, Nagelkerke R^2 (multi-adjusted + SHBG & TT): 0.152 and Nagelkerke R^2 (multi-adjusted + SHBG

& DHT): 0.162. AIC value for multi-adjusted + SHBG & TT: 953.2, AIC value for multi-adjusted + TT: 970.7, AIC value for multi-adjusted + SHBG &

DHT: 976.1, AIC value for multi-adjusted + SHBG: 977.3 and AIC value for multi-adjusted + DHT: 978.9

SHBG, sex hormone-binding globulin; Odds Ratio, OR; DHT, dihydrotestosterone; E2, oestradiol, AIC; Akaike's information criterion

Men without T2D (n=892) and men with incident T2D (175) were included in the analysis

‡ Men without T2D (n=787) and men with incident T2D (150) were included in the analysis

Appendix 6. *Effect of medication usage on the observed regression estimates for associations between SHBG (and sex steroids) and incident CVD in multi-adjusted logistic binomial regression models*

Medications		Incident CVD				
		%(n)	OR	95% CI		R ² change
Lipid lowering (including Statin)	Baseline	8.3(78)	1.543	1.160	2.053	
	+/-	6.2(82)	1.555	1.165	2.075	+0.004
	+/+	5.9 (83)	1.546	1.159	2.063	+0.001
	-/+	8.1(77)	1.499	1.097	2.049	+0.039
	-/-	87.4(1319)	Ref	-	-	
Hypoglycaemic	Baseline	1.4(22)	1.536	1.154	2.045	
	+/-	1.2(19)	1.526	1.146	2.030	0.000
	+/+	2.7(41)	1.530	1.150	2.037	+0.001
	-/+	1.2(19)	1.526	1.147	2.032	0.000
	-/-	92.9(1402)	Ref	-	-	
Anti-hypertensive	Baseline	50.6(764)	1.562	1.170	2.085	
	+/-	28.8(436)	1.541	1.158	2.050	+0.052
	+/+	18.4(279)	1.555	1.170	2.068	+0.029
	-/+	65.3(986)	1.541	1.158	2.050	+0.052
	-/-	41.7(630)	Ref	-	-	
Anti-depressants	Baseline	6.69(101)	1.536	1.153	2.048	
	+/-	1.72(26)	1.528	1.147	2.035	+0.002
	+/+	2.18(33)	1.557	1.167	2.079	+0.004
	-/+	4.5(68)	1.531	1.150	2.037	0.000
	-/-	97.7(1475)	Ref	-	-	

Data presented are OR (95% CI) from binomial regression of incident CVD. Medication usage assessed through the Pharmaceutical Benefits Scheme linkage. Data include those men who were found to take selected medications up to 6 months prior to initial visit (baseline), had ceased taking selected medications between baseline and follow-up (+/-), those who had commenced taking selected medications after baseline visit and up to 6 months prior to follow-up visit (-/+), those who had been taking selected medications after baseline visit and up to 6 months prior to follow-up visit (+/+) and those who were found to have not used the selected medications (-/-) exposure referent category.

Appendix 7. Effect of anxiety, sleep duration, OSA and shift work on the observed regression estimates for associations between SHBG and incident CVD in multi-adjusted logistic binomial regression models

Predictors	Unadjusted		Adjusted					
	OR (95%CI)	p-value	+Age		+Age + SHBG +TT		Full multi-adjusted model	
			OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value
SHBG (nmol/L)	1.40(1.17,1.68)	<0.00	1.13(0.91,1.40)	0.27	-	-	1.53(1.14,2.04)	0.004
Testosterone (nmol/L)	0.76(0.61,0.96)	0.02	0.79(0.63,1.00)	0.05	-	-	0.72(0.53,0.98)	0.04
Anxiety (Yes/No)	1.03(0.46,2.28)	0.94	1.07(0.48,2.41)	0.86	1.01(0.45,2.29)	0.98	-	-
Sleep duration, hours (≤5.9, 6.0-8.5 & ≥8.6) ^a	0.87(0.35,2.12)	0.76	1.03(0.41,2.60)	0.94	1.24(0.48,3.21)	0.65	-	-
OSA (Yes/No) ^b	0.93(0.27,3.23)	0.91	0.97(0.27,3.45)	0.96	0.97(0.27,3.50)	0.96	-	-
Shift work, Years (Never, 0.1-2.9 years & ≥3.0) ^c	1.30(0.90,1.87)	0.16	1.29(0.89,1.88)	0.17	1.29(0.88,1.89)	0.19	1.32(0.85,2.06)	0.22

All values represent the standardised coefficients of regression analyses. Statistically significant associations (P <0.05) are shown in bold. A full multi-adjusted model including age, current smoking status, physical activity, diabetes, WC; hypertension with BP medication, HDL-C, hS-CRP, SHBG and TT.

Abbreviations: BP, Blood pressure; CVD, Cardiovascular disease; HDL-C, High-density lipoprotein cholesterol; hS-CRP, High sensitivity C-reactive protein; OR, Odds ratio; OSA, Obstructive sleep apnoea; SHBG, Sex hormone-binding globulin; TT, Total testosterone; WC, Waist circumference

^a510 ^b415 ^c526

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