

**Effects of Testosterone Levels on Mortality and Cardiovascular  
Risk in Men with Type 2 Diabetes**

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**By**

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## **Summary of findings**

There is increased prevalence of low testosterone level in men with type 2 diabetes. There is growing evidence that low testosterone levels has adverse cardio-metabolic outcome in the long term. The mortality and morbidity in men with type 2 diabetes and low testosterone has not been studied previously.

Chapter 1 reviews the relationship between low testosterone and cardiovascular mortality and morbidity and discusses about metabolic pathways which could be affected in low testosterone state.

Chapter 2 is the first study to look into the mortality of type 2 diabetic men with low testosterone. The results demonstrated an increase in mortality with low testosterone state and a beneficial effect of testosterone replacement therapy in hypogonadal men with type 2 diabetes on long-term follow up.

Chapter 3 and 4 looked at the longitudinal and cross sectional studies studying the effects of low testosterone on cardio-metabolic risk profile. The results demonstrated an adverse cardio-metabolic risk profile in type 2 diabetic men with low testosterone and an inverse relationship between the SHBG levels and cardio-metabolic risk profile in men with type 2 diabetes independent of testosterone levels.

Chapter 5 examined at the relationship between androgen receptor sensitivity as assed by CAG repeat polymorphism (AR CAG) and cardio-metabolic risk profile in men with type 2 diabetes. The results showed no significant direct relation between AR CAG and cardio-metabolic risk profile. However there was a significant correlation between the ratio of testosterone and AR CAG (T/AR CAG or BioT/ CAG) and cardio-metabolic risk profile with

the higher ratio predicting favourable cardio-metabolic profiles for glycaemic control and anthropometric measurements.

In chapter 6 the different effects of testosterone on metabolic pathways in key tissues were investigated using testicular feminised mouse (Tfm) model which has an inactive AR and low circulating testosterone levels. The results demonstrated that a low testosterone state was associated with adverse cardio-metabolic profile by affecting major enzymes and receptors involved in the carbohydrate and lipid metabolism in liver, muscle and adipose tissues. Physiological replacement of testosterone partially reversed the expression of some of these parameters which provide evidence that some of the actions of testosterone in metabolic pathways are independent of androgen receptor.

Chapter 7 draws together the results and conclusions are discussed.

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## **Publications and presentations arising from the thesis**

### **Publications**

1. Testosterone Deficiency is Associated With Increased Risk of Mortality and Physiological Testosterone Replacement Improves Survival in Men With Type 2 Diabetes. **Vakkat Muraleedharan**,; Hazel Marsh, Dheeraj Kapoor, Kevin S Channer; T Hugh Jones, in European Journal of Endocrinology. 2013 Oct 21; 169(6):725-33. doi: 10.1530/EJE-13-0321. Print 2013 Dec (*One of the most cited paper in 2014, 2015 and 2016 and 2018 (Thomson reuters web of science) with 263 citations so far*)
2. Testosterone and mortality. **Muraleedharan V**, Jones TH. Clin Endocrinol (Oxf). 2014 Oct; 81(4):477-87. DOI: 10.1111/cen.12503.
3. Testosterone and Metabolic Syndrome. **Vakkat Muraleedharan** and T. Hugh Jones; Therapeutic Advances in Endocrinology and Metabolism December 2010 1:207-223.
4. Testosterone differentially regulates targets of lipid and glucose metabolism in liver, muscle and adipose tissues of the testicular feminised mouse. Daniel M. Kelly, Samia Akhtar<sup>1</sup>, Donna J. Sellers, **Vakkat Muraleedharan**, Kevin S. Channer, T. Hugh Jones. Endocrine. November 2016, Volume 54, Issue 2, pp 504–515  
DOI: 10.1007/s12020-016-1019-1
5. Testosterone protects against early atherogenesis by beneficially modulating tissue-specific metabolic function in liver, muscle and adipose of male mice. D.M. Kelly, S. Akhtar, D.J. Sellers, **V. Muraleedharan**, K.S. Channer, T.H. Jones. Atherosclerosis Volume: 244 Pages: E7-E7; JAN 2016 DOI: <http://dx.doi.org/10.1016/j.atherosclerosis.2015.10.072>
6. Testosterone suppresses the expression of regulatory enzymes of fatty acid synthesis and protects against hepatic steatosis in cholesterol-fed androgen deficient mice. Kelly DM,

Nettleship JE, Akhtar S, **Muraleedharan V**, Sellers DJ, Brooke JC, McLaren DS, Channer KS, Jones TH. Life Sci. 2014 Jul 30;109(2):95-103. doi: 10.1016/j.lfs.2014.06.007.

