

REVIEW



# Role of sex hormone-binding globulin in the free hormone hypothesis and the relevance of free testosterone in androgen physiology

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## Abstract

According to the free hormone hypothesis, biological activity of a certain hormone is best reflected by free rather than total hormone concentrations. A crucial element in this theory is the presence of binding proteins, which function as gatekeepers for steroid action. For testosterone, tissue exposure is governed by a delicate equilibrium between free and total testosterone which is determined through interaction with the binding proteins sex hormone-binding globulin and albumin. Ageing, genetics and various pathological conditions influence this equilibrium, hereby possibly modulating hormonal exposure to the target tissues. Despite ongoing controversy on the subject, strong evidence from recent in vitro, in vivo and human experiments emphasizes the relevance of free testosterone. Currently, however, clinical possibilities for free hormone diagnostics are limited. Direct immunoassays are inaccurate, while gold standard liquid chromatography with tandem mass spectrometry (LC–MS/MS) coupled equilibrium dialysis is not available for clinical routine. Calculation models for free testosterone, despite intrinsic limitations, provide a suitable alternative, of which the Vermeulen calculator is currently the preferred method. Calculated free testosterone is indeed associated with bone health, frailty and other clinical endpoints. Moreover, the added value of free testosterone in the clinical diagnosis of male hypogonadism is clearly evident. In suspected hypogonadal men in whom borderline low total testosterone and/or altered sex hormone-binding globulin levels are detected, the determination of free testosterone avoids under- and overdiagnosis, facilitating adequate prescription of hormonal replacement therapy. As such, free testosterone should be integrated as a standard biochemical parameter, on top of total testosterone, in the diagnostic workflow of male hypogonadism.

**Keywords** Free hormone hypothesis · Free testosterone · Male hypogonadism · Free steroid diagnostics · Androgens

## Introduction

Steroid hormones are biological signal transducers that are vital in coordinating diverse developmental and physiological processes. From fetal life into adulthood, steroid hormones regulate different important functional axes in

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the human body. Physiologically, steroid hormones are divided into three categories: sex steroids (androgens, estrogens and progestogens), corticoids (mineralocorticoids and glucocorticoids) and vitamin D [1]. While all of these steroid hormones serve a different physiological purpose, their steroidogenesis all starts with the same molecule, cholesterol. Hence, structurally, each type of steroid hormone is based on the classic cyclopentanophenanthrene four-ring structure, or a derivative [2]. Because of their lipophilic properties, transportation of steroid hormones to target organs and tissues via the bloodstream is mediated through binding proteins (BPs). In fact, the majority of steroid hormone molecules adhere to these BPs, leaving only a small fraction that circulates freely in human plasma [3]. The free hormone hypothesis delivers a rational approach on biological activity of these different fractions, thereby redefining hormonal exposure in terms of free steroid hormones.

Notwithstanding emerging evidence on the subject, the free hormone hypothesis remains somewhat controversial [4]. Multiple elegant manuscripts reviewing free fraction hormones have been published in the last decade, each one conveying a slightly different message to its readers [3–5]. Accordingly, the debate around free testosterone (T), and its potential use in clinical setting, caused the formation of two movements. While the majority of societies on endocrinology have implemented free T into its guidelines on male hypogonadism, the Australian Endocrine Society does not recommend its usage for clinical decision making [6, 7]. The purpose of this review is to update current views on the free hormone hypothesis using recent knowledge. In this review, we will highlight recent results from important *in vitro*, *in vivo* and human studies from our group and others, thereby refocusing the use of free T and driving its implementation into clinical context. As such, we will demonstrate the need for free T in both male and female patient populations, for example in the diagnosis of male hypogonadism and polycystic ovarian syndrome (PCOS). Furthermore, we will discuss the methodology on measured and calculated free T in great detail, including recent data showing that the Vermeulen formula deserves clinical recommendation as the free T calculator of choice. After highlighting biological relevance and added diagnostic value of free T in the clinical setting, we will conclude with a research agenda giving an overview of open questions and on obstacles yet to be cleared.

### Steroid hormone-binding proteins: gatekeepers of hormonal exposure

An essential factor to be considered in the free hormone hypothesis, is the contribution of plasma BPs. In human

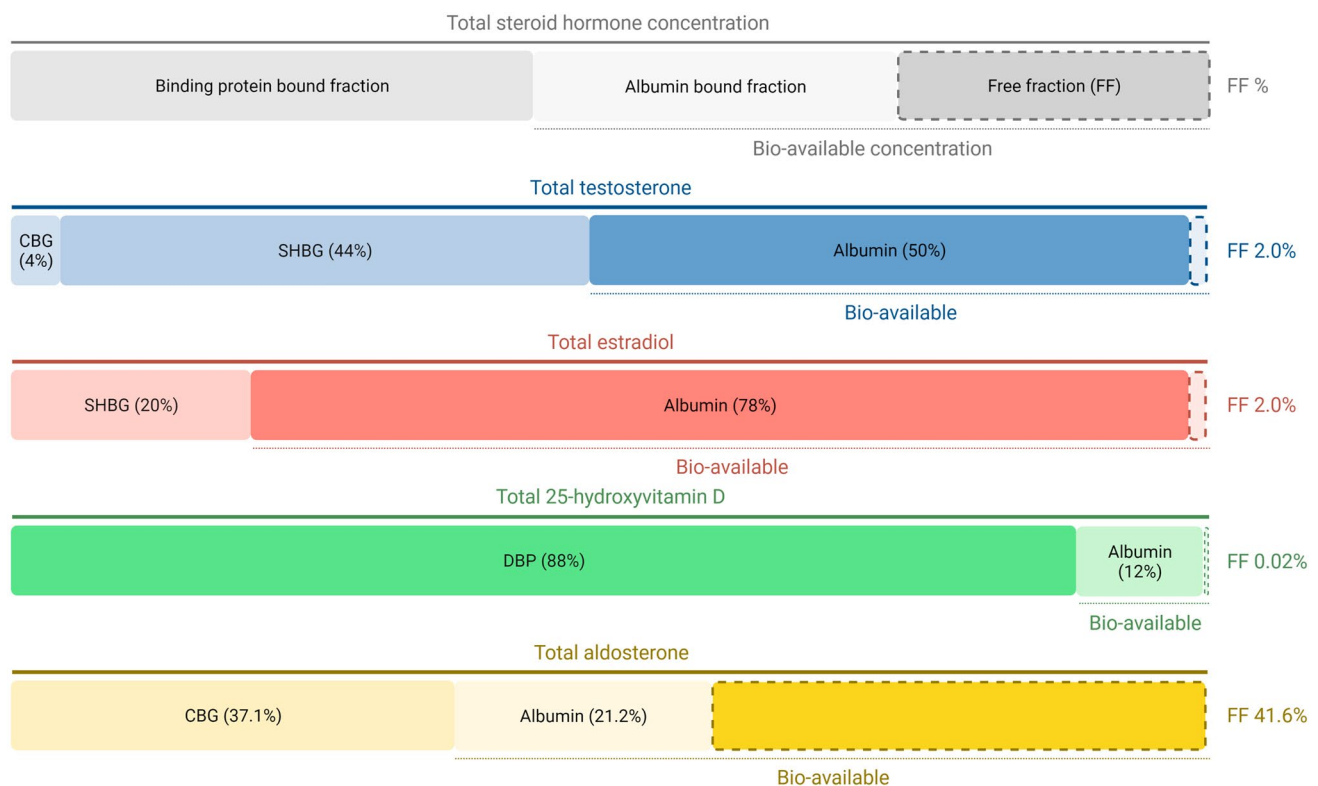
steroid physiology, several BPs have been identified. Not only do these BPs provide transport of steroid hormones from their production to their target site, but they also contribute considerably to the equilibrium between the free and protein-bound fractions in plasma. As such, BPs function as primary gatekeepers of steroid action [8].

### Binding proteins define steroid hormone fractions

Production of steroid hormones occurs at different sites in the human body, delineating a specific process for each hormone. Following biosynthesis, transport to target organs and tissues is facilitated by a group of plasma BPs that are either specific or non-specific carriers. Specific steroid carriers include SHBG which preferentially binds T, dihydrotestosterone (DHT), estradiol and other sex steroids, vitamin D-binding protein (DBP) for vitamin D and its metabolites and cortisol-binding globulin (CBG) that preferentially binds cortisol, aldosterone and progesterone. Additionally, thyroid-binding globulin (TBG) and transthyretin act as specific carriers for thyroid hormones. Next to specific transport, the most abundant human plasma protein, albumin, provides non-specific means of transportation for all previously mentioned components. As a result, three plasma hormone fractions can be defined: the non-protein-bound (free) fraction, the fraction bound to specific BPs (e.g., SHBG) and the albumin-bound fraction (Fig. 1). The free hormone concentration is determined by the level of total hormone, as well as the level of BP and albumin in circulation and the binding affinity of a hormone for its carrier(s), thereby characterizing the equilibrium state between the free and the bound fractions.

### SHBG and albumin define a delicate equilibrium in testosterone fractions

The bulk of T transportation is operated by three carriers: SHBG, CBG and albumin, respectively, representing 44, 4 and 50% of the total T fraction in human male plasma [9]. While the vast majority of circulating T is protein-bound, on average only 2% circulates freely [9]. Human SHBG is a glycoprotein that is mainly produced by the liver. It circulates as a 90–100 kDa homodimeric protein and is able to bind all androgens and estrogens, with the exception of dehydroepiandrosterone sulfate (DHEAS) and androstenedione. Recent studies on UK biobank data indicated that the median plasma SHBG concentration is 36.89 nmol/L in men. In women, median SHBG plasma concentration was valued at 60.34 and 53.56 nmol/L for pre- and postmenopausal age, respectively [10, 11]. This carrier protein is encoded by the *SHBG* gene, found on the



**Fig. 1** Steroid hormone fractions in human circulation—Illustration of the relative contribution (in %) of the binding-protein bound, the albumin bound and the free steroid fraction (FF) to the total steroid hormone pool. For each hormone, a color gradient is applied. The

least abundant fraction is graphically presented by the lightest color, while the most abundant fraction is the brightest. Free steroid fractions are framed by a correspondingly darker colored dashed line. Created with BioRender.com

short arm (p12-13) of chromosome 17, consisting of eight exons [12]. Besides expression in the liver, the gene is also transcribed in the brain, uterus, breast, ovary, placenta, prostate and testis [13]. Binding of sex steroid metabolites is accomplished in a binding pocket of the homodimeric protein, formed through association of two monomers, stabilized by the bivalent cations zinc and calcium, with each homodimeric protein holding up to two molecules. A serine residue at position 42 in the binding pocket is responsible for formation of hydrogen bonds with functional groups of *T* and estradiol [5, 14, 15]. The dynamic relationship between *T* and SHBG is characterized through their binding affinity, a measure for strength of interaction between metabolite and carrier. Among steroid hormones, *T* has the second highest association constant ( $K_a$ ), valued at  $1-2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , whereas DHT has the highest  $K_a$  for SHBG ( $2.69 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ) [16]. Therefore, binding between *T* and SHBG occurs rapidly and is very strong with little tendency of spontaneous dissociation. Binding affinities of SHBG, and all other previously mentioned BPs, have been subject to extensive experimental research, summarized in Table 1 [9, 16].

