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Sex hormone binding globulin: A review of its interactions with testosterone and age, and its impact on mortality in men with type 2 diabetes.

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Abstract.

Introduction. The age-related fall in male testosterone levels can have clinical consequences. The concentration of serum free testosterone, the putative bioactive moiety, is mediated by carrier proteins especially sex hormone binding globulin (SHBG).

Aim. We consider the nature of hormone binding to carriers with new insights into determining calculated free testosterone levels and review how SHBG and testosterone influence age-related mortality.

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Introduction.

The importance, particularly in men with type 2 diabetes (T2DM), of adult onset testosterone deficiency (TD), a combination of low serum testosterone and symptoms, is illustrated by research showing associations between low hormone levels and various pathologies and even mortality. [1] The mechanisms of these associations appear to involve serum proteins that transport testosterone and mediate the amounts of hormone that are in free solution or bioavailable. Serum sex hormone binding globulin (SHBG) has attracted particular attention as it appears critical in determining the serum level of these moieties which have been proposed as determinants of androgen activity (Figure 1). [2] Due to continuing uncertainties regarding some testosterone and SHBG assays and methods used to determine free and bioavailable testosterone, it seems clear that gaining a better understanding of the relationship between the hormone, SHBG and clinical phenotypes should help improve the diagnosis and management of men with symptoms of hypogonadism.

We firstly review data describing the clinical importance of maintaining adequate levels of testosterone and secondly consider the nature of hormone binding to its carriers and methods to estimate free and bioavailable testosterone. We thirdly, review studies indicating that SHBG is a clinically useful marker focusing on the finding that SHBG is independently associated with mortality in men with T2DM. Since age is strongly associated with mortality and SHBG and, appears to mediate the impact of SHBG and testosterone on mortality, we describe further analysis of data from our published study showing the impact of combinations of these predictors on the probability of age-related mortality. [3] The combination of low total testosterone and high SHBG in men aged>66 years is a principal risk category.

Clinical Importance of testosterone.

Testosterone has varied functions in humans, perhaps the best recognized being in development of the male reproductive system [4]. Some of its other pleiotropic functions may become evident in older men as serum levels typically decline by about 30% between ages 40-90 years [5,6,7,8,9]. Thus, symptoms of adult onset TD may include decreases in bone mineral density, muscle strength, cognitive and sexual function.[4,5,6,7,8,9] Adult onset TD is also associated with increased central obesity and insulin resistance and consequently, the metabolic syndrome (MetS) and T2DM. [4,10,11,12,13,14,15,16] The prevalence of adult onset TD is high in T2DM and MetS patients; in our cohort about 70% of men with T2DM had low serum total testosterone levels (<12nmol/L) and sexual symptoms [12] though other studies have shown a lower prevalence of up to 40%. [13,17] Importantly, both adult onset TD and erectile dysfunction are independently associated with increased mortality [18,19,20] and, T2DM is linked with cardiovascular disease (CVD), sexual dysfunction and reduced life expectancy. [16]

Independent studies show an association between low serum testosterone and adverse CVD events and all-cause mortality. [21,22,23,24,25,26,27,28] For example, the European Male Ageing study (2599 men, 7% withT2DM, aged 40-79 years) showed that adult onset TD with total testosterone <8nmol/l was significantly associated with increased total and CVD-related mortality [22]. That the reduced availability of testosterone is a causative factor is supported by data showing testosterone replacement (TTh) can lead to improved sexual health and reduced all-cause mortality [6,19,23,24,25]. Shores et al showed TTh was associated with significantly reduced mortality in men with T2DM aged over 40 years and with total testosterone ≤8.7nmol/l but not in non-diabetic counterparts.[24] This finding was

confirmed by 2 longitudinal studies; Muraleedaran et al studied the effects of low testosterone (<10.4 nmol/l) and TTh on mortality in 581 men with T2DM. [6] After adjustment for confounders, mortality was higher in the low testosterone group. Further, the 174 men not on TTh were at significantly higher risk of mortality than the 64 men receiving TTh. We found similar results in 857 men with T2DM using a total testosterone cut-off of 12.0 nmol/l and free testosterone of 0.25 nmol/l. [19,25]. Compared with men with low total or calculated free testosterone and not on TTh, mortality was lower in men with normal testosterone (Hazard Ratio (HR): 0.62, CI: 0.41 - 0.94) and men with low testosterone on TTh (HR: 0.38, CI: 0.16 - 0.90). This benefit was independent of changes in cardiovascular/metabolic risk factors [29].