7. Testosterone deficiency and severity of erectile dysfunction are independently associated with reduced quality of life in men with type 2 diabetes. Brooke JC, Walter DJ, Kapoor D, Marsh H, **Muraleedharan V**, Jones TH. Andrology. 2014 Mar;2(2):205-211

### **Oral presentations at international conferences**

1. Invited speaker at the South Asian Regional Conference of World Organization of Family Doctors, WONCA SAR 2016, 11th to 14th February 2016 in Colombo Sri Lanka  
“Testosterone and Type 2 Diabetes”
2. Invited speaker at Ceylon College of physicians 47th Annual meeting 6th Oct 2014  
“Testosterone and Cardiovascular Mortality in Men with T2 Diabetes”
3. Endo2011 Boston (Endocrine society Annual meeting) “Testosterone inhibits mRNA expression of key enzyme Fatty Acid Synthase in liver and Protects against Hepatic Steatosis”
4. Endo2011 Boston (Endocrine society Annual meeting) "Long Term Testosterone Replacement Improves Survival in Men with Type 2 Diabetes and Hypogonadism”
5. Endo 2010 San Diego ( Endocrine society Annual meeting) "Low Testosterone Level Is Associated with Significant Increase in All Cause, Cardiovascular and Cancer mortality In Men With Type 2 Diabetes"
6. Men’s Health World Congress, Nice France, 2010 "Testosterone Deficiency is Associated with Increased Mortality in Men with Type 2 Diabetes – A Six Year follow up Study"

### **Other oral presentations**

1. Grand rounds presentation King's Mill Hospital " Testosterone, metabolic syndrome and diabetes" 26th Oct 2016
2. Regional SPR teaching at Derby " Testosterone and type2 diabetes" 25th Nov 2015
3. Grand round presentation King's Mill Hospital Diabetes and Testosterone 29th Jan 2014.
4. 3rd year postgraduate presentation at University of Sheffield, 6th June 2013 Role of testosterone on fat storage and metabolism and its relation to atherosclerosis.
5. Mellanby centre internal seminars University of Sheffield Oral presentation, 28th Oct 2011 "Testosterone Replacement Therapy Improves Survival in Men with Type 2 Diabetes and Hypogonadism".
6. Mellanby centre internal seminars University of Sheffield Oral Presentation, 25th Nov 2011 "Testosterone Inhibits mRNA Expression of Fatty Acid Synthase in the Liver and Protects Against Hepatic Steatosis in Tfm Mouse"

### **Poster presentations as first author**

1. Endo2017 Orlando Florida (Endocrine society annual meeting) Poster "SHBG Is an Independent Predictor of Cardio-Metabolic Risk Profile on Long Term Follow up in Men with Type 2 Diabetes" 1st April 2017.
2. Endo2017 Orlando Florida (Endocrine society annual meeting) Poster "Testosterone and SHBG Show Independent Negative Correlation with Cardiovascular Risk Profile in Men Which Improves with Testosterone Replacement in Men with Type 2 Diabetes" 1st April 2017.

3. Endo 2015 San Diego (Endocrine society annual meeting) Testosterone Deficiency Is Associated with Greater Deterioration of Glycemic Control during 7-Year Follow up in Men with Type 2 Diabetes
4. BES 2011 Birmingham (British Endocrine society annual meeting), Poster “Low Testosterone Predicts Increased Mortality and Testosterone Replacement Therapy Improves Survival in Men with Type 2 Diabetes”
5. BES 2011 Birmingham (British Endocrine society annual meeting) Poster “Testosterone Undecanoate has a beneficial effect on Lipid Profile in Men with Hypogonadism in Routine Clinical Practice”.
6. ENDO2010 San Diego CA (Endocrine society annual meeting) Poster Presentation “The Effect Of Testosterone Undecanoate On Cardiovascular Risk Factors In Men With Hypogonadism In Clinical Practice”
7. BES2010 Manchester (British Endocrine society annual meeting) “Poster presentations- Testosterone Replacement May Be Beneficial In Hypogonadal Men With Cardiovascular Disease”.
8. BES 2010 Manchester (British Endocrine society annual meeting) “Poster presentations- Low Testosterone Levels are Associated with Increase in Mortality in Patients with Type 2 Diabetes”.
9. East Midlands Endocrine society meeting 2010 oral presentation “Low endogenous Testosterone Predicts increased mortality in men with type 2 diabetes – a six year follow up study”
10. Sheffield Medical School Research meeting 2010- Poster presentation “Low Testosterone Level Is Associated with Significant Increase in All Cause and Cardiovascular Mortality In Men With Type 2 Diabetes”