Albumin, designated as a non-specific transporter of *T* in the context of the free hormone hypothesis, is the most abundant protein in human plasma. It is a 66.5 kDa protein, circulating at a concentration of 35.0 to 50.0 g/L in both men and women and is mainly responsible for maintaining colloid osmotic pressure [17]. It also plays a major role in the transportation and homeostasis of diverse ligands (electrolytes, fatty acids, hormones, vitamins, drugs...) [18]. Synthesis of albumin only occurs in the liver, initiated by transcription of the *ALB* gene located on chromosome 4 (chr4q13.3) [19]. The binding affinity of *T* for albumin ( $K_a$  of  $\pm 4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) is five orders of magnitude smaller and weaker than binding affinity for SHBG. However, genetic variation in albumin could contribute to altered plasma albumin concentrations and/or binding affinity for *T*. To date, 71 genetic variants of albumin have been identified. One of these albumin variants, Roma c.1033G > A, has been linked to reduced binding of *T* [20]. Lower binding affinity for albumin, which has also been demonstrated for all other steroid and thyroid hormones, has raised discussion about the relevance of this albumin-bound fraction. Indeed, the albumin-bound fraction could, because of more probable spontaneous dissociation, potentially also have an effect on

**Table 1** Characteristics of binding proteins and their fractions for different hormones

Characteristics of binding proteins and fractions per hormone

Hormone	Specific carrier			Aspecific carrier			Free fraction (%)	Reference
	Binding Protein	Binding affinity (in $M^{-1} s^{-1}$ )	Bound fraction (in %)	Binding Protein	Binding affinity (in $M^{-1} s^{-1}$ )	Bound fraction (in %)		
Testosterone	SHBG	$2.0 \times 10^9$	44.0	Albumin	$4.0 \times 10^4$	50.0	2.0	[31]
				CBG	$5.3 \times 10^6$	4.0		[9]
Estradiol	SHBG	$7.0 \times 10^8$	20.0	Albumin	$6.0 \times 10^4$	78.0	2.0	[31]
25(OH)D	DBP	$7.0\text{--}9.0 \times 10^8$	88.0	Albumin	$6.0 \times 10^5$	12.0	0.02–0.04	[252]
1,25(OH) <sub>2</sub> D	DBP	$4.0 \times 10^7$	85.0	Albumin	$5.4 \times 10^4$	15.0	0.3–0.4	[253]
Cortisol	CBG	$8.0 \times 10^7$	90.0	Albumin	$3.0 \times 10^3$	7.0	4.0	[254]
Aldosterone	CBG	$2.0 \times 10^6$	37.1	Albumin	$2.0 \times 10^3$	21.2	41.6	[9]
T4	TBG	$1.0 \times 10^{10}$	70.0	Albumin	$1.5 \times 10^6$	5.0	0.03	[255]
				TTR	$2.0 \times 10^8$	20.0		
T3	TBG	$1.0 \times 10^9$	75.0	Albumin	$2.0 \times 10^5$	20.0	0.3	[255]
				TTR	$1.0 \times 10^6$	5.0		

This table shows binding proteins and their respective binding affinity constants (in  $M^{-1} s^{-1}$ ) for several hormone fractions found in human serum. Affinity constants were experimentally determined under normal physiological circumstances

SHBG sex hormone-binding globulin, CBG cortisol-binding globulin, 25(OH)D 25-hydroxyvitamin D, DBP vitamin D-binding protein, 1,25(OH)<sub>2</sub>D 1,25-dihydroxyvitamin D, T4 thyroxine, TBG thyroid-binding globulin, TTR transthyretin, T3 triiodothyronine

biological activity [21, 22]. Hence, the sum of the free and the albumin-bound fraction was determined to constitute the biological available (or bioavailable) amount in circulation (Fig. 1). More recently, however, interaction between the albumin-bound and free fraction has become even more complex. Binding dynamics of *T* to albumin have been generally believed to be in a 1:1 stoichiometric way. However, structural location of the binding sites in albumin by two-dimensional nuclear magnetic resonance has shown that the carrier protein possesses at least three binding sites for *T*, each with its own association constant. Moreover, evidence has been provided that these binding sites are allosterically coupled and, importantly, shared with free fatty acids and some commonly used drugs, such as naproxen, warfarin and exogenous glucocorticoids [23, 24]. Binding site competition and allosteric coupling can in certain conditions, especially postprandial and in circumstances of heightened levels of fatty acids and drug usage, result in displacement of *T* from its binding site thereby potentially affecting *T* bioavailability and/or metabolism [25, 26].

### The link between binding proteins and the free hormone hypothesis

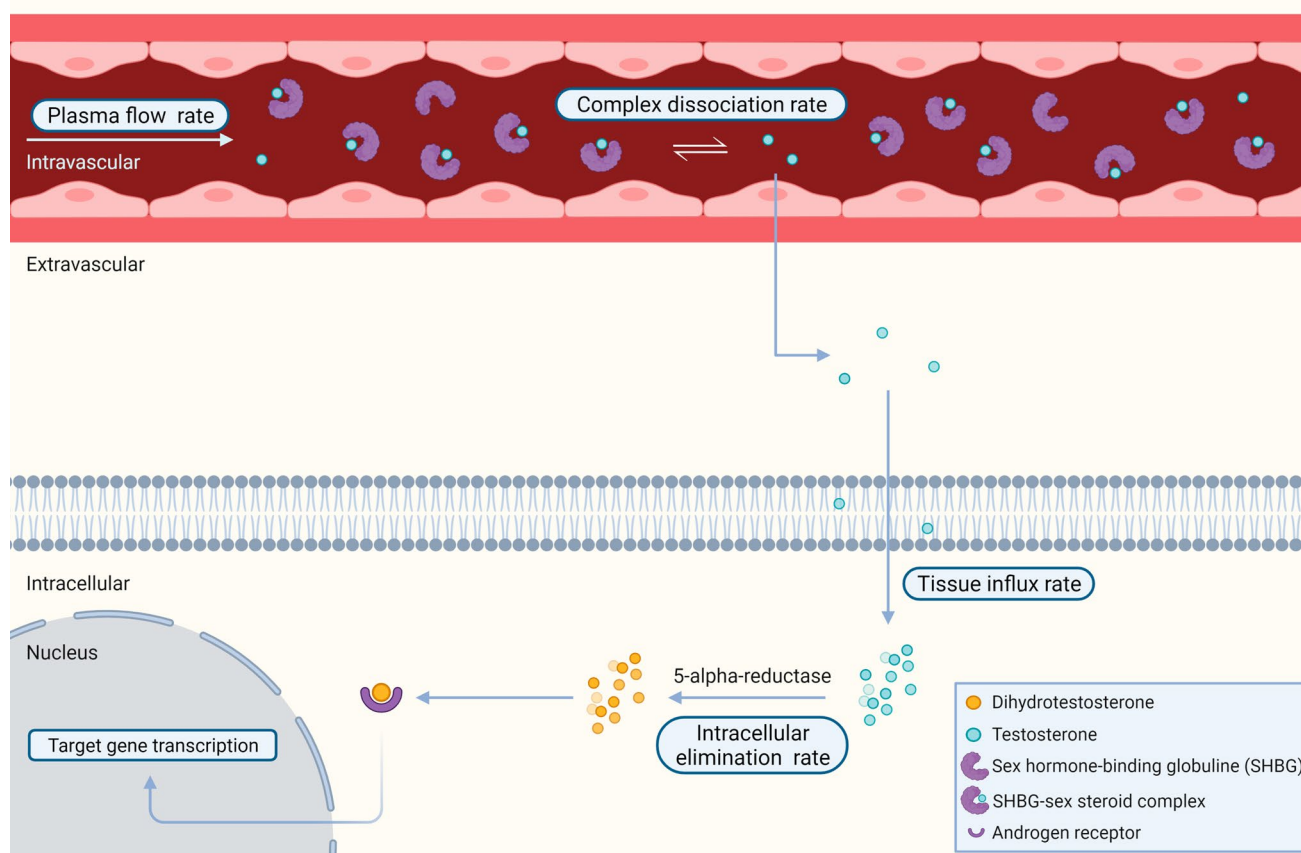
All BPs, whether specific or non-specific, together control tissue availability and metabolic clearance rate of a given hormone through regulation of the free hormone fraction. In the free hormone hypothesis, it is mostly this free fraction, and by extension the free hormone concentration, that accounts for the biological activity of hormone action.

### Unraveling the free hormone hypothesis: a model-based approach

The free hormone hypothesis asserts that biological activity of a given hormone is affected by the unbound (free) rather than the protein-bound plasma concentration [27]. In 1952, Recant and Riggs observed that patients suffering from nephrotic syndrome were euthyroid, without clinical signs of hypothyroidism, while in contrast their serum levels of thyroid hormone were far below the normal range. They, therefore, hypothesized the existence of an, at that moment analytically undetectable, unbound fraction exerting biological activity rather than the protein-bound fraction. While suffering from hypoproteinemia and concordant low protein-bound thyroxine, patients would thus still have a sufficient supply of free, unbound, thyroxine [28]. This observation was later formalized by Robbins and Rall in 1957, stating that the concentration of free thyroid hormone in the blood is the main determinant of thyroid hormone action. In accordance, the amount of thyroxine metabolized may also be a function of the level of free thyroxine in serum [29]. In the following years, major advances were made as BPs were characterized and knowledge of a free hormone pool, which is in continuous equilibrium with the protein-bound pool, became well established [30]. Additionally, similar experiments on the sex steroids *T* and estradiol were conducted by Heyns and De Moor. They succeeded to isolate the free from the protein-bound fraction, hereby enabling them to study binding kinetics of these sex steroids and their respective BPs [31].

Although many insights were achieved in terms of free serum hormones, it was not until 1989 that a sound mathematical and physiological model for the hypothesis was introduced by Mendel, referring to the hypothesis now generally accepted as the free hormone hypothesis [27]. Despite broadly dispersed and acknowledged, it is commonly confounded with the assumption that only the non-protein-bound (free) fraction of hormones is able to cross the cell membrane to the intracellular compartment, referred to as the free hormone transport hypothesis [32]. While the free hormone transport hypothesis might be valid, it does not then automatically follow that only the free hormone concentration in the plasma can affect intracellular hormone concentrations and, therefore, exert biological activity. Indeed, this last statement was illustrated by Mendel's mathematical models [27]. These models characterize the process of cellular steroid hormone uptake through four parameters: plasma flow rate, hormone dissociation rate from its BP, tissue influx rate and intracellular elimination rate. In each of the proposed models,

all representing different tissue types, one of these four factors operates as the rate-limiting factor in the uptake process. This in turn affects the validity of the free hormone hypothesis (Fig. 2). For instance, whenever intracellular elimination is considered the rate-limiting step in the net tissue uptake, the free hormone hypothesis is valid. This implies that intracellular hormone concentration, and, therefore, biological activity of this hormone, is predominantly determined by its free fraction. The hypothesis is, however, invalid when flow or dissociation from the BP is rate-limiting. In case of rate-limitation by influx, the free hormone hypothesis may be either valid (influx via the free hormone pool) or invalid (influx via the protein-bound hormone pool). Providing this knowledge, some general observations can be stated. If a particular hormone exerts an intracellular effect, but its structure is not metabolically altered by the cell, uptake of that hormone is by definition elimination-limited, pleading a valid hypothesis. In contrast, tissues that rapidly metabolize and thus alter the hormonal structure, for instance metabolic clearance by



**Fig. 2** Free hormone hypothesis—Illustration of the free hormone hypothesis for testosterone as proposed by Mendel. With respect to net tissue uptake of hormones, four rate-limiting factors were defined:

plasma flow rate, complex dissociation rate, tissue influx rate and intracellular elimination rate. Created with BioRender.com



hepatic tissue, are not elimination-limited pleading against the hypothesis. In summary, these findings highlight that the free hormone hypothesis is not expected to be valid under all possible sets of conditions. A single steroid hormone can be subjected to different models depending on the tissue type [27].