These data show that low serum testosterone levels have clinical implications, and accordingly, serum total and free testosterone treatment thresholds for TTh are identified; guidelines issued by the British Society for Sexual Medicine have indicated that men with total testosterone<8nmol/l or free testosterone<0.180nmol/l usually require TTh while those with total testosterone 8-12nmol/l may require TTh depending on symptoms [30]. However, the relationship between extent of reduction in serum testosterone and particular clinical symptoms is unclear and, the establishing of universal, clinically useful cut-off values is problematic. Firstly, an assay standardisation approach may be required as marked between-method variation in the accuracy and precision of SHBG and testosterone assays has been identified (https://birminghamquality.org.uk/eqa-programmes/ster/ - accessed 20/05/2019) [31] and secondly, none of the commonly used guidelines include corresponding values for variables such as SHBG that mediate free testosterone levels.

Sex Hormone Binding Globulin

Hydrophobic steroids such as testosterone are largely transported in serum by carrier proteins including SHBG and albumin. [32,33]. SHBG is central because its ability to bind testosterone mediates transport, serum levels of free/bioavailable and cellular uptake of testosterone and appears to offer in some men, a clinically useful insight into androgen status. [2,32,33,34,35]

SHBG, a homodimeric glycoprotein, is largely synthesised in the liver. [2] Each monomer has one steroid binding site allowing transport of two steroid molecules/dimer. In serum, SHBG binds steroids such as dihydrotestosterone and testosterone with high affinity; K_a~10^9 l/mol while albumin, present in human serum at high concentration (typically ~40 g/l) offers substantial capacity but low affinity (K_a; ~3X10^4 l/mol). [35,36,37,38,39] Cortisol binding globulin (K_a 5.3X10^6 l/mol) and orosomucoid (K_a 3.0X10^5 l/mol) also bind the steroid weakly. [32,38,39,40,41] Accordingly, they often ignored in the context of testosterone and phenotype.

Serum SHBG, total and free/bioavailable testosterone

Various algorithms have been used to calculate free or bioavailable testosterone. [38,39,40,41] The widely used Vermeulen et al model is based on mass-action binding and assumes one molecule of testosterone binds to one SHBG molecule [38]. Other more recent models are based on each SHBG monomer having a binding site that can receive one testosterone molecule. [42]

Figure 1 shows, for 3 total testosterone concentrations (median, 25% and 75% percentile of our cohort of men with T2DM) [19] how changes in SHBG mediate levels of calculated bioavailable and free testosterone. By changing SHBG values within the observed distribution in men with T2DM, the free testosterone concentration can fall considerably when SHBG increases from the 25th to 75th

percentile. For serum albumin, a protein that usually demonstrates much less variation, the extent of change in free testosterone is modest (data not shown). Thus, even allowing for possible errors in the binding parameters and, differences in the approaches used to derive the algorithms, it may be reasonable to consider values of free or bioavailable testosterone in “local units” as showing patterns of change.

Because of its high serum concentration, barely 1% of albumin binding sites in men are generally occupied while for SHBG, 36% of sites are occupied by testosterone, 20% by androgen metabolites or oestradiol and only 44% are unoccupied. [36] Dunn et al proposed that SHBG largely determines the serum distribution of sex steroids and that changes in its level will alter testosterone distribution [36].

Albumin-bound testosterone may also be bioavailable because binding is weak and interactions with the vascular wall may allow dissociation of steroid during flow through capillaries. [43,44]. Bioavailable may be a more useful measure of hormone status than total testosterone; for example, its fall during male aging is steeper and associated with features of adult onset TD. [45,46,47,48]

Issues in Quantifying Free and Bioavailable testosterone.