10. BES 2009 (British Endocrine society annual meeting) “Testosterone replacement therapy in Prader-Willi Syndrome (PWS) with diabetes markedly improves glycaemic control”

### **Presentations as co author**

1. Endo2018 Boston (Endocrine society annual meeting) – Poster: Significance Of AMS And IIEF-5 Questionnaires In Hypogonadal Men With Type 2 Diabetes Mellitus
2. Endo2018 Boston (Endocrine society annual meeting) – Poster: Impact Of Testosterone Replacement Therapy In Hypogonadal Men With Diabetes Mellitus On Quality Of Life
3. Endo 2015 San Diego (Endocrine society annual meeting) Poster “Evidence That Testosterone Improves Glucose Utilisation and a ‘Buffering’ Effect of Subcutaneous Fat to Protect Against ‘Overspill’ of Lipid Deposition into Visceral Fat and Non-Adipose Tissues”
4. BES 2014 (British Endocrine society annual meeting), Poster “Testosterone regulates glucose control in liver and muscle of Tfm mice as a mechanism to improve type 2 diabetes”
5. Endo 2013 San Francisco (Endocrine society annual meeting), Poster, “Testosterone effects the expression of liver X receptor and targets of lipid and glucose metabolism in the testicular feminised mouse as a potential mechanism to improve insulin resistance”
6. Endo 2013 San Francisco (Endocrine society annual meeting), Poster, “Testosterone Replacement Therapy Has Beneficial Effects on Cardiovascular Risk Factors and Liver Function in Hypogonadal Men”
7. Endo 2013 San Francisco (Endocrine society annual meeting), Poster, “Testosterone replacement therapy is safe for use in treating hypogonadism in men”
8. ECE 2013 Copenhagen Denmark, Poster, “Testosterone differentially regulates liver X receptor expression and targets of lipid and glucose metabolism in liver, muscle and adipose tissue of the testicular feminised mouse”
9. BES 2013 (British endocrine society annual meeting) Harrogate, Poster “Testosterone differentially regulates lipid and glucose metabolism in visceral and subcutaneous fat in the testicular feminised mouse”
10. Endo 2012 Houston Oral presentation (Endocrine society annual meeting) “Testosterone Increases Hepatic Liver X Receptor and ApoE Expression and Improves Lipid Metabolism in the Testicular Feminized Mouse: A Potential Protective Mechanism Against Atherosclerosis and Fatty Liver Disease”

11. Endo 2012 Houston (Endocrine society annual meeting) poster “Cardiovascular Safety and Testosterone Replacement Therapy in Male Hypogonadism Including Men with Type 2 Diabetes and Cardiovascular Disease”
12. Endo 2012 Houston(Endocrine society annual meeting) poster “Long-Term Effects of Testosterone Replacement Therapy on Cardiovascular Risk Factors in Hypogonadism, Including Men with Cardiovascular Disease and/or Type 2 Diabetes”
13. BES 2012 Harrogate (British endocrine society annual meeting), Poster “Testosterone replacement therapy has beneficial effects on Cardiovascular risk factors and liver function in hypogonadal men”
14. BES 2011 Birmingham(British endocrine society annual meeting), Poster: “Low testosterone and severity of erectile dysfunction (ED) are independently associated with poor health related quality of life ( HRQoL) in men with type 2 diabetes”.
15. Endo2011 Boston (Endocrine society annual meeting), Poster, Men with Low Testosterone and Type 2 Diabetes Have Reduced Health-Related Quality of Life (HRQoL)
16. Endo2011 Boston (Endocrine society annual meeting), Poster, Severity of Erectile Dysfunction and Testosterone Deficiency Are Associated with Reduced Quality of Life (HRQoL) in Men with Type 2 Diabetes Mellitus

**Schematic representation of studies conducted over the course of PhD and personnel contribution to each section**

**Effects of Testosterone Levels on Mortality and Cardiovascular Risk in Men with Type 2 Diabetes**

**Clinical studies**

**Mortality Study** Long term effect of testosterone on mortality in men with type 2 diabetes – Study design, data collection, analysis, presentation and publication.