### The megalin/cubilin model does not impair the free hormone hypothesis

Although well established by Mendel, various researchers challenging the free hormone hypothesis have tried to demonstrate that the biological activity is mainly exerted by the protein-bound rather than the free fraction. The proposed mechanism for uptake of the hormone–BP complex is through the action of megalin and cubilin, via the process of receptor-mediated endocytosis. Megalin and cubilin are both large, multiligand, endocytic-membrane glycoproteins that are expressed in a wide variety of tissues, including but not limited to the kidneys, brain, endocrine glands and gonads. Functioning either individually or as a cooperative unit, they are imperative for the endocytic uptake of low molecular weight molecules such as DBP, hormones, enzymes and lipoproteins [33]. A megalin-knockout mouse model was used to provide evidence for the existence of a megalin-mediated cellular uptake pathway for the internalization of the T-SHBG complex. When megalin was absent, mice would exhibit an impaired testicular descent into the scrotum, delivering evidence that the uptake of the protein-bound fraction is also relevant for intracellular hormone action [34]. This research has, however, been challenged because of multiple flaws in the research design, hence not supporting the “bound steroid” hypothesis [35]. One of the counterarguments stated that mice lacking megalin suffer from severe vitamin D deficiency, accounting for the impaired gonadal development and function, rendering deductions for the T-SHBG complex incorrect [35].

While not applicable to *T* and SHBG, megalin- and cubilin-mediated endocytosis is, however, relevant in vitamin D metabolism. Being mainly expressed on the luminal brush border membrane of the renal proximal convoluted tubule, megalin and cubilin bind and reabsorb the vitamin D–DBP complex from the glomerular ultrafiltrate, hereby preventing uncontrolled loss of vitamin D metabolites in urine. Additionally, megalin-knockout mice as well as patients with diabetes or chronic kidney disease (CKD), in whom renal megalin expression and function is reduced, show extensive loss of both vitamin D metabolites and DBP in urine leading to a higher risk of vitamin D insufficiency [36, 37]. It is, however, important to state that the kidney is one of the major metabolizer organs in vitamin D biotransformation. Hydroxylation of 25(OH)D to its most active form, 1,25(OH)<sub>2</sub>D, by CYP27B1 is performed in renal tissue with

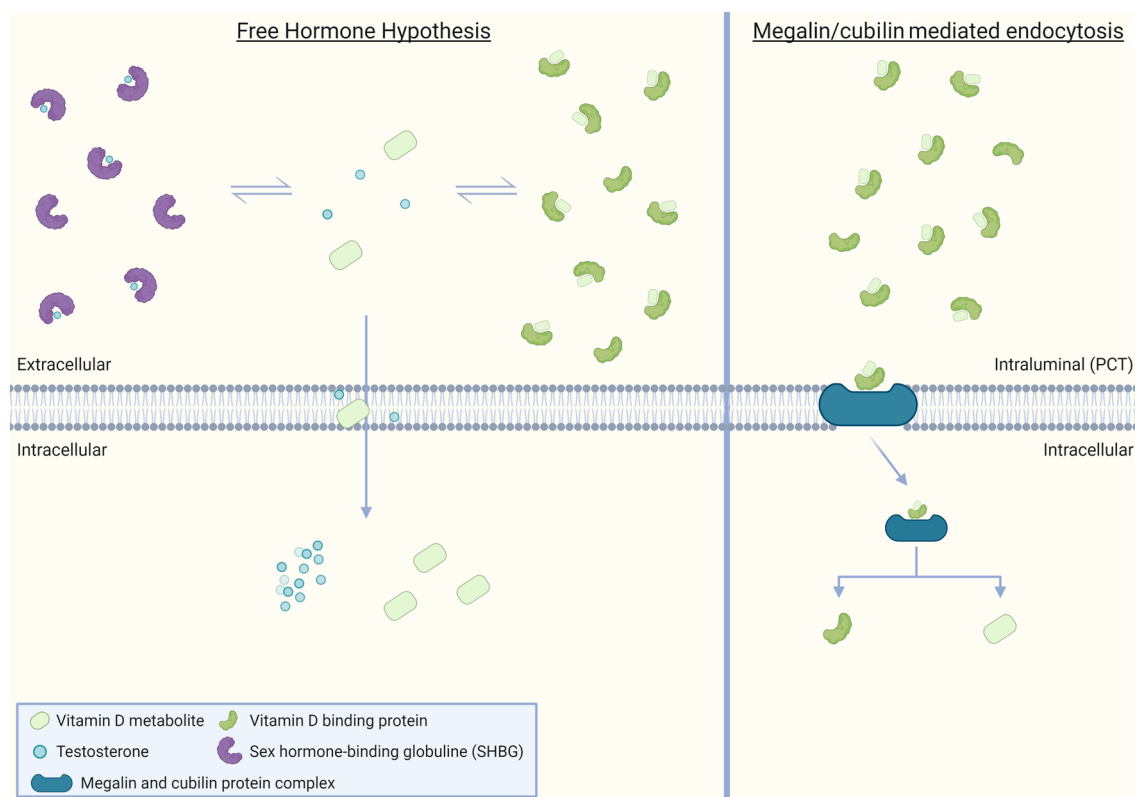
supply of 25(OH)D depending on megalin-/cubilin-mediated endocytosis [38]. Mendel’s mathematical model for the free hormone hypothesis describes that metabolizer organs or cells, in this case cells of the proximal convoluted tubule, have by definition a non-elimination limited uptake and, therefore, are not subject to the free hormone hypothesis. Hence, megalin-/cubilin-mediated uptake of the vitamin D–DBP complex is not a valid counterargument to the free hormone hypothesis [27]. Summarizing, while of crucial importance in vitamin D physiology, action of the megalin and cubilin complex is not applicable on sex steroids and SHBG (Fig. 3).

### Core support to the free hormone hypothesis: in vitro and in vivo data

Since its postulation by Mendel in 1989, understanding of the free hormone hypothesis has advanced significantly. New insights could possibly contribute to the expertise of the daily management of many endocrine disorders. Clinical relevance and utility of this concept is determined by the relationship between the different fractions of the plasma hormone pool and the clinical manifestation of the corresponding affected endocrine axis. Currently, serum total *T* concentration is still most often used as the primary determinant for the assessment of androgen status in clinical practice. However, consensus in the discussion on the most clinically useful measurement of sex steroids, total or free concentration, is yet to be reached [39]. In what follows, we present in vitro and in vivo data, delivering arguments in favor of the free hormone hypothesis, emphasizing the importance of SHBG and relevance of determination of the non-protein-bound fraction. Hence, this review pitches measurement of free *T* as an added value on top of total *T*.

#### In vitro

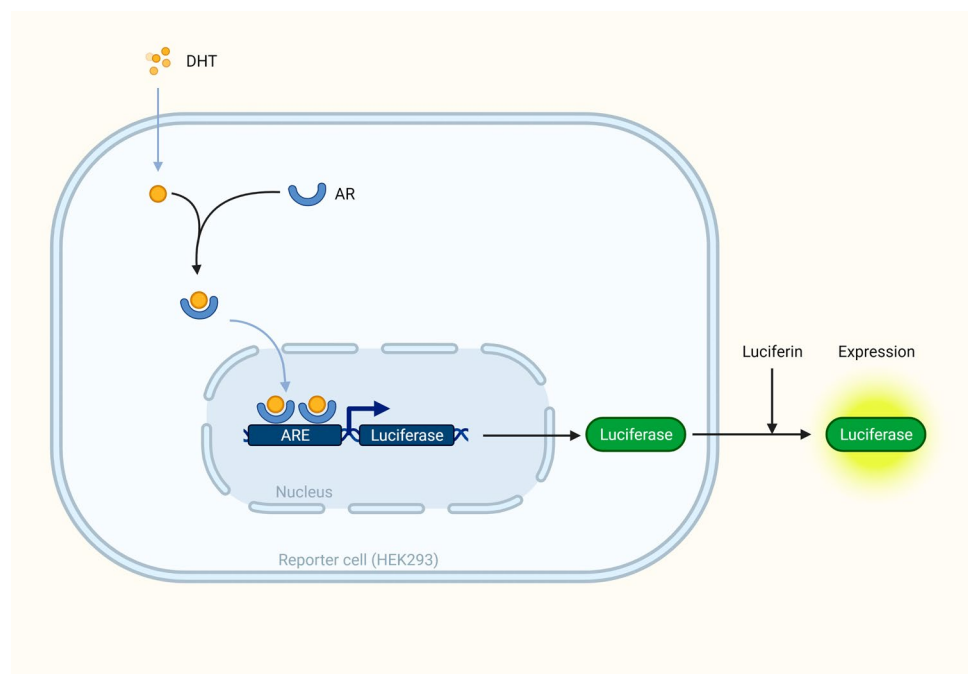
Assessment of in vitro androgen bioactivity was studied by our research group using a stable HEK-293 cell line transfected with a human androgen receptor cDNA construct [40]. Expressing the human androgen receptor, androgen activity is reported through a luciferase-coupled androgen response element (Fig. 4). Using this reporter assay, it was demonstrated that adding recombinant human SHBG to SHBG-deficient serum significantly reduces androgen bioactivity in vitro, hereby confirming the inhibitory effect of SHBG on androgen exposure [41]. In an extension of this first experiment, the impact of genetic variants of SHBG on androgen bioactivity was also investigated in vitro. As mentioned before, SHBG genetic variants affect SHBG and total *T* concentrations. However, genetic variation in SHBG also alters the binding affinity constant for *T* and DHT [42]. According to the free hormone hypothesis it should then



**Fig. 3** Free hormone hypothesis vs megalin/cubilin-mediated uptake—Left pane illustrates intracellular influx of free fraction testosterone and vitamin D metabolites by passive diffusion according to the free hormone hypothesis. Right pane represents uptake of the

vitamin D-binding protein–vitamin D complex mediated by megalin and cubilin in the proximal convoluted tubule (PCT) of the nephron. Created with BioRender.com

**Fig. 4** Cellular androgen reporter assay—to design the luciferase androgen reporter assay, HEK-293 cells were stably transfected with a cDNA construct of the luciferase enzyme coupled to an androgen responsive element (ARE) and minimal promoter. Following binding of androgens to the androgen receptor (AR), the complex translocates to the cell nucleus and activates the transcriptional machinery, which will result in a light reaction that can be measured. Created with BioRender.com



follow that an alteration in binding affinity influences the equilibrium between the SHBG-bound and free fraction and thus ultimately impacting androgen activity. Indeed, usage of genetic SHBG variants in combination with the luciferase androgen reporter assay modifies *in vitro* androgen bioactivity and reveals increased activity for genetic variants that lower binding affinity [41]. These results are in line with previously conducted *in vitro* studies that already demonstrated the inhibitory effect of SHBG on *T* action or uptake [43, 44]. Furthermore, we confirmed these findings in primary cultures of seminal vesicles [45]. In conclusion, these *in vitro* experiments highlight the importance of SHBG and its inhibitory effect on androgen bioactivity, conveying a major role to the carrier protein and further fortifying the validity of the free hormone hypothesis.

### In vivo

Although rodents express an SHBG homologue locally in the testis, the androgen-binding protein, hepatic expression of SHBG and SHBG secretion into circulation is absent postnatally. This deficit in circulating SHBG explains low levels and susceptibility to fluctuation of *T* in rodents [46]. As such, to study the regulation of SHBG expression *in vivo*, a mouse model expressing a human SHBG transgene has been developed [47]. These transgenic animal models expressing human SHBG had higher concentrations of *T* compared to wildtype mice [47]. In more recent work from our research group, SHBG concentration in SHBG transgenic mice has been shown to increase with age and is remarkably higher in males. In addition, total serum *T* and DHT are increased approximately 200-fold as opposed to wildtype and multiple *T* precursors (e.g., pregnenolone, androstenedione) were also found to be increased [45]. Curiously, although androgen levels were raised in SHBG transgenic mice, free *T* concentrations, measured directly by liquid chromatography with tandem mass spectrometry (LC–MS/MS) following equilibrium dialysis (ED), were not different compared to controls. However, despite similar free *T* levels in SHBG transgenic mice, luteinizing hormone (LH) levels were significantly higher than in controls. Moreover, following orchiectomy and implantation of a continuous-release *T* implant, free *T* was lower in transgenic compared to wildtype mice [45]. With these findings, we provided evidence that the rise in LH was explained by a SHBG-mediated decrease in free *T*, thereby triggering hypothalamic-pituitary feedback. The activated axis on its turn regulates and again raises free *T* concentrations by increasing testicular *T* production. Ultimately, free *T* and not total *T* is apparently the biologically active signal which directly feedbacks the hypothalamic-pituitary axis.