Determination of free or bioavailable testosterone experimentally or using algorithms is relatively demanding because of inter- and intra-laboratory imprecision of available methods and different values for binding parameters. Discrepancies between free testosterone values calculated using the Vermeulen et al approach and a variety of other algorithms or determined experimentally are reported. [37,38, 42,49,50,51,52] Recently, Zacharov et al proposed a more complex model of testosterone binding. [53] Thus, while it had been believed that testosterone

associated to each SHBG site with the same affinity, Zacharov et al proposed that testosterone binding to one monomer affects the interaction of the next testosterone molecule with the unoccupied, second site and the second molecule is bound with a different affinity to the first indicating the process is allosteric. [53] Zakharov et al argued that SHBG binding of testosterone cannot be described by available algorithms and instead used an allosteric model to calculate free testosterone. [53] In samples from Framingham Heart Study subjects, levels calculated using a traditional algorithm were markedly lower than those obtained using an allosteric model; in subjects aged<30 years, free testosterone using a traditional model was 145.35±45.8 (SD) pg/ml and the allosteric model, 238.339±71.77 (SD) pg/ml. The allosteric model gave values similar to those obtained using equilibrium dialysis. [42,53]

While there is no universally accepted method for determining free and bioavailable testosterone, an international expert consensus conference in 2015 stated that this analysis was important in the assessment of men with adult onset TD [54]. As clinicians (SR, GIH), we agree and consider free testosterone together with SHBG and total testosterone integral in routine patient screening and monitoring. Free testosterone measurements are particularly useful in the investigation of men with adult onset TD symptoms and a borderline total testosterone of 8-11 nmol/l, though perhaps less so in men with total testosterone<8 nmol/l. [55,56]

SHBG: Wide range of values in human serum

The symptoms and characteristics of hypogonadism are nonspecific and varied and not always associated with low testosterone levels prompting interest in

the relationship between SHBG (as an independent variable) and clinical phenotype [57]. While SHBG may not have a direct effect on clinical outcomes we might expect higher serum SHBG levels to be associated with greater morbidity/mortality as free/bioavailable testosterone levels would presumably be lower. Thus, increased SHBG causes decreased free testosterone, triggering luteinizing hormone release and increased testosterone production until free hormone levels are restored. An insight into the biological importance of SHBG may be gained by considering individuals who have no peptide. Vos et al described an extremely rare missense mutation that encoded a secretion defective SHBG variant that accumulated in cells. [58] A brother and sister, homozygotes for the mutant allele, had no detectable serum SHBG. In the brother, serum total testosterone levels were low but free levels appeared adequate. Gonadal development and sperm production and function appeared normal though he had symptoms of hypoandrogenism. The sister reported a late menarche and irregular menstrual cycles. These findings suggest SHBG is required for some of testosterone's anabolic activities but is not essential for male reproductive development [58].

It is noteworthy that considering a physiological role for SHBG is not helped by the finding that its serum concentrations vary during life and demonstrate marked inter- and intra-individual variation even in apparently healthy adults. [2] For example, Krakowsky et al [34] found a mean SHBG concentration in 1000 consecutive patients of 31.8±15.2 nmol/l (range 6-109 nmol/l) while we found in 857 men with T2DM, concentrations between 7.9 - 185.5 nmol/l [59]. Further, during adulthood, SHBG levels increase with age though there is a wide variation in individuals stratified by young/old age. [34,59]

SHBG level and clinical phenotype.

Much research shows the importance of SHBG in determining clinical phenotype. [3,57,59,60,61,62,63,64] For example, the European Male Aging Study showed men with normal total but low free testosterone (using the algorithm of Vermeulen et al [38]) had higher LH levels, more sexual and physical symptoms than men with normal total and free testosterone or men with low total and normal free testosterone levels. [21] Thus, low free testosterone, even with a normal total testosterone, was associated with adult onset TD, while low total testosterone with normal free was not. Studies in transgenic mice that overexpressed SHBG also show that androgen deficiency is apparent in individuals with normal or borderline levels of serum total testosterone. [61]

Many factors mediate serum peptide levels; hormones including thyroxine, insulin, insulin like growth factor-1, prolactin and, non-hormonal factors including physical activity, body weight, body-mass-index (BMI), waist-to-hip ratio, some drugs and diet modify SHBG levels. [2,59] SHBG levels are lower with increasing obesity and in patients with T2DM and a low level of SHBG is associated with increased risk of developing MetS, gestational diabetes and T2DM and the cardiovascular disease linked with these conditions. Furthermore, SHBG genetic variation has been proposed to contribute to the development of T2DM.