**Longitudinal study** - Using the original screening biochemical data from the previous study and the current clinical data assessed the long term effect of baseline testosterone and SHBG levels on cardiovascular risk profile and cardiovascular events – Study design, clinical assessment, data collection, analysis and presentation.

**Cross sectional study**- using the follow up clinical data and the follow up biochemical data from the same cohort, assessed the effect of current testosterone and SHBG levels on cardiovascular risk factors including carotid intima media thickness in a cross sectional study – Study design, clinical assessment, including carotid ultrasound, data collection, analysis and presentation.

**Role androgen receptor in cardio vascular risk profile** – Studied the relationship between the androgen receptor CAG polymorphism, ratio of testosterone to AR CAG and cardiovascular risk profile in men with type 2 diabetes in a cross sectional study – Study design, clinical assessment, including carotid ultrasound, some of the DNA extraction and AR CAG measurement, data collection and analysis.

**Animal study - Tfm Mouse model**

**The molecular mechanism of non-androgen receptor mediated actions of testosterone on the metabolic pathways** - The effect of testosterone on the glucose and lipid metabolism in liver, muscle and adipose tissue - Study design, tissue collection, RNA extraction and QPCR analysis of target molecules, data analysis, presentation and contribution to publication

## **Chapter One**

### **General Introduction**

#### **1.1 Introduction**

Testosterone is a steroid hormone produced by testes and adrenal cortex. Men have a higher concentration as the testes produce a much larger quantity of testosterone. The testosterone is released in a circadian rhythm with circulating levels peaking between 06.00 and 08.00 and reaching a nadir between 18.00 and 20.00. Testosterone is metabolised to further active hormones  $5\alpha$ - dihydrotestosterone (DHT) and  $17\beta$  oestradiol. DHT is responsible for many virilising effects of testosterone.

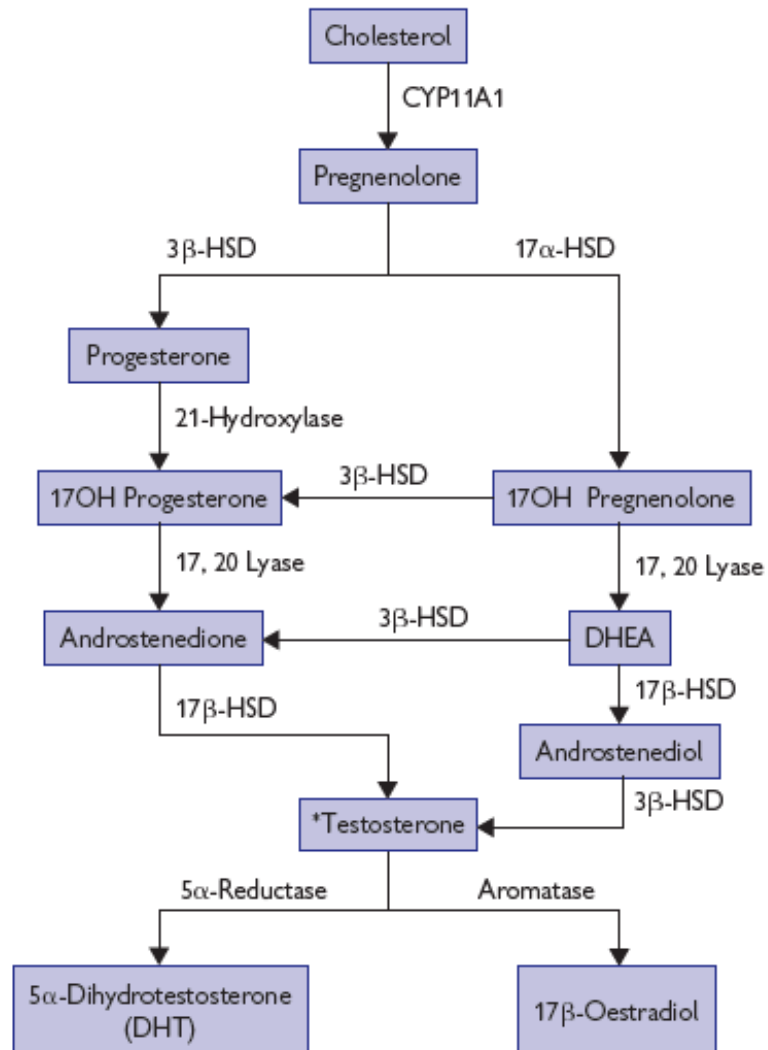
Oestradiol in men has an important role in gaining and maintaining bone mass, closing of the epiphyses and the feedback on gonadotropin secretion. It has also reported to have significant role in modulating the sexual functions including libido, erectile function and spermatogenesis in men (Schulster, Bernie et al. 2016). Disruption in oestrogen receptors have been shown to result in delayed skeletal maturation and osteoporosis along with other metabolic abnormalities including insulin resistance, glucose intolerance, and acanthosis nigricans (Smith, Boyd et al. 1994). Similarly human aromatase deficiency has reported to be resulting in skeletal dysmorphism and metabolic abnormalities including insulin resistance, type 2 diabetes and steatohepatitis (Maffei, Murata et al. 2004). Oestrogen receptor knockout (ERKO) mice showed disruption of skeletal development, subfertility or infertility and impaired glucose tolerance (Couse and Korach 1999). Aromatase inhibitor therapy has been reported to be effective in treatment of idiopathic short stature, constitutional delayed puberty and precocious puberty in boys. There are also studies showing benefits of aromatase inhibitor therapy in men with late onset hypogonadism, and in obese men to improve the