Phenotyping of the SHBG transgenic mice identified a partially compensated hypogonadism reflected in an

increased LH together with a minor, but significant, weight reduction of the seminal vesicles and pelvic bulbos cavernosus and levator ani muscles. Despite an extremely high amount of total *T* in these animals, weight reduction of the most androgen-sensitive organs provides *in vivo* evidence of androgen deficiency attributed to the inhibitory effects of high levels of SHBG [45]. More profound, administration of anabolic doses of *T* or DHT led to seminal vesicle hypertrophy in wildtype mice that is, however, prevented by SHBG in transgenic mice. Next to the androgen suppressing effect of SHBG, injection of castrated mice with *T*, DHT or mibolerone (a synthetic androgen with negligible binding affinity for SHBG) revealed that SHBG prolonged the circulating half-life of *T* and DHT, but not of mibolerone [45]. This confirms that SHBG has the capacity to bind and lengthen half-life of its ligands.

In summary, our group contributed a considerable amount of data from *in vitro* and *in vivo* experiments that lend direct and convincing support to the core statements that make up the free hormone hypothesis. First of all, the ability of SHBG to suppress androgen bioactivity was confirmed in both *in vitro* and *in vivo* studies. Second, this suppression is advocated by high affinity binding between SHBG and *T*, thereby rendering the SHBG-*T* complex biologically inert. Free *T* on the other hand appears to be able to cross the cell membrane and carry out its androgenic biological effect. Finally, regulation of this non-SHBG-bound fraction is under tight control of a negative feedback loop through the hypothalamic-pituitary axis. These arguments underline the physiological importance of SHBG and brand it as the gatekeeper of intracellular concentrations of *T* and its biological action in target tissues.

### Absence of binding proteins in human subjects favors biological activity through free hormones

Apart from *in vitro* and *in vivo* data, compelling human data in support of the free hormone hypothesis is also available. As discussed above, BPs are intrinsically important in establishing and maintaining the equilibrium between the protein and the non-protein-bound fraction. In fact, BPs themselves bear evidence in favor of the free hormone hypothesis. A family with a missense mutation within the SHBG gene was identified, which resulted in unmeasurably low SHBG concentrations in plasma. SHBG-deficient serum was obtained from a male subject in this family who suffered from low libido and erectile dysfunction, symptoms related to decreased *T*. This person, however, had normal male gonadal and pubertal development as well as reproductive function. Despite his extremely low total *T*, biochemical analysis uncovered normal free *T* [48]. The absence of elevated LH in this individual indicated a normal pituitary perception of *T* availability. The subjects symptoms (e.g.,



low libido and erectile dysfunction), possibly due to late onset hypogonadism, could in this case not be completely explained by *T* deficiency alone, since free *T* was normal. An analogous finding recently emerged for DBP and vitamin D. A patient was found to have a novel pathogenic variant of the GC-gene, encoding for DBP, resulting in undetectable levels of DBP but without any clinical signs of vitamin D deficiency. Biochemical evaluation of vitamin D metabolites indicated lowered values of total 25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D), but normal range values of the corresponding free fractions of these metabolites [49]. Both clinical cases provide support for the free hormone hypothesis and deliver additional arguments for the relevance of free metabolites as preferred indicators for clinical manifestations of hormone deficiencies.

## The impact of variability in SHBG on free hormones

### Life cycle evolution of SHBG levels

As mentioned previously, free hormone concentration not only depends on binding affinity of a given hormone for its carrier proteins, but also on BP concentrations. SHBG levels change during different stages of human life [50]. SHBG can be measured in fetal blood and amniotic fluid from 13 to 20 weeks of gestation and does not significantly differ at term (36–37 weeks) [51]. Human SHBG levels do not differ between male and female fetuses [52, 53]. Maternal SHBG levels on the other hand are subject to a significant five- to tenfold increase from early to mid and late pregnancy [54]. This gain in SHBG might serve as a mechanism to protect the mother against the pregnancy-induced increase of androgens [13]. This assumption seems indeed confirmed by a rare observation of a woman with extremely low SHBG, caused by a combination of two rare single nucleotide polymorphisms (SNPs), who exhibited symptoms of hyperandrogenism during pregnancy, whereas the twin daughters she gave birth to did not show any signs of virilization [55].

In neonates, SHBG increases shortly after birth, attributed to a parallel postnatal increase in thyroid hormones [56, 57]. This increase persists and SHBG remains high throughout childhood. During puberty, the concentration of SHBG falls in both sexes, with a stronger twofold decrease in men, resulting in adult men having only half of the SHBG concentration as compared to women of the same age [58, 59]. The reason for this remarkable decline during puberty remains poorly understood. In the last decade, however, several factors have been associated with this decline in SHBG. In a large cross-sectional study of 903 healthy subjects aged 6 to 20 years, body mass index (BMI), *T*, estradiol and DHEAS were found to be negatively correlated with evolution of SHBG levels from prepuberty into adolescence in men. In

women, however, this negative association with SHBG was only observed for BMI and DHEAS [60].

In contrast to declining SHBG levels throughout male puberty, androgen concentrations increase. However, in adult and elderly men the opposite occurs: SHBG concentrations increase whereas androgen levels decline. Indeed, the European Male Aging Study (EMAS) showed different cross-sectional age trends concerning SHBG and androgen status in community-dwelling men aged 40–79 years [61]. While SHBG is significantly higher in older age groups, total *T* and free *T* both display a decreasing trend with older age. This negative trend is, however, markedly stronger for free *T*, with a decrease of 1.3% per annum as compared to a decrease of only 0.4% per year for total *T*. These observations of decreased androgen levels and increased SHBG have been confirmed in the longitudinal Siblos Extension Study (SIBEX) [62]. In conclusion, age is an important contributor to variance in total *T*, free *T* as well as SHBG [61, 62].

### Genetic variability in SHBG influences hormonal status

Production of SHBG is a complex process that is influenced by hormonal, nutritional, metabolic and pharmacologic factors [63, 64]. Additionally, interindividual variation in SHBG levels has been shown to be highly heritable (between 30 and 80%). SNPs are important coding and/or regulatory sequence modifications in the *SHBG* gene that alter transcription, translation and thus production of SHBG in general. These SNPs can explain the relationship between genetic background and interindividual variation of SHBG and by extent, sex steroid status. Various polymorphisms in the *SHBG* locus and the X chromosome have been linked to substantial variations in SHBG and/or *T* concentrations (Table 2). Analysis of genome-wide association data of more than 8000 men identified that two SNPs within the *SHBG* locus are independently associated with serum *T* concentrations. Rs12150660, located 11.5 kb upstream of the major transcription start site of *SHBG*, represented the SNP with the strongest genotype-dependent difference in *T*. Mean serum *T* concentrations are found to be highest in men with the TT genotype, lowest for the GG genotype and situated in between for the GT genotype of this SNP. For the second SNP, rs6258, a rare missense polymorphism located in exon 4 of *SHBG*, lower concentrations of *T* were observed for the CT vs CC genotype. Additionally, as rs12150660 and rs6258 are located within the *SHBG* locus, a relevant association with SHBG concentration was demonstrated for both SNPs [65–67]. Differences in serum *T* concentrations for both SNPs, however, were still significant after adjusting for these altered SHBG concentrations [65]. Furthermore, the CT genotype for the rs6258 polymorphism results in reduced binding affinity of SHBG for testosterone. In fact, because of lower binding affinity of SHBG, the percentage free *T* was

**Table 2** Non-exhaustive overview of polymorphisms affecting SHBG and total testosterone levels

Polymorphism	Location	Reference allele	Alternative allele	ALFA allele frequency (alternative allele) (in %)	Physiological effect of the alternative allele		Reference
					SHBG	Total testosterone	
rs12150660	chr17:7,618,597	G	T	22.8	Increase	Increase	Ohlsson et al. [65]
rs6258	chr17:7,631,360	C	T	0.6	Decrease	Decrease	Ohlsson et al. [65]
rs727428	chr17:7,634,474	T	C	56.9	Increase	Increase	Grigorova et al. [66]
(TAAAA) <sub>n</sub>	5'-prime UTR	6 repeats	≥ 7 repeats	NA	Decrease	Decrease	Eriksson et al. [69]
rs1799941	chr17:7,630,105	G	A	23.9	Increase	Increase	Eriksson et al. [69]
rs6257	chr17:7,630,399	T	C	9.7	Decrease	–	Ding et al. [75]
rs6259	chr17:7,633,209	G	A	10.7	Increase	–	Ding et al. [75]
rs5934505	chrX:8,945,785	T	C	27.3	No effect	Increase	Ohlsson et al. [65]
rs10822186	chr10:63,590,623	A	G	50.4	–	Decrease	Jin et al. [77]

Polymorphism location and ALFA allele frequency were acquired from dbSNP: the NCBI database of genetic variation

22% higher in individuals with the CT vs the CC genotype [65]. For a third SNP in the *SHBG* locus, rs727428, the T allele accounted for a decrease in both SHBG and T levels, without affecting calculated free T [66, 68].

Important genetic polymorphisms have also been identified in the promotor region of *SHBG*, adjusting its expression levels. First, total T, and not free T, was elevated in subjects with six repeats, as compared to seven or more, of the (TAAAA)<sub>n</sub> repeat polymorphism. This effect occurred independently from SHBG levels [69]. Other studies confirmed this observation, although their data also suggest an increase of free T in subjects with nine or more repeats [70, 71]. Furthermore, the number of (TAAAA)<sub>n</sub> repeats was also inversely related to SHBG levels [69, 70]. Second, for the rare homozygous AA genotype of rs1799941, an increase in both serum SHBG and T was observed as opposed to the reference genotype [66, 69, 72, 73]. Although more recently the same AA genotype of rs1799941 was linked to lower calculated free T [74], this was not preceded by findings in earlier studies [66, 69, 73]. Finally, for carriers of two other SNPs, rs6257 and rs6259, a decrease and, respectively, an increase of SHBG was observed [75].

Similar to the polymorphisms specified above, all located on chromosome 17 within the *SHBG* gene or its promotor, associations to serum androgen levels have been reported for loci on other chromosomes. Rs5934505 is a SNP located at Xp22, positioned 79 kb downstream and 145 kb upstream of, respectively, *FAM9B* and *FAM9A* (*family with sequence similarity 9, member B and A*), two genes that are expressed exclusively in the testis [76]. *FAM9A* and *FAM9B*, are believed to be involved in, among other, the formation of the synaptonemal complex and DNA replication/recombination during cell division. Nonetheless, the physiological importance of these genes remains largely unclear [76]. In men presenting with a T versus C allele of rs5934505, a decrease in total and calculated free T, but not in SHBG

concentrations was observed [65, 77]. Another SNP, rs10822186 at 10q21, has also been associated with changes in T levels in men. This locus was connected to *JMJD1C*, a gene that might be involved in testosterone synthesis. A genetic variation in this gene may cause a change in circulating androgen levels [77].

Analogous to SHBG, comparable experiments have been carried out for DBP. As the major carrier for vitamin D in circulation, it serves a primordial role in determining total and free concentrations of vitamin D [3]. Similar to SHBG, over 120 genetic variants of DBP have been discovered of which three major polymorphic alleles are the most relevant ones (GC1f, GC1s and GC2), yielding six allelic combinations and three corresponding phenotypes [78]. A variation in geographical distribution is seen in these phenotypes, as for instance in Africans, GC1f is the most common allele while in Europeans GC1s is most frequently found [79]. This genetic variation has been deemed the main argument for the observation of lower levels of both DBP and 25(OH)D in subjects of African versus European origin, while levels of bioavailable 25(OH)D were similar in both ethnic groups [80]. However, as no racial difference in DBP was observed when using a polyclonal assay or applying LC–MS/MS, this conclusion was most likely the result of a monoclonal assay bias, confirming poor vitamin D status in subjects of African origin [81, 82].