The above data raise issues of how closely serum SHBG levels are regulated and which of the varied factors that affect expression are most important. [65] Gyawali et al in a cross-sectional and longitudinal study identified various determinants of serum SHBG in men. [66] Baseline SHBG levels were positively associated with age, thyroxine and total testosterone and inversely with abdominal fat mass, triglycerides and oestradiol. Low SHBG levels largely reflect obesity. It was

suggested that variation in SHBG reflects *de novo* hepatic lipogenesis and insulin resistance and that thyroid hormone may modulate circulating SHBG. High levels of serum sex steroids are associated with higher SHBG levels. [66]

SHBG: An independent factor determining mortality?

A range of metabolically active factors influence serum SHBG levels many of which are risk factors for clinically important phenotypes. Accordingly, establishing the independence of associations between SHBG and patient characteristics is important.

Recently, Tint et al [60] showed that elevated levels of SHBG and low levels of free testosterone were significantly and independently associated with all cause-mortality after adjustment for diabetes-related risk factors. The association of total testosterone with mortality weakened after such adjustment. We also investigated the impact of SHBG, age and related variables on mortality in 364 men with T2DM. [3] Age, SHBG and testosterone were significantly and independently associated with mortality. SHBG was associated with age, testosterone, statin use and MetS features. Mortality rate and age increased with SHBG quartiles. When pairwise combinations of age and SHBG dichotomized by median values were considered, the association of SHBG with mortality was age-dependent; firstly, age >66 years/SHBG >35 nmol/l was significantly associated (HR: 9.37, p<0.001) with greater mortality (22.45%) compared to age ≤66 years/SHBG ≤35 nmol/l (mortality: 3.23%) and secondly, SHBG ≤35 nmol/l was associated with decreased mortality in men >66 years (HR: 0.41, p=0,037, reference: SHBG > 35 nmol/l) but in men ≤66 years there was no significant difference in those with SHBG >35 nmol/l (HR:1.73,

p=0.56, reference: SHBG ≤ 35 nmol/l). By contrast, total testosterone<12 nmol/l was significantly associated with increased mortality in both age groups. Thus, while the association of SHBG with mortality in T2DM men is mediated by age, that with total testosterone<12 nmol/l was found irrespective of age < or >66 years.

These analyses demonstrate the cumulative effects of the risk phenotypes; age>66 years, total testosterone<12 nmol/l and SHBG>35 nmol/l. Men with two of factors demonstrated high mortality; age>66 years/SHBG>35 nmol/l: mortality 22.5% and age>66 years/total testosterone<12 nmol/l: mortality 25.0%. In men with all three risk variables, age>66 years/total testosterone<12 nmol/l/SHBG>35 nmol/l, mortality was 36.8%. Thus, SHBG is linearly associated with age but its association with mortality is more evident in men aged>66 years (who also suffer higher mortality). In men with T2DM, the combination of low total testosterone, high SHBG and age> 66 years is a principal risk category compared with other combinations. [3]

SHBG, testosterone and age-related mortality.

As both SHBG and mortality increase with age while both total and free testosterone levels fall (https://uroweb.org/wp-content/uploads/EAU-Guidelines-Male-Hypogonadism-2015.pdf - accessed on 20/05/2019) establishing prognosis (eg. mortality) in individual patients is difficult. One approach that demonstrates the association of inter-related variables with mortality at different ages is based on Benjamin Gompertz's observations that mortality increases exponentially with age. [67,68,69], More recently we found in our cohort of men with T2DM that the relationship between age and mortality was as described by Gompertz. [25]

To determine how SHBG and testosterone influence age-related mortality in T2DM men who had not received TTh we determined the probability of each of the

previously described 364 men with T2DM categorised by SHBG or testosterone, living or dying at a particular age. [3] We used a logistic regression model to derive the probability of mortality (dependent variable) with age (continuous variable) and SHBG and/or testosterone (dichotomized) as independent variables. Probability of mortality and 95% confidence intervals were plotted against age. If no overlap of the 95% confidence intervals between treatment/no treatment lines was observed, we considered the treatment significantly altered the relationship between age and mortality. The approach allows a graphic demonstration of the impact of stratified levels of SHBG and/or testosterone (stratified by median values; SHBG 35nmol/l, total testosterone 12nmol/l) on mortality over a range of ages. Since age is the most significant predictor of death, finding that a variable alters this relationship gives an indication of its biological importance. The regression analyses (Table 1) were carried out sequentially, building with each successive model on the previous analysis. We ended with mortality as the dependent variable and age and a dichotomised combination of SHBG and total testosterone as independent variables (model 8). None of these men had received TTh and, accordingly, median values for SHBG (35nmol/l) and total testosterone (12nmol/l) are different from previous distributions where baseline values of the total cohort that included men who received TTh were used.