testosterone levels (de Ronde and de Jong 2011). The metabolism of testosterone is shown in figure 1.

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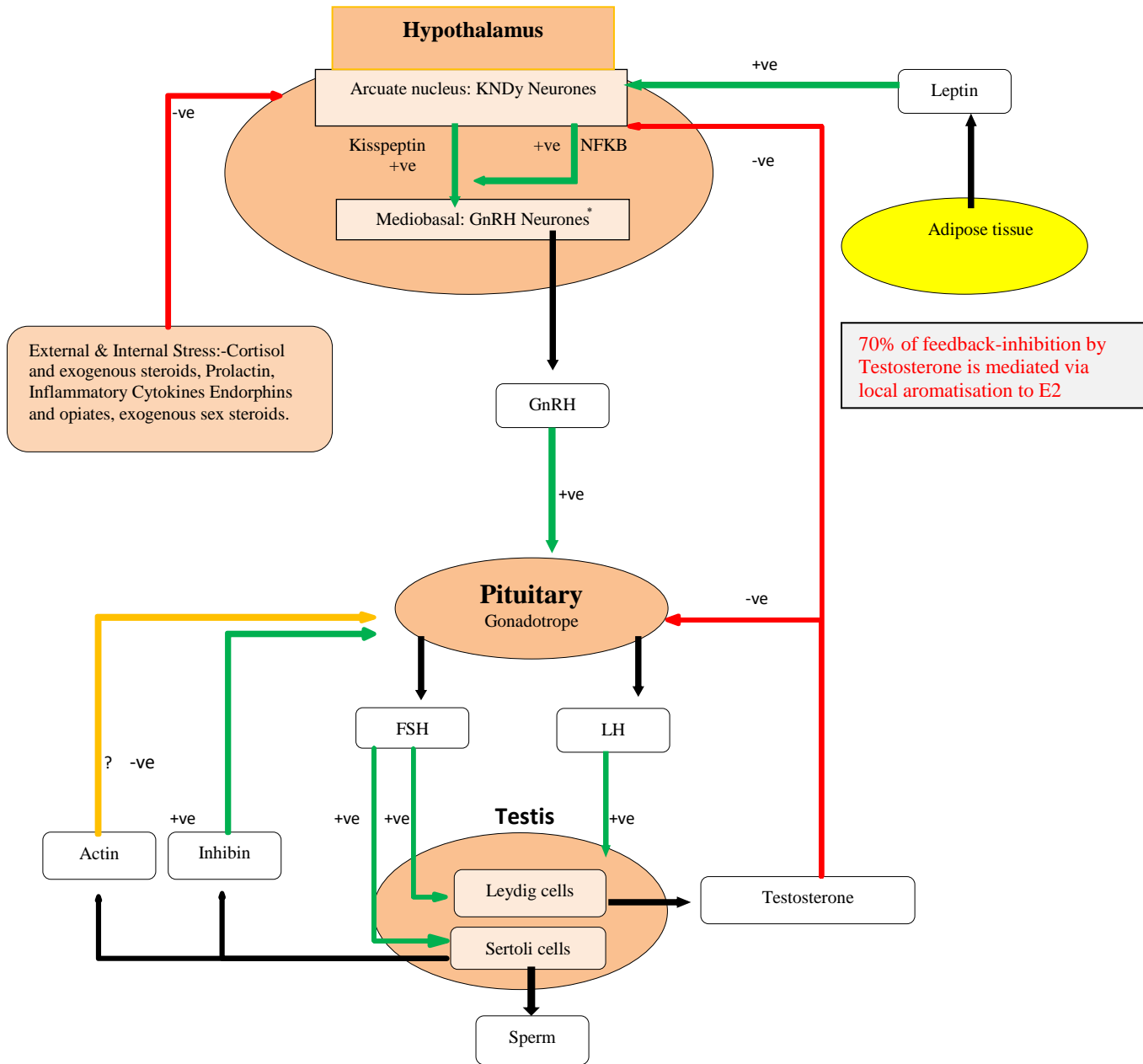
**Figure 1.1 Metabolism of testosterone**