## Regulation of SHBG levels

Biosynthesis of SHBG is mainly situated in hepatocytes and transcriptional control of the *SHBG* gene is, as discussed in the previous section, influenced by various factors [83, 84]. A mouse model with a gene addition of the 4.3 kb human *SHBG* uncovered that upstream of exon 1, a sequence of approximately 0.9 kb suffices for high gene expression levels

[47]. Further characterization of this upstream region identified binding sites for chicken ovalbumin upstream promoter-transcription factor (COUP-TF) and hepatocyte nuclear factor-4 alpha (HNF-4 $\alpha$ ) [85]. Competing for a mutual binding site, HNF-4 $\alpha$  and COUP-TF create a functional antagonism, respectively, actively recruiting or blocking the transcriptional machinery, which is essential for expression of SHBG in the liver. Their interaction serves as a molecular ‘on–off switch’ mechanism that explains the acute response of SHBG expression to changes in metabolic states [85].

Levels of SHBG in circulation are governed and affected by several determinants, summarized in Table 3. A consistent finding is the negative association between SHBG and BMI [86, 87]. Other indices used in assessment of central obesity, such as waist circumference and waist–hip ratio, also exhibit this negative association with SHBG [88]. Indeed, male and female subjects suffering from obesity show lower levels of SHBG compared to their non-obese peers [87, 89]. In fact, a negative association between obesity and SHBG is even present in overweight and obese children [90]. Moreover, following bariatric surgery, SHBG levels rapidly increase [91, 92]. Furthermore, this relationship between plasma SHBG levels and obesity is stronger than the positive association of SHBG with age described earlier. SHBG concentrations are highest in non-obese older men and lowest in obese younger men [87]. In contrast, in female patients with anorexia nervosa, SHBG concentrations were lower compared to normal weight subjects and increased to normal range after adequate weight gain, thus being a reliable index of nutritional status [93]. Apart from BMI, other conditions influence SHBG levels. For instance, in both sexes, HIV-positive individuals have increased SHBG concentrations compared to their seronegative peers [94–96]. Thyrotoxicosis is also featured by an increase in SHBG as well as increased total *T* concentrations [97]. Hypothyroidism on the other hand is accompanied by a decrease in both

SHBG and total *T* [98], which is reversible with adequate treatment [99].

Fluctuation of SHBG can also arise from administration of exogenous substances that display estrogenic and/or androgenic properties. Among others, estrogen-containing products, like combined oral contraceptives (COC) but not their progesterone-only counterparts, are associated with an increase in SHBG concentration and other major changes in several plasma protein profiles [100]. Tamoxifen, mainly used in treatment of breast cancer predominantly displays an estrogen-like effect on hepatic tissue, stimulating SHBG production [101, 102]. Similarly, the use of mitotane in the treatment of adrenal carcinoma, is associated with an increase in plasma SHBG levels [103]. Contrary to drugs with estrogenic effect, danazol, mainly used in context of endometriosis, is a compound of androgenic nature. Binding to SHBG with high affinity, it is capable of altering binding affinity of SHBG for *T* thereby displacing it from its carrier, subsequently increasing free *T*, and eventually leading to decreased concentrations in SHBG [104–106]. Affecting sex steroid balance in a different way, drugs without innate estrogenic and/or androgenic properties also exert their effect on SHBG levels. Administration of some antiepileptic drugs (e.g., phenobarbital, phenytoin and carbamazepine) can induce hepatic microsomal enzymes. This results in an increase of SHBG concentration that in turn influences free circulating androgen levels, lowering *T* bioavailability [107–109]. Administration of statins is associated with a decrease in SHBG [110, 111]. In another example, treatment with synthetic glucocorticoids, for instance dexamethasone, has also led to decreased SHBG levels in women with PCOS and in children with acute lymphoblastic leukemia [112, 113]. Clearly, SHBG levels in circulation respond to different endocrine and metabolic signals. This in turn affects circulating *T* levels and hence designates SHBG as a potential biomarker for disease.

**Table 3** Conditions associated with alteration of SHBG concentrations

Conditions associated with alterations in SHBG concentrations	
Increased SHBG	Decreased SHBG
Aging	Obesity
Thyrotoxicosis	Type 2 diabetes mellitus
Liver disease (e.g., cirrhosis, hepatitis)	Hypothyroidism
HIV infection	Nephrotic syndrome
Anti-convulsants	Increase in growth hormone and acromegaly
Therapeutic drugs with estrogenic properties (e.g., combined oral contraceptives)	Therapeutic drugs with androgenic/progestagenic properties
Selective estrogen receptor modulators (SERM) (e.g., tamoxifen)	Use of exogenous glucocorticoids
Malnutrition	SHBG gene polymorphisms
SHBG gene polymorphisms	

### SHBG: a potential biomarker of disease?

In the last decade, SHBG has been the center of attention in research on type 2 diabetes mellitus (T2D), obesity, PCOS, male hypogonadism, liver disease, bone health and even cancer. All these disorders have been associated with altered SHBG levels [114]. As a result, clinical usefulness of SHBG and its potential as a biomarker of disease has been questioned. To assess this potential, the classical definition of a biomarker is of great importance: “A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention [115].” Additionally, to be able to classify SHBG as an ideal marker, among other it should be specific to a particular disease and able to differentiate between different physiological states [116]. For the following reasons SHBG fails to meet this qualification. First, almost all SHBG associations result from cross-sectional study designs. As no prospective studies have been conducted, statements on causation are lacking. Second, SHBG shows a strong correlation to T, making it difficult to observe an independent SHBG effect. Finally, vice versa, designing a study to reveal an independent effect of SHBG proves challenging, as SHBG and T are tightly bound. In summary, while SHBG may not classify as an ideal diagnostic biomarker, it shows associations with different disorders. As such, and for further research purposes, we deem it useful to provide a concise overview of these associations.

### Glycemic control and progression to metabolic syndrome

SHBG has been implicated as a contributing factor to T2D and insulin resistance (IR), entities closely related to obesity. In a large longitudinal cohort study, lower SHBG was an independent risk factor for the development of T2D, inferring a five- or ninefold higher risk for male and female subjects, respectively, as compared to the reference group, hereby confirming findings from smaller studies [117–119]. The same inverse association between SHBG and T2D has been confirmed in multiple other studies in both men and women [120–123]. Importantly, the association between SHBG and T2D also holds after adjusting for BMI and other common risk factors, implying lower SHBG as a predictor for T2D is at least partially independent of BMI [118, 121, 124]. In addition, SHBG was also found to be negatively associated with plasma glucose, insulin levels and IR [125–127]. All taken into account, low SHBG concentrations could serve as an early indicator for T2D, or at least, for the underlying pathophysiological processes.

Although the inverse association of SHBG and plasma insulin levels in the obese population clarified the link between SHBG and T2D, the mechanism by which insulin acts on SHBG has been subject to decades-long debate. The

assumption was made that hyperinsulinemia, by an unknown mechanism, had a direct inhibitory effect on hepatic SHBG expression in vitro and in vivo [128, 129]. This direct inverse link between insulin and SHBG has proven to be invalid [130]. Experimental research in rodents demonstrated that the presence of monosaccharides, and not insulin, quickly regulates hepatic SHBG expression by altering hepatic HNF-4 $\alpha$  levels. Monosaccharides, also abundantly present in a state of IR, thus explain low levels of SHBG in this state of disease [131]. Additionally, monosaccharides seem to decrease SHBG levels in a dose-dependent manner after supplementation of either glucose or fructose by reducing SHBG promoter activity. In fact, in transgenic mice expressing human SHBG, fructose, which as opposed to glucose fails to stimulate pancreatic insulin secretion, can effectively further reduce SHBG levels below decreased SHBG levels by glucose, confirming the limited role of insulin in this process [131]. A decrease in SHBG can, therefore, characterize metabolic disturbance caused by dietary overconsumption of monosaccharides. While clearly evident that HNF-4 $\alpha$  functions as the central switch between SHBG and IR, the directionality of this relationship remains open for discussion as no prospective studies on the subject have been conducted. However, most cross-sectional study results seem to support the hypothesis that IR initiates a reduction in HNF-4 $\alpha$  that eventually leads to lower SHBG expression [132–134].

The pathophysiologic process of decreased SHBG attributed by excessive intake of monosaccharides is also involved in metabolic syndrome (METS) [133]. A relationship between SHBG and METS is particularly relevant as progression to this clinical syndrome involves multiple cardio-metabolic risk factors, among others visceral obesity and IR. As we have discussed, both obesity/BMI and T2D share an inverse association with SHBG concentrations. In line with these findings, low levels of SHBG seem to be predictive of developing METS at an older age [135, 136]. Furthermore, low SHBG levels also contribute to long term consequences of METS. Associations between low SHBG and higher risk of coronary and peripheral artery disease have been found, independent of other cardiovascular risk factors [137, 138]. All previous data show a convincing association between obesity/BMI, T2D, METS and SHBG. Focusing on metabolic disturbances, inclusion of SHBG in diagnostic evaluation protocols could be an asset.

### SHBG in nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease, possibly progressing to cirrhosis or hepatocellular carcinoma. NAFLD is mainly characterized by accumulation of triglycerides and fatty acids in the liver, in the absence of a secondary cause of fatty liver such as excessive alcohol use or viral hepatitis. Association



between NAFLD and total  $T$  is sex dependent. Whereas men with NAFLD have lower total  $T$  compared to their healthy peers, this association was not seen in women [139]. In fact, men with higher total  $T$  have a reduced risk of developing NAFLD, while the opposite association was seen for women [139–141]. Regarding calculated free  $T$ , literature remains undecided describing either an inverse, positive or no association with NAFLD [140–143]. For SHBG on the other hand, subjects of both sexes with NAFLD showed lower values compared to non-affected subjects, with a stronger inverse association in women than in men. Moreover, for both sexes, odds ratios showed a reduced risk of NAFLD with higher SHBG levels [139, 141]. Furthermore, exploring possible treatment options and prevention strategies for NAFLD, experiments in mice have shown that overexpression of human SHBG prevents fat accumulation in the liver [144]. While the link between SHBG and NAFLD progression is not yet fully elucidated, SHBG clearly shows associations to NAFLD.

### SHBG and bone health

A link between SHBG levels and osteoporosis has also been reported. [145]. A higher serum SHBG was observed in male patients suffering from primary or secondary osteoporosis [146–148]. SHBG also correlates, in both sexes, with an increase in bone resorption markers such as C-telopeptide of type I collagen and N-telopeptides, reflecting greater bone loss [146, 149]. Moreover, in men, bone mineral density (BMD) at the greater trochanter, femoral neck and the lumbar spine is negatively correlated with concentrations of SHBG [146, 150–152]. Multiple studies report a correlation between SHBG levels and fractures in elderly men as well [153–155]. In fact, according to a meta-analysis, each increase of 1  $\mu\text{g/dL}$  SHBG would lead to an additional fracture risk of 22% [156]. In addition, forementioned genetic studies on polymorphisms in the promotor region of SHBG confirmed the (TAAAA)<sub>n</sub> repeat and rs1799941 as independent predictors of BMD at the hip [69]. In women on the other hand, SHBG levels inversely correlate with BMD at various body sites, ranging from distal radius, proximal femur and greater trochanter to lumbar spine and even the whole body [157–159]. Interestingly, associations between total  $T$  levels and low bone density have been reported, but results are conflicting [146, 160–162]. For free  $T$ , lower values were reported in association with secondary osteoporosis [146], while another study could not confirm an association with BMD [160]. More importantly, free  $T$  levels have also showed to be able to predict osteoporosis-related fractures, even independently of BMD [163, 164]. All of these studies, both in men and in women, suggest an association between SHBG, bone health and fracture risk.