In logistic regression analyses we showed that individually, age (Model 1) and age and SHBG entered as independent variables (model 2) were significantly associated with mortality. When total testosterone was entered (with age) it was not significantly associated with mortality (model 3) though in an analysis (model 4) comprising all three variables, each was independently, significantly associated with mortality. As expected, increasing age and SHBG were associated with increased

mortality with total testosterone inversely associated with mortality. Dichotomizing SHBG and total testosterone by their median values showed SHBG>35 nmol/l and total testosterone<12 nmol/l were individually and independently significantly associated with increased mortality (model 4). We then further studied the association between SHBG and mortality in men stratified by the median total testosterone of 12nmol/l. Interestingly, the association of SHBG >35 nmol/l with mortality was not observed in men with testosterone ≥12 nmol/l (Model 6b) unlike in men with total testosterone < 12nmol/l (Model 6a). Accordingly, we used combinations of SHBG and total testosterone (model 7) with age as independent variables and found men with total testosterone<12nmol/l and SHBG>35nmol/l (reference) were at risk compared to the other 3 combinations. Model 8 shows that relative to other combinations of SHBG and testosterone, men with SHBG>35 nmol/l and testosterone<12 nmol/l had the highest mortality risk. The model was used to calculate the probability of death and 95% confidence intervals for each man. Figure 2 shows the association between probability of mortality and age in these two groups. Mortality is greater in men with the combination of SHBG >35nmol/l and testosterone<12 nmol/l though there is some overlap of 95% CI values after about 69 years indicating that, despite relatively small patient numbers, the combination of elevated SHBG and low total testosterone alters the relationship between age and mortality.

Conclusions

A paradox is apparent. While it is noteworthy that SHBG and T2DM and the MetS are positively associated with mortality, both conditions are negatively associated with SHBG. Thus, while high levels of SHBG are associated with

increased all-cause mortality, levels of the peptide are inversely associated with insulin resistance and this may mediate the association between low serum testosterone and increased mortality in men with T2DM. [3,6,19,60,70]

Both SHBG and low total testosterone are identified as independent variables that mediate age-related mortality in men with T2DM. The more limited data available for free testosterone derived using a traditional algorithm are supportive of these findings. In a T2DM context free/bioavailable testosterone is integral in the development of disease phenotypes including mortality; low free testosterone was associated with mortality more strongly than either total testosterone or SHBG. [60] Clearly, apart from inter-individual variation in SHBG and testosterone levels, other host characteristics such as androgen sensitivity and the functionality of androgen receptors are potential factors affecting outcome. [71,72]

A further complication may be reports indicating SHBG acts as more than just a carrier protein in specific instances. It has also been suggested that SHBG could affect mortality by a mechanism that is independent of its role in testosterone metabolism such as regulation of cell proliferation. [42,74] These data remain to be assessed.

This review has demonstrated associations between SHBG and clinical phenotypes with particular focus on mortality. While the mechanism is uncertain, it might be speculated that the association is determined by the actions of the globulin in determining serum levels of free/bioavailable testosterone. However, the association of SHBG with mortality is independent of free and total hormone perhaps hinting at further functions of the globulin and/or the need for better methodologies to quantify these analytes and/or a role for other steroid hormones transported by SHBG. [31,42,73,74,75,76]

Given the association of SHBG with mortality, it is tempting to speculate on the feasibility and usefulness of clinical modification of serum globulin levels. Given the association of SHBG with mortality, it is tempting to speculate on the feasibility and usefulness of clinical modification of serum globulin levels. A wide range of factors including widely used drugs such as statins, mediate SHBG levels. For example, in men with T2DM, Stanworth et al. showed that atorvastatin therapy (but not simvastatin) was associated with significantly reduced total testosterone and a trend towards reduced SHBG levels and we reported that statin therapy was associated with a significant reduction in SHBG levels. [3,77]

Alternatively, vegetarian, vegan and low calorie diets may be associated with higher serum SHBG concentrations though changes in testosterone levels have varied between studies. [78] It is not known if any of these approaches have clinical relevance in the context of adult onset TD and, in our view use of therapy to modify SHBG levels is, given current uncertainties, inappropriate.