**Pathway of testosterone synthesis and metabolism.**(CYP11A1=cholesterol side chain cleavage mitochondrial P450 enzyme; HSD=hydroxysteroid dehydrogenase; DHEA=dehydroepiandrosterone); Testosterone is also broken down to form inactive metabolites.

Testosterone secretion is regulated by gonadotrophins mainly luteinising hormone (LH) which in turn is regulated by gonadotrophin releasing hormone (GnRH). In plasma, testosterone circulation is mostly protein bound mainly to the sex hormone binding globulin (SHBG 60-80%) and some to albumin (20-40%). About 1-3% of testosterone circulates as free hormone. The half-life of free testosterone is 10 minutes. SHBG has high affinity for testosterone and testosterone bound to it is thought to be not available for use by tissues. Testosterone bound to albumin is readily available as it is weak bond. The free testosterone and albumin bound fraction make up the 'bioavailable testosterone' (Pardridge 1986). Bioavailable and free testosterone levels provide stronger correlations with parameters such as bone mineral density and muscle strength than the total testosterone (van den Beld, de Jong et al. 2000).

**Figure 1.2 Hypothalamo – pituitary - gonadal axis**



FSH- follicle stimulating hormone, LH- Luteinising Hormone, GnRH- Gonadotropin releasing hormone., NFKB- nuclear factor kappa B,

KNDy- kisspeptin/neurokinin B/dynorphin.

\*There is sexual dimorphism in regulation of GnRH secretion, in that males lack the "feed-forward" oestrogen-mediated positive feedback pathway that cause the pre ovulatory LH surge. Also, whereas both glucocorticoids and obesity tend to suppress the axis in males, they do not do so in females; rather causing a PCOS-like phenotype.

There is a steady decline in testosterone levels with age (Harman, Metter et al. 2001, Feldman, Longcope et al. 2002). Bioavailable testosterone tends to fall slightly more (2-3%) in comparison with total testosterone levels (1.6%) per year. A more recent study (Handelsman 2013) suggested age itself is not associated with a decrease in testosterone levels. The study suggests the decline in testosterone is due to the comorbidities accumulated with age rather than the age itself. The prevalence of hypogonadism in male populations is not known with certainty. There are different criteria adapted for diagnosis of hypogonadism adopted by different professional societies and "expert" panels, with a clear disparity between classical endocrinology, which emphasises the centrality of making an overarching diagnosis to explain the cause of hypogonadism, and Andrology/Sexual medicine, which is more focused on serum T levels and patient symptoms. This contributes to the uncertainty of the prevalence regarding hypogonadism.

In one of the large study looking at the prevalence of hypogonadism, Baltimore Longitudinal Study of aging reported up to 30% prevalence of low testosterone in men over 70 years and above (Harman, Metter et al. 2001). A more recent study of nearly 3000 community dwelling men aged 40-79, reported a much lower prevalence of late onset hypogonadism of 2.1% (Tajar, Huhtaniemi et al. 2012). The Boston Area Community Health (BACH) survey, reported a crude prevalence of hypogonadism of 5.6% in men aged 30-79 years and was not significantly related to race and ethnic group (Araujo, Esche et al. 2007). There was a marked increase in prevalence after the age of 70 years. Among men with type 2 diabetes up to 40% have low levels of testosterone (Kapoor, Aldred et al. 2007).