### Diagnosing hypogonadism in men: total versus free testosterone

Male hypogonadism is a clinical condition that can be subdivided into two types. Organic hypogonadism is characterized by a structural, congenital or destructive disturbance of the hypothalamic–pituitary–gonadal (HPG) axis, leading to the inability of the gonads to produce a sufficient amount of androgens resulting in clinical signs of androgen deficiency. Functional hypogonadism on the other hand refers to identical clinical signs and coexisting androgen deficiency without an intrinsic pathology of the HPG axis [165]. These androgens are, during the male life span, needed to promote masculine development/virilization and growth, and are of vital importance in reproductive functioning [166]. Manifesting differently between male individuals, symptoms of androgen deficiency vary according to age of onset, severity of  $T$  deficiency, androgen sensitivity and previous and/or current use of  $T$  replacement therapy [166]. These symptoms cover a broad clinical spectrum (Table 4). It is, however, clear that a number of these complaints overlap with other chronic diseases such as obesity and diabetes mellitus. In fact, multiple studies confirmed the association between low or inappropriate  $T$  levels in men and T2D and obesity [167–170]. Furthermore, because of high prevalence of cooccurrence of T2D and obesity, low  $T$  was unsurprisingly also associated with metabolic syndrome [171, 172].

While these clinical symptoms are the main motivation for patients to visit a physician, diagnostic emphasis of androgen deficiency has heavily shifted onto biochemical measurement of circulating  $T$ . Confirmation of deficient gonadal production of  $T$  is, therefore, reflected in the measurement of low total  $T$  in serum [173]. Despite being the first line test in diagnosis of hypogonadism in all practice guidelines, there is no agreement between these guidelines concerning differentiating low from normal circulating  $T$  (Table 5). Although there are no issues concerning test sensitivity and picking up low range concentrations of total  $T$ , a precise definition of the lower limit of ‘normal’ total  $T$  remains ambiguous. Multiple studies attempted to establish a reference range to achieve generally accepted values for total  $T$  in young men, ending in diverse intervals for all different cohorts [174–177]. A collaboration of European and American researchers and clinicians argued that these differences could be attributed to geographic region, interlaboratory assay validation as well as biological and environmental factors. In their effort to harmonize reference ranges, they established 264 to 916 ng/dL as a normal interval for total  $T$  in European and American non-obese men aging from 19 to 39 years. Using a reference method from the Centers for Disease Control and Prevention (CDC) they concluded that previous cohort inter-variability was mainly due to a substantial amount of inter-assay variation [178]. The use of this



**Table 4** Symptoms of androgen deficiency

Symptoms of androgen deficiency
<i>Fetal onset</i>
Absence or underdevelopment of male genitalia
Presence of female genitalia
Ambiguous genitalia
Cryptorchidism
<i>Pre- and pubertal onset</i>
Decreased volume of male reproductive organs (penis, testes, prostate)
Diminished secondary male characteristics (muscle mass, body and facial hair, voice pitch)
Hypopigmented scrotum
Gynecomastia
Low hair line
Decreased bone mass—eunuchoid stature
<i>Post pubertal onset</i>
Erectile dysfunction
Infertility
Decreased libido
Decreased energy and motivation
Symptoms of depression
Diminished secondary male characteristics (muscle mass, body and facial hair)
Gynecomastia
Decreased bone mass

**Table 5** Diagnosing male hypogonadism according to different guidelines, societies and research studies

Guideline/Society/Study	Year	Clinical diagnosis	Biochemical diagnosis		Reference
			Total testosterone (nmol/L)	Total testosterone (ng/dL)	
International Society of Andrology International Society for the Study of the Aging Male Association of Andrology	2009	At least one symptom or sign	< 8.0	< 231.0	Lunenfeld B. et al. [256]
European Male Aging Study (EMAS)	2010	Decreased libido Less spontaneous morning erections Erectile dysfunction	< 11.0	< 320.0	Wu F.C.W. et al. [257]
Endocrine Society Australia	2016	At least one symptom or sign	Young men < 7.4 Elderly < 6.4	Young men < 213.0 Elderly < 185.0	Yeap B.B. et al. [6]
Endocrine Society (ES)	2018	At least one symptom or sign	< 9.2	< 264.0	Bhasin S. et al. [7]
American Urology Association	2018	At least one symptom or sign	< 10.4	< 300.0	Mulhall J.P. et al. [258]
European Academy of Andrology	2020	At least one symptom or sign	< 8.0	< 231.0	Corona G. et al. [259]
European Association of Urology	2021	At least one symptom or sign	< 12.0	< 346.0	Salonia A. et al. [260]
Society for Endocrinology	2021	At least one symptom or sign	< 8.0	< 231.0	Jayasena C. et al. [261]

reference method should allow for interlaboratory harmonization of total  $T$  reference ranges in men. Caution, however, is still advocated when classifying men according to total  $T$  values. Currently, obesity is one of the leading major health problems, with over 650 million people affected worldwide [179]. Imposed by their obese status, these men are prone to lower total  $T$  as well as lower SHBG concentrations, implying a substantial risk for misclassification when using ‘normal’ reference ranges [180, 181].

While we agree that measurement of total  $T$  is and should remain the first step in diagnosing male hypogonadism, results of an observational study conducted by our research group confirmed the potential added value of free  $T$  in this context [182]. In our study, despite men having normal total  $T$  levels, a low calculated free  $T$  serum level was associated with androgen deficiency-related symptoms (sexual, physical and psychological functioning, biochemical measurements, body composition and bone measurements). Moreover, cases of low total  $T$  in combination with normal calculated free  $T$  were not associated with hypogonadal signs. With regard to our results, we recommend that free  $T$  should be included on top of total  $T$  in the evaluation of hypogonadism in men, especially in obese subjects [182]. Moreover, in HIV-positive men, underdiagnosis of male hypogonadism would have occurred in one third to half of the study subjects if only total  $T$  was measured as opposed to combining it with free  $T$  [183, 184]. These data underline the importance of free  $T$  values and adding this parameter to biochemical testing could, therefore, increase diagnostic sensitivity of male hypogonadism. Using this knowledge, the Endocrine Society (ES) decided to amend its guidelines on the diagnosis of hypogonadism in men, including free  $T$  as a novel parameter [7]. In the diagnostic approach proposed by the ES, total  $T$  should be measured in a fasting subject on two separate mornings. Additionally, determining free  $T$  is either advised when men are subject to conditions that alter SHBG levels or when initial measurement of total  $T$  was below or close to the lower limit of normal range. Furthermore, recommendations on determining free  $T$  are either direct measurement by ED or through calculation using total  $T$ , SHBG and albumin values [7].

### Free sex steroids in women with polycystic ovarian syndrome

PCOS is one of the most common endocrine disorders worldwide, with, depending on the criteria used, a prevalence rate of 4% up to 18% [185]. PCOS leads to infertility, menstrual problems and clinical manifestations of hyperandrogenism in women of childbearing age [186]. Women with PCOS are often obese, develop IR and are at risk of developing T2D [187, 188]. The pathological mechanism by which PCOS arises is very complex, multifactorial and still remains

largely unclear, but appears to be a disbalance in hormones, androgens and insulin in particular [189]. Furthermore, obesity may exacerbate features of PCOS by worsening IR and stimulating hyperandrogenism [190, 191].

In the diagnostic workup of PCOS, androgen excess is usually assessed by measurement of total  $T$  [187]. In routine clinical practice, total  $T$  is most frequently measured by immunoassay methods. However, some caution is warranted as commercially available immunoassays may not be suited to accurately and precisely determine low  $T$  concentrations in women [192]. As a consequence, total  $T$  by direct immunoassay misclassifies PCOS in 1 out of 3 subjects [193]. Hence, acquisition of total  $T$  results in women should be done by chromatography-mass spectrometry, which has become the gold standard for measurement of sex steroids [39, 187]. Notwithstanding more accurate total  $T$  results can be achieved with LC-MS/MS, total  $T$  may be an insensitive marker for hyperandrogenism in women [193]. In contrast to total  $T$ , free  $T$  seems to be a better marker to determine hyperandrogenemia in women with PCOS [187], provided that accurate and high quality methodology is used for measured or calculated free  $T$  [193, 194].

Together with free  $T$ , SHBG has recently been recognized a useful diagnostic marker and therapeutic target for managing women with PCOS [195]. A meta-analysis showed that subjects with PCOS had remarkably lower levels of SHBG and that this effect was even more pronounced in subjects with concomitant obesity [196]. In the evaluation of androgen excess in women suspected with PCOS, it is thus important to evaluate both free  $T$  and SHBG, as they reflect both metabolic and ovarian disturbances associated with this disease [197].

### Methodology on calculated and measured free testosterone

Provided the evidence that free  $T$  gains clinical importance in the diagnostic landscape of male hypogonadism, several methods for the assessment of this free fraction have been developed and are currently available. Moreover, not only can free  $T$  be of value in the clinical context of hypogonadism, but, as discussed in another section of this review, due to alterations in SHBG and total  $T$ , free  $T$  might be of interest in several other pathologies. In clinical setting, assessment of free  $T$  can be carried out either by direct measurement or through calculation.

### Measurement of free testosterone

Measurement of serum free  $T$  through ED is, as recommended by ‘The Princeton Consensus Statement’ and many other publications, the well-established reference method [198]. This technique allows for dialyzing a patient’s serum

or plasma across a semipermeable membrane, retaining protein-bound  $T$  in the process. Whereas SHBG and albumin-bound  $T$  are withheld from the dialysate, unbound (free)  $T$  can freely pass. After reaching equilibrium, the dialysate can subsequently either be analyzed by a direct or indirect method, thereby describing the technique as direct or indirect ED. In direct ED, measurement of the free hormone concentration in the dialysate is directly performed by LC–MS/MS. Indirect ED on the other hand uses a labeled (usually tritium) tracer steroid, and the measured total steroid concentration, to proportionally determine the free steroid concentration. Indirect ED, as compared to direct ED, is subject to several limitations, such as tracer instability, tracer purity and need of a dilution step, that all introduce possible sources of added total error to the free steroid measurement [199, 200]. Hence, direct ED using LC–MS/MS is the preferred method for measurement of free serum hormones.

Different other methodologies for assaying free fraction steroids exist, but these are beyond the scope of this review. Briefly, LC–MS/MS coupled ultrafiltration (UF) constitutes a comparable alternative to ED. UF has also previously been described in the validation of a reference measurement procedure for free  $T$  [200]. Little data exist on the validation of UF versus ED, yet UF is a relatively convenient technique and when properly validated can display equal performance to ED [199, 201, 202]. Similar like ED, UF deals with its own technical issues. Most common pitfalls of UF are due to non-specific binding, steroid adsorption to the filtration membrane and protein leakage through the filtration membrane [200, 203]. Additionally, an analog-based immunoassay has been developed. These methods claim to measure free hormone directly in serum. Nevertheless, inadequate accuracy, bias and dependency on  $T$  rather than free  $T$  are major drawbacks in this analog-based assays [204–206]. Comparative studies of this analog-based immunoassay and ED confirmed that this technique does not detect nor quantify free  $T$  and that is should, therefore, not be used as an assay for free  $T$  [205, 207, 208]. This led the ES to recommend against application of this assay in clinical settings [39]. While use of this analog-based technique is clearly decreasing, surprisingly, a survey conducted in Northern America acknowledged that in 2011 approximately 33% of queried clinical laboratories still used this analog-based test as primary test to measure free  $T$  [209].