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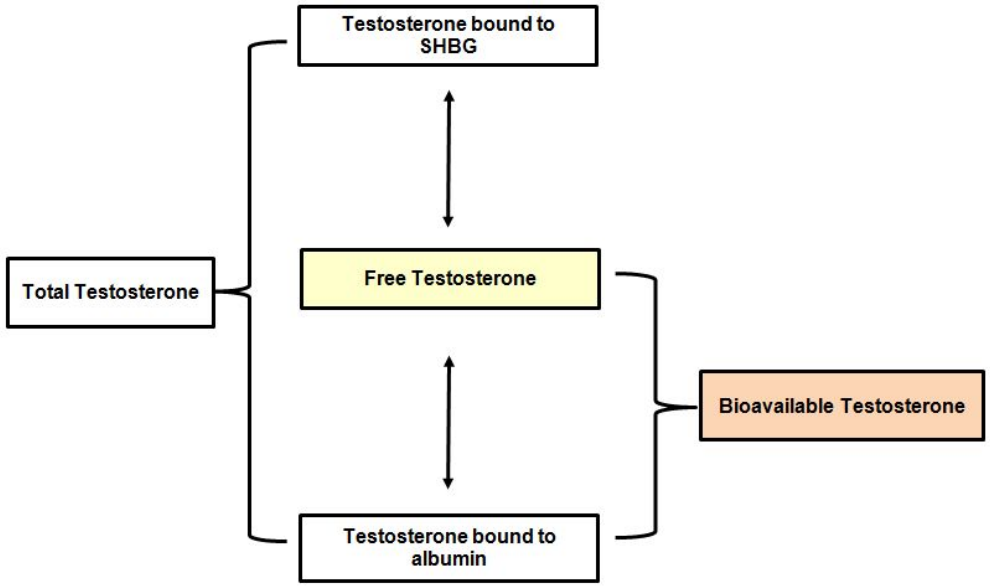
Figure 1. Calculated free testosterone and bioavailable testosterone levels at median (32.5 nmol/l) and inter quartile range (24.2 / 45.1 nmol/l) of SHBG and median (11.4 nmol/l) and inter quartile range (8.3 nmol/l / 14.8 nmol/l) of total Testosterone concentrations in men with T2DM [19]



Total Testosterone (nmol/l)	8.3	11.4	14.8
SHBG (nmol/l)		24.2	
Free Testosterone (nmol/l)	0.19	0.27	0.36
Bioavailable Testosterone (nmol/l)	4.5	6.3	8.5
SHBG (nmol/l)		32.5	
Free Testosterone (nmol/l)	0.16	0.23	0.31
Bioavailable Testosterone (nmol/l)	3.8	5.4	7.2
SHBG (nmol/l)		45.1	
Free Testosterone (nmol/l)	0.13	0.19	0.24
Bioavailable Testosterone (nmol/l)	3.1	4.4	5.9

Calculated free testosterone and bioavailable testosterone levels were estimated using the algorithm by Vermeulen et al [26] with albumin concentrations at a default value of 43g/l.

Other proteins (CBG / orosomucoid) are not included in the figure as they are not included in the Vermeulen algorithm.

Figure 2: Association between probability of mortality and age. Estimated mortality probability and 95%CI (in men with T2DM between 65 – 80 years) from the fitted logistic regression (Table 1) was calculated from the logistic regression analysis (Model 8) and plotted against age at death or final visit.