As mentioned before, it is not only possible to assess the free fraction, but also the bioavailable fraction of sex steroids. The bioavailable fraction of  $T$  consists of both the free  $T$  fraction and the albumin-bound  $T$  fraction. In this definition of bioavailable  $T$ , the albumin-bound fraction is designated part of the usable  $T$  pool because of the low  $K_a$  between  $T$  and albumin, making the binding between ligand and carrier prone to dissociation [21, 22]. Gold standard for measurement of bioavailable  $T$  is a direct precipitation

method with either ammonium sulphate or concanavalin A. Both substances form a complex with SHBG, resulting in precipitation of the carrier and by extension the SHBG-bound  $T$ . Addition of a tracer element,  $^3\text{H}$ -testosterone, to the sample or direct measurement by immunoassay or LC–MS/MS, allows for assessment of both the free and albumin-bound fraction in the supernatant [208, 210]. However, this method is rarely used in diagnostic laboratories as it is not automated, cumbersome to execute and may involve radioactive tracer elements. Furthermore, because it is not standardized, large variability in bioavailable  $T$  results may exist between laboratories [208]. Additionally, measurement or calculation of bioavailable  $T$  is not mentioned in any clinical guidelines [6, 7]. As such, bioavailable  $T$  is only used in a limited way and evidence of clinical associations is scarce. For now, we would not recommend using bioavailable  $T$  as an alternative for free  $T$ .

### Calculation-based free testosterone

Because of limited availability of ED in clinical settings and the unsuitability of direct free testosterone assays, values for free  $T$  are frequently obtained through calculation. Multiple calculation algorithms, using other serum-measured biochemical variables, e.g., SHBG, albumin and total  $T$  concentrations, have been computed. These formulas depend on the precision and accuracy of methodologies of their included variables. Immunoassays for total  $T$ ; SHBG and albumin are validated diagnostic techniques. Despite their high availability and improvements over the last 30 years, performance issues with regard to precision and accuracy still exist. On the other hand, LC–MS/MS provides a more accurate measuring method for total  $T$  [39, 211]. A wide spectrum of algorithms for free  $T$  exists, which can broadly be divided into three categories (Table 6). First, several equations were derived from the general law of mass action. Association constants for albumin and SHBG used in these formulas were obtained from in vitro experiments by linearizing data through Scatchard analysis [16, 208, 212]. A second category contests the principle of Scatchard analysis, refuting the assumption that SHBG expresses linear binding kinetics [213]. This model takes into account allosteric interaction between binding sites for  $T$  on the SHBG homodimer. Empirical models exploiting bootstrap regression methods on cohort datasets comprise the third and last category [214, 215]. All these models have respective strengths and intrinsic limitations, as described in an extensive review [5].

An alternative method for free fraction  $T$  calculation is the use of the free androgen index (FAI). The FAI is calculated by the ratio of total  $T$  to SHBG, delivering an alternative way of estimating free androgens. Because of its accessibility, the FAI is widely utilized in clinical laboratories and clinical studies spanning different disciplines in medicine [216, 217].

**Table 6** Different calculation models for free testosterone

Method	Equation variables				Binding affinity SHBG (in M <sup>-1</sup> s <sup>-1</sup> )	Binding affinity albumin (in M <sup>-1</sup> s <sup>-1</sup> )	Binding affinity CBG (in M <sup>-1</sup> s <sup>-1</sup> )	Reference
	TT	SHBG	Albumin	CBG				
<i>Algorithms based on linear models of binding between testosterone and SHBG</i>								
Vermeulen	x	x	x	—	1.00 × 10 <sup>9</sup>	3.60 × 10 <sup>4</sup>	—	[208]
Mazer	x	x	x	x	1.00 × 10 <sup>9</sup>	3.60 × 10 <sup>4</sup>	0.313 × 10 <sup>9</sup>	[16]
Sodergard	NA	NA	NA	NA	5.97 × 10 <sup>8</sup>	4.06 × 10 <sup>4</sup>		[212]
<i>Algorithms based on non-linear models incorporating allosteric interaction for SHBG</i>								
Zakharov	x	x	x	—	First testosterone molecule: 3.82 × 10 <sup>7</sup> or 0.25 × 10 <sup>9</sup> Second testosterone molecule: 1.50 × 10 <sup>9</sup> or 1.80 × 10 <sup>9</sup>	0.28 × 10 <sup>4</sup>	—	[213]
<i>Algorithms derived from empiric bootstrapping</i>								
Ly/Handelsman	x	x	—	—	—	—	—	[214]
Sartorius	x	x	—	—	—	—	—	[215]

Algorithms are divided into three categories depending on how they were derived: linear binding of testosterone to SHBG, non-linear binding taking into account allosteric interaction and empiric bootstrapping. For each algorithm an indication is made as to which variables are used in the equation. Binding affinity for these variables is also displayed. In case of Zakharov method, both the first and second molecule of testosterone can have two binding affinities. The applicable binding affinity for each molecule is governed by two inter-converting microstates in unliganded SHBG

TT total testosterone, SHBG sex hormone-binding globulin, CBG cortisol-binding globulin

Additionally, in many research designs on hyperandrogenism in women, FAI is a key parameter of interest [218, 219]. Caution, however, should be exercised, as the FAI is highly impacted by SHBG concentrations. In human subjects, male or female, in whom SHBG concentrations are altered, FAI could increase or decrease dramatically. This results in an artificial over- or underestimation of free fraction androgens [220]. As dissented previously in this review, SHBG concentrations are subject to change in pathologies such as obesity, diabetes and metabolic syndrome. It is, therefore, not advised to use FAI as a clinical decision maker.

### Agreement between measured and calculated free testosterone

Regrettably, all the methods described above leave researchers and clinicians with the critical question of which method provides the best fit to delimit free  $T$  values. To address the matter, a Belgian research consortium including some of the authors of this review, assayed over 300 serum samples of human males and females for free  $T$  [221]. Measurement of free  $T$  on all samples was performed by gold standard LC–MS/MS coupled ED. In addition, free  $T$  for each sample was calculated using the Vermeulen, Zakharov and Ly/Handelsman model. Median bias, expressed by the ratio of calculated to measured free  $T$ , was 1.19, 1.0 and 2.05 for the Vermeulen, Ly/Handelsman and Zakharov model, respectively. Our data also demonstrated that both Ly/Handelsman and Zakharov methods do not operate independent of SHBG,

total  $T$  and to a lesser extent albumin values [221]. This is particularly important in distinct conditions where SHBG and total  $T$  are in the lower range, increasing probability of underestimating free  $T$  by calculation. Underestimation may pose a problem, especially in clinical settings of hypogonadal men (low total  $T$ ), obesity (low SHBG) and women with PCOS (low SHBG). Paradoxically, assessment of these patient groups is also commonly the most relevant reason for requesting free  $T$ . In contrast we concluded that, although overestimating free  $T$  by 20–30%, the formula according to Vermeulen was independent of SHBG, total  $T$  and albumin [221]. Taken into consideration the moderate bias level, but most importantly less influenced by the latter three variables, the Vermeulen formula, for the time being, still appears to be the most robust approximation and deserves our recommendation as free  $T$  calculator for clinical use.

### Free testosterone in clinical practice: limitations and approaches

Direct measurement of free  $T$  using LC–MS/MS coupled ED is currently, as previously discussed, the gold standard. Essentially obtaining accurate results, execution of this assay is, however, very labor intensive and technically challenging, resulting in limited availability of the method in everyday clinical routine [222]. In fact, currently non-automated, the process is subject to operator-dependent errors. Apart from these technical difficulties, assay performance is also affected by various conditions and error sources, governing

high assay variability. Adhesion of components to the membrane or the dialysis chamber, dilution, assay temperature and buffer characteristics such as volume, pH and composition all impose differential effects to binding parameters and equilibrium conditions leading to distinct quantifications [39, 200, 208, 223–226]. In addition to these technical issues, deployment of an ED technique adds extra costs to the clinical laboratory practice [227]. As such, LC–MS/MS coupled ED is mostly only available in academic laboratories. Additionally, measurement of (free)  $T$  is of less commercial interest, unlike (free) thyroid hormones. Whereas thyroid diagnostics make up a large part of daily laboratory tests requested by almost all clinicians,  $T$  is mostly only requested by endocrinologists and urologists. Hence, in the past decades, more resources have been invested in clinical thyroid diagnostics, enabling commercialization of kits for the clinical laboratory allowing direct measurement of free thyroid hormones. In contrast, less funds were, and still are, directed on development of assays for free  $T$ .

There is, however, a fairly good agreement between measured and free  $T$  calculated by the Vermeulen formula, if validated against ED [221, 228]. In fact, in clinical settings where direct measurement of free  $T$  is not available, the Vermeulen formula can serve as an acceptable clinical alternative. In addition, calculation as opposed to direct measurement also presents as a more convenient and cheaper quantitative metric. Summarized in Table 7, users of free  $T$  calculators should on the other hand also consider the different imperfections of these clinical utilities. Most importantly, in these models, free  $T$  is calculated through values for SHBG, albumin and total  $T$ . Consequently, calculation quality depends on performance characteristics of the assays used to determine these three variables. Second, concerning the interaction between SHBG and  $T$ , variability and assumptions in terms of binding affinity, stoichiometry and allosteric interaction produce different quantities of free  $T$

in different models [5, 208]. Third, all of these models are in fact simplified versions of the true binding milieu and may potentially not include all variables affecting the equilibrium between total and free  $T$  (e.g., role of CBG). Finally, for both measured and calculated free  $T$ , reference ranges may not be universally valid. Depending on the method to assess free  $T$  as well as characteristics of the cohort, different intervals may be generated [177, 229]. In extension, there is no consensus on a universal cut-off between low and normal values for free testosterone. Further research efforts on harmonizing these reference ranges are, therefore, needed.

In summary, we would suggest a pragmatic and practical approach on assessing androgen status in a clinical setting. Measurement of total  $T$  and SHBG is standard of care, which in any case also allows for additional calculation of free  $T$ . In an altered SHBG-binding milieu, or when total  $T$  is borderline normal or below the lower limit of normal, accurate determination of free  $T$  can subsequently be carried out using LC–MS/MS coupled ED.

## Linking clinical endpoints to free testosterone

### Androgen-related clinical endpoints

Illustrated in the previous sections of this review, free  $T$  has its role in the diagnosis of male hypogonadism. In the assessment of quality of life in hypogonadal patients, physicians mostly rely on androgen-dependent clinical endpoints. While these endpoints have been profoundly studied in terms of total  $T$  concentrations, evidence on the link between free  $T$  and these endpoints is more scarce. Interestingly, while demonstrated that, in case of the Vermeulen formula, calculation of free  $T$  leads to a constitutive positive bias of 20–30% compared to direct measurement using ED, almost all current research uses one of the previously mentioned calculation models to study associations with clinical endpoints.

**Table 7** Assets and liabilities of free testosterone calculators

Assets of calculated free testosterone	Liabilities of calculated free testosterone
When validated methods are used to measure total $T$ and SHBG, calculated free testosterone is in good agreement with measured free testosterone	Highly dependent on quality of SHBG and total testosterone assays
Calculation methods are convenient, accessible and inexpensive	Reference ranges are method-specific and due to variability in analyzed cohort samples may not be universally applicable
If direct measurement is unavailable, calculators are an acceptable clinical utility	Variability in calculation of free testosterone between different calculators
Population data supporting associations with androgen-sensitive clinical endpoints is available	Different binding affinity constants and stoichiometry for SHBG-testosterone are used in the different models
	SHBG polymorphisms, although rather rarely observed on population level, may impact binding affinity for testosterone
	Suboptimal accuracy with a positive bias is observed in concordance with directly measured free testosterone



The Swedish Mister Osteoporosis (MrOS) study in a cohort of 2908 men, aged 75.4 years on average, demonstrated that calculated free  $T$  is a modest independent predictor of BMD at predominantly cortical bone sites. Moreover, a free  $T$  concentration below the median served as an independent predictor for osteoporosis-related fractures [163]. Related to these markers of bone health is frailty, which is of particular interest in the elderly. Several adverse outcomes such as disability, falling and death are associated with the concept of frailty [230]. In the European Male Ageing Study, frailty was assessed using two approaches: frailty index and frailty phenotype. In both cases, after adjusting for age, smoking, BMI and other comorbidities, low levels of calculated free  $T$  and not total  $T$  concentrations were associated with a greater likelihood of frailty [231]. These findings confirmed earlier conclusions by an Australian research group that prospectively assessed androgen status and frailty in community-dwelling men aging 76–93 years. Calculated free  $T$  was found to be independently associated with frailty in both a cross-sectional and a longitudinal way [232]. The Framingham Offspring Study included 1445 men with a mean age of 61 years that were examined for physical performance and mobility limitation. Higher calculated free  $T$  was associated with a lower incidence in mobility limitation. This was reflected in higher walking speed, a firmer grip and better subjective health perception for community-dwelling men with a higher baseline free  $T$  [233]. Additionally, bioavailable  $T$  has also been studied in relation to frailty. Assessing 1469 community-dwelling men aged 65 years and older, the US MrOS study revealed an independent association of bioavailable  $T$  with frailty status. After multivariate adjustment, men in the lowest quartile of bioavailable  $T$  were subject to a 1.39-fold increased risk of greater frailty compared to men in the highest quartile [153].