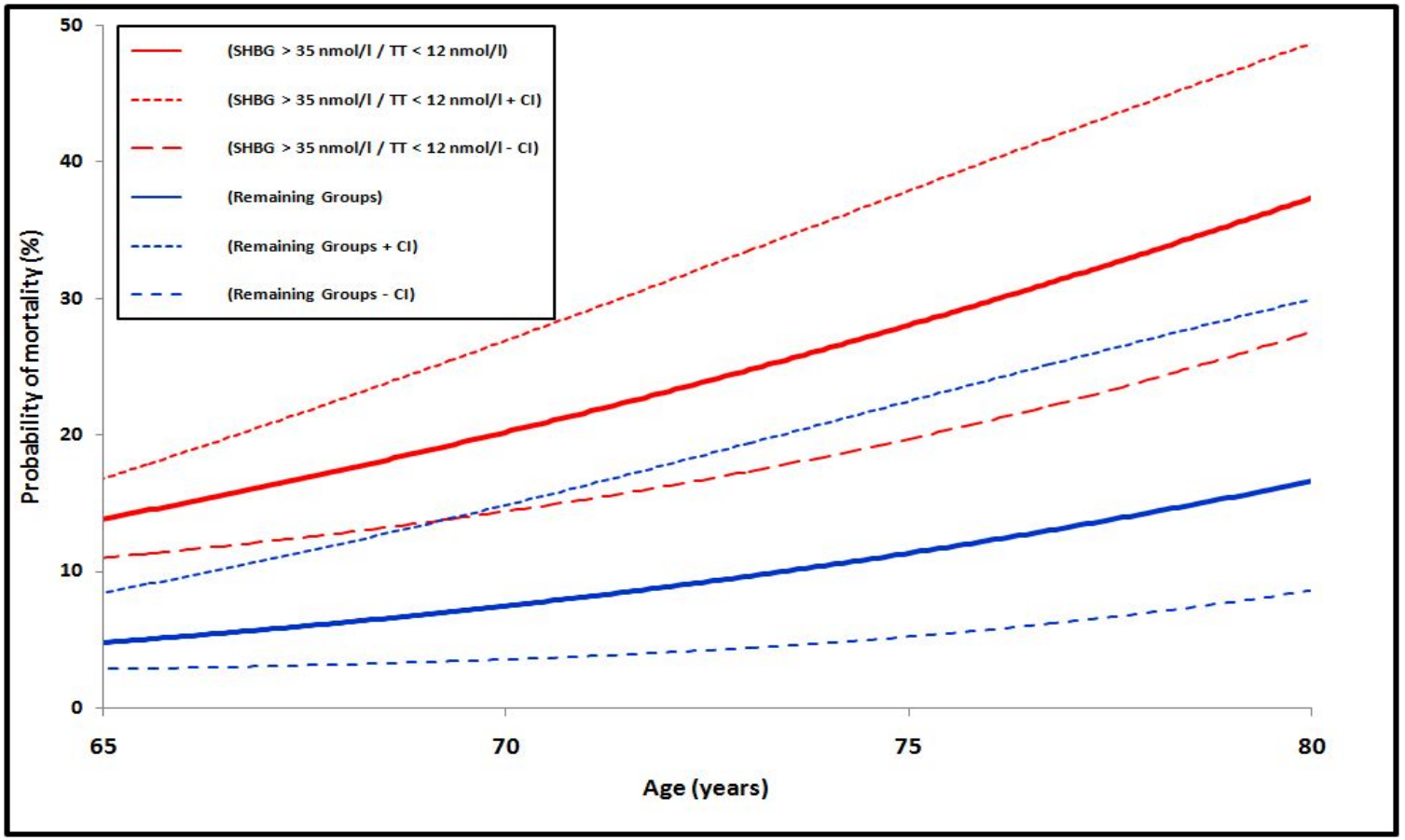


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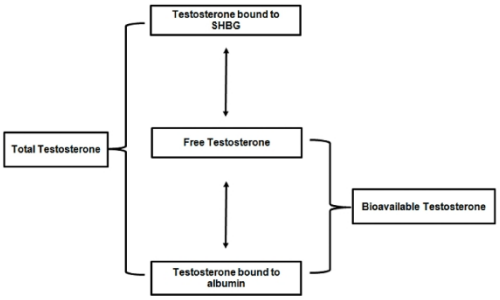
Table 1: Logistic regression models showing association between age and mortality (dichotomous dependent variable) in men with T2DM in the presence of SHBG and / or TT (either as continuous variables or stratified categories) in the total cohort (Models 1, 2, 3, 4, 5, 7 and 8) or when stratified by TT of 12 nmol/l (Models 6a, 6b).

-	OR (95% CI)	p
Model 1, n=364		
Age (years)	1.10 (1.07-1.13)	<0.001
Model 2, n=364		
Age (years)	1.10 (1.05-1.14)	<0.001
SHBG (nmol/l)	1.02 (1.00-1.03)	0.015
Model 3, n=362		
Age (years)	1.10 (1.06-1.15)	<0.001
TT (nmol/l)	0.97 (0.91-1.04)	0.38
Model 4, n=362		
Age (years)	1.08 (1.04-1.13)	<0.001
SHBG (nmol/l)	1.03 (1.01-1.05)	0.001
TT (nmol/l)	0.91 (0.85-0.98)	0.015
Model 5, n=362		
Age (years)	1.08 (1.04-1.13)	<0.001
SHBG ≤ 35 nmol/l	reference	
SHBG > 35 nmol/l	2.54 (1.14-5.66)	0.022
TT <12 nmol/l	reference	
TT ≥ 12 nmol/l	0.37 (0.17-0.81)	0.013
Model 6a (TT < 12nmol/l), n=181		
Age (years)	1.09 (1.03-1.15)	0.001
SHBG ≤ 35 nmol/l	reference	
SHBG > 35 nmol/l	2.83 (1.15-6.97)	0.023
Model 6b (TT ≥ 12nmol/l), n=181		
Age (years)	1.09 (1.03-1.16)	0.006
SHBG ≤ 35 nmol/l	reference	
SHBG > 35 nmol/l	1.75 (0.36-8.37)	0.49
Model 7		
Age (years)	1.09 (1.05-1.14)	<0.001
SHBG > 35 nmol/l / TT < 12 nmol/l	**reference**	
SHBG ≤ 35 nmol/l / TT < 12 nmol/l	0.35 (0.14 - 0.86)	0.022
SHBG ≤ 35 nmol/l / TT ≥ 12 nmol/l	0.19 (0.04 - 0.95)	0.043
SHBG > 35 nmol/l / TT ≥ 12 nmol/l	0.34 (0.14 - 0.80)	0.013
Model 8		
Age (years)	1.08 (1.06-1.11)	<0.001
SHBG > 35 nmol/l / TT < 12 nmol/l	3.05 (1.43 - 6.53)	0.004
Remaining groups	**reference**	
SHBG ≤ 35 nmol/l / TT < 12 nmol/l		
SHBG ≤ 35 nmol/l / TT ≥ 12 nmol/l		
SHBG > 35 nmol/l / TT ≥ 12 nmol/l		



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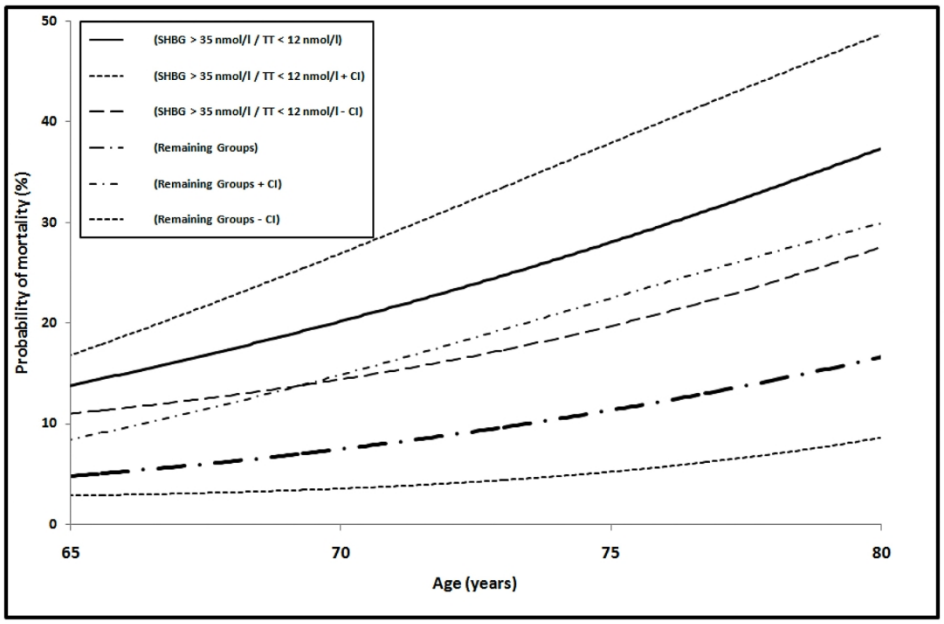

Figure 2: Association between probability of mortality and age. Estimated mortality probability and 95%CI (in men with T2DM between 65 – 80 years) from the fitted logistic regression (Table 1) was calculated from the logistic regression analysis (Model 8) and plotted against age at death or final visit.

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