Besides bone health, other clinical endpoints were found to be associated with free  $T$ . An Australian cross-sectional study in 2932 men, aged 70 to 89 years, unveiled that calculated free  $T$  levels are positively associated with cognitive performance, independent of age, mood and physical comorbidity [234]. In the same cohort of men, a weak inverse association between calculated free  $T$  and depressive mood was observed, independent of physical comorbidity [235]. In a study on hematopoiesis, parameters such as hemoglobin, hematocrit and red blood cell count seem to be positively correlated with calculated free  $T$ , with the effect remaining significant after correction for patient age [236].

### Clinical outcomes in other diseases

In related research, calculated and/or measured free  $T$ , as opposed to total  $T$ , were found to correlate stronger with clinical outcomes of NAFLD, PCOS and hypertension [143, 237, 238]. For example, in oligomenorrheic subfertile

women with a suspicion of PCOS, total  $T$  as well as calculated free  $T$  correlated closely with ovarian volume and antral follicle count. While calculated free  $T$  also correlated with metabolic parameters such as BMI, insulin and IR, this association became insignificant after correction for BMI [197]. Inversely related to and impacted by BMI, SHBG is a linking entity and can partly explain the relationship between calculated free  $T$  and these metabolic parameters [87]. In contrast to obese men, in whom SHBG and total  $T$  concentrations decrease but free  $T$  remains stable, obesity in women causes, next to a decrease in SHBG, a higher free  $T$  [182, 239]. In PCOS and obesity setting, this fall in SHBG is unable to buffer rising free  $T$  concentration, leading to androgen excess and clinical consequences in these women [197]. Additionally, free  $T$  positively correlated with LH as well as the LH/follicle stimulating hormone (FSH)-ratio, independent of BMI. Favoring LH over FSH synthesis in the pituitary gland, this disturbance of the hypothalamic–pituitary–ovarian axis by androgen excess hinders cyclic production of estradiol and progesterone, causing oligomenorrhea and ovulatory dysfunction [197, 240]. Hence, in comparison to men in whom hypothalamic–pituitary feedback regulates free  $T$ , women lack this regulatory feedback mechanism making them more susceptible to decreasing SHBG concentrations.

Similar findings were made for other steroid hormones. For instance, activation and reserve capacity of the hypothalamic–pituitary–adrenal (HPA) axis in critically ill patients undergoing surgery was found to correlate better with serum free than total cortisol concentrations. The use of serum total cortisol alone could lead to a misdiagnosis of HPA axis insufficiency in up to 23% of these patients [241, 242]. Valorization of a patient's vitamin D status in clinical settings where DBP levels are altered, such as CKD, obesity and liver problems, has also been deemed more appropriate when using free serum 25(OH)D as compared to total serum 25(OH)D [243–246].

Although free  $T$  has been clearly associated with several clinical endpoints of interest, the fact that these conclusions are based upon calculated, and not directly measured, free  $T$  is a less potent element of the free hormone hypothesis statement. As discussed before in this review, free  $T$  calculations have several limitations. However, repeating these clinical studies using direct ED measurement would impose technical and logistical challenges, as well as require a great deal of effort from all scientific collaborators. While, however, not available at this moment, endeavors are underway to optimize the implementation of free  $T$  in clinical practice.

### The need for free testosterone in clinical setting: why and when?

Men with established organic hypogonadism are usually treated with  $T$  replacement therapy. Available in various

formulations, androgen replacement serves the goal of establishing and/or sustaining secondary sexual characteristics in these men. Detailed specifics on administration, intended and adverse effects of  $T$  replacement therapy are beyond the scope of this review and have been discussed elsewhere [166]. Prescription of  $T$  supplements has increased phenomenally during the past 2 decades, especially in the larger group of men suffering from functional hypogonadism [247]. Nevertheless,  $T$  supplementation is not without risk and could potentially be harmful concerning cardiovascular risk in the elderly [248]. Currently, the path to diagnosing hypogonadism is still directed towards biochemical dosing of total  $T$ . However, conditions that affect SHBG levels unequivocally affect total  $T$  concentrations. Free  $T$  on the other hand is not necessarily impacted by this change in SHBG. The question rises how free  $T$  can be integrated into a clinical workflow concerning male hypogonadism. More systematic use of free  $T$  on top of total  $T$  could reduce overtreatment and  $T$  prescriptions.

The performance of total  $T$  as a predictor for free  $T$  was assessed in a cohort study in 3672 men. In this study, diagnosis of hypogonadism was based solely on biochemical values, disregarding any clinical symptomatology. Overall, with an area under the receiver operator curve of 0.93, total  $T$  was, in general, found to be a good predictor of calculated free  $T$ . In contrast, however, 61.7% of cohort subjects with decreased total  $T$  levels had normal calculated free  $T$  [249]. This was reflected in values for sensitivity and specificity at clinically useful thresholds. In this study, at the assays lower limit of normal (total  $T$  of 280 ng/dL), sensitivity and specificity of predicting low calculated free  $T$  were only 91.0 and 73.7%, respectively. Acceptable levels for sensitivity, 95.0% and up, were only acquired at a total  $T$  cut-off of 350 ng/dL, while on the other hand specificity only reached satisfactory levels of 98.0% and more at a total  $T$  cut-off of 150 ng/dL [249]. This study, therefore, demonstrated that only when total  $T$  is very low (< 150 ng/dL) or above 350 ng/dL, calculated free  $T$  is reliably predicted and hypogonadism can, respectively, be confirmed or excluded. These findings highlight that total  $T$  in the lower range of normal is actually a poor predictor of low calculated free  $T$ . Indeed, the ES recommends that for these men (e.g., lower range of normal total  $T$ ) additional biochemical confirmation by free  $T$  is performed [7]. Furthermore, in accordance with ES guidelines, added value of free  $T$  is also evident when SHBG levels are altered [7]. As discussed earlier, in conditions where SHBG is either increased or decreased, total  $T$  levels vary accordingly, hereby limiting the diagnostic utility of total  $T$ . Reduction of total  $T$  by lowered SHBG could, therefore, lead to overdiagnosis of male hypogonadism as free  $T$  levels may be unaffected by this change. However, underdiagnosis of hypogonadism may also occur when SHBG and concomitantly total  $T$  are increased, hereby leading to total  $T$  in

normal reference range while free  $T$  may be still decreased [250]. Both situations illustrate that reliance solely on total  $T$  is suboptimal and that free  $T$  should be included on top of total  $T$  in the evaluation of hypogonadism in men. This particularly applies to situations in which total  $T$  is borderline low and/or SHBG concentration is altered.

Discrepant results between total and free  $T$  were also observed in our analyses of the EMAS cohort of 3334 community-dwelling men aged 40–79 years [182]. In EMAS, in contrast to the previous study mentioned, we also included clinical symptomatology, and not only biochemical diagnosis, in our methodology. When only total  $T$ , as opposed to the total  $T$  calculated free  $T$  combination, is used in the biochemical identification of male hypogonadal patients, there is chance of false positive and negative findings of 23.1 and 9.5%, respectively, [182]. Furthermore, symptoms of male hypogonadism were most prominent in men with low total  $T$  and low calculated free  $T$ . Compared to men with normal concentrations of total  $T$  and calculated free  $T$ , men with normal total  $T$  and low calculated free  $T$ , and not men with low total  $T$  and normal calculated free  $T$ , also showed signs compatible with androgen deficiency. Additionally, in normal total  $T$ /low calculated free  $T$  men, concentrations of SHBG and LH were higher whereas low total  $T$ /normal calculated free  $T$  men had lower SHBG but unchanged LH levels [182]. These biochemical findings of normal total  $T$ , low free  $T$ , elevated SHBG and LH, together with clear clinical symptoms, are associated with an age-related profile of male hypogonadism. The other combination of variables, low total  $T$ , normal calculated free  $T$ , low SHBG and normal LH, without clinical symptoms suggestive for hypogonadism, are most probably obesity-related hormonal changes [182]. Therefore, our study concluded that in addition to total  $T$ , and especially when total  $T$  is borderline low and in the elderly and obese, calculated free  $T$  should be assessed as it can offer an improved precision in the evaluation of hypogonadal symptoms. We later also confirmed these data following a longitudinal analysis of the same EMAS cohort [251]. Only a small portion of men (27.3%) with low total  $T$  truly have low free  $T$  and develop the accompanying clinical symptoms of hypogonadism. This in contrast to the larger portion of men (72.7%) that develop an apparent biochemical hypogonadism. While this latter proportion of men have low total  $T$  values, their free  $T$  was in fact in normal range, exempting them from experiencing sexual symptoms [251]. With these combined results of the cross-sectional and longitudinal analysis, we clearly highlight that the sole use of total  $T$  could lead to either under or overdiagnosis of hypogonadism. To avoid these pitfalls of misdiagnosis, in particular when total  $T$  is borderline low and/or SHBG concentration is altered, addition of free  $T$  to the diagnostic landscape of male hypogonadism, is of incontrovertible value.

**Table 8** Research agenda highlighting major remaining research problems and questions

1	Optimization of current (commercially available) assays for total <i>T</i> against an international total <i>T</i> reference method (e.g., CDC)
2	International harmonization of total <i>T</i> reference ranges
3	Development of an international reference method for the measurement of SHBG
4	Optimization of current (commercially available) assays for SHBG
5	International harmonization of SHBG reference ranges
6	Validation and finetuning of the direct measurement method for free <i>T</i>
7	Reappraisal of current free <i>T</i> calculators to improve accuracy and agreement with direct measurement methods
8	Defining reference ranges for free <i>T</i> in healthy subjects and specific patient populations

*T* testosterone, *CDC* centers for disease control and prevention, *SHBG* sex hormone-binding globulin

## Conclusion

This review delivers strong arguments from in vitro, in vivo and human experiments in support of the free hormone hypothesis. While we also acknowledge particular shortcomings in the hypothesis, we believe free fraction hormones as described by the free hormone hypothesis provide clinical utility in patient healthcare. Assessing free *T* on top of total *T* as a standard biochemical parameter in the diagnostic workflow of male hypogonadism is clinically relevant when total *T* is borderline low and/or SHBG concentration is altered. Furthermore, measurement of free *T* in PCOS and other endocrinological conditions can be of diagnostic value. As LC–MS/MS coupled ED is not routinely available, for now, calculated free *T* by means of the Vermeulen formula is preferential to assess andrological status in the clinical setting. However, several barriers remain in the implementation of free testosterone into routine clinical practice (Table 8). More in-depth research is needed on the interaction between steroid hormones and their BPs. Further exploration of the steroid binding milieu and development of corresponding models (e.g., reappraisal of free hormone calculators) can establish clinical relevance of these free fraction hormones. In addition, efforts should be made to develop new or improve existing free hormone methodologies. Improving availability and accessibility to direct measurement methods are important milestones and will deliver added value to the diagnostic landscape of hormonal exposure in various endocrinological conditions.

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