Late onset hypogonadism was originally defined broadly as “a clinical and biochemical syndrome associated with advancing age and characterised by typical symptoms and a

deficiency in serum testosterone levels. It may significantly reduce quality of life and adversely affects the function of multiple organ systems” (Jones 2009).

The European Male Ageing Study (EMAS)(Wu , Tajar et al. 2010) found that low testosterone levels mediated by gonadotrophin suppression in older men was overwhelmingly mediated by effect of concomitant obesity or ill health. Age-related effect was seen in less than 5% of men and characterised by raised LH and FSH indicating primary gonadal insufficiency presumably mediated by accumulation of age-related molecular damage. Based on the new evidence a new definition of LOH is formulated which defines LOH as “a clinical and biochemical syndrome associated with advancing age and characterized by symptoms and a deficiency in serum T levels (below the young healthy adult male reference range” (Wang, Nieschlag et al. 2009). However these definitions are still may not clearly define the state because it fails to explicitly redefine LOH as a state of primary gonadal insufficiency, rather than of non-specifically low testosterone.

A study on patients with type 1 diabetes has shown that prevalence of hypogonadism may be similar to type 2 diabetic patients (Chandel, Dhindsa et al. 2008). A recent review by Zaortsky et al critically analysed the studies on the associations and consequences of male hypogonadism (Zarotsky, Huang et al. 2014). After reviewing 53 qualifying studies they concluded that the important factors which predicted and correlated with hypogonadism were advanced age, obesity, a diagnosis of metabolic syndrome and a poor general health status. Type 2 diabetes mellitus correlated with hypogonadism in most studies, but was not established as a risk factor. Although diseases such as coronary heart disease, hypertension, stroke and peripheral arterial disease did not predict hypogonadism, they did correlate with incidental low testosterone. The data reviewed on potential consequences suggest that low testosterone levels may be linked to earlier all-cause and cardiovascular related mortality among men.

Over last two decades there have been a large number of studies looking at the actions of testosterone, clinical as well as molecular, suggesting a larger role of testosterone over and above its action as a sex hormone.

There is growing evidence that low testosterone levels are associated with greater morbidity and mortality (Zarotsky, Huang et al. 2014). However it is not clear if the suppression of pituitary gonadal axis is adaptive, maladaptive or neutral. Studies mainly correlated low testosterone with all-cause mortality. The increase in mortality appears to be related mainly to cardiovascular diseases, COPD and cancer. There is also evidence that obesity is a pro-inflammatory metabolic state. There is an increase in secretion of pro-inflammatory cytokines and adipokines free fatty acids from adipose tissue. These parameters are considered to be important risk factors in the development of metabolic syndrome and type 2 diabetes. The increased oestrogen secretion from adipose tissues could contribute to the development of androgen deficiency in men (Traish, Feeley et al. 2009). There is a high prevalence of low testosterone and symptomatic hypogonadism in men with type 2 diabetes and/or metabolic syndrome (Dhindsa, Prabhakar et al. 2004, Agarwal and Oefelein 2005, Corona, Mannucci et al. 2006, Ding, Song et al. 2006, Kapoor, Aldred et al. 2007, Corona, Mannucci et al. 2009). There also evidence that low testosterone state increases the deposition of visceral adipose tissue.(Corona, Mannucci et al. 2006, Corona, Mannucci et al. 2009). There is growing evidence that testosterone levels (total and free testosterone) and SHBG levels are closely linked with risk of developing metabolic syndrome (Dhindsa, Prabhakar et al. 2004, Allan and McLachlan 2010, Faris and Smith 2010, MacDonald, Herbison et al. 2010, Brand, van der Tweel et al. 2011). Data from Massachusetts Male aging study showed that low total testosterone was associated with increased risk of developing metabolic syndrome on long term follow up in men with normal

BMI (Kupelian, Page et al. 2006). A more recent cross-sectional study in men with type 1 and type 2 diabetes compared to healthy controls reported a strong correlation between central obesity and serum testosterone and a weaker correlation with erectile dysfunction or other symptoms of testosterone deficiency (Biswas, Hampton et al. 2012).

There are many recent epidemiological studies showing an increase in mortality with low testosterone levels as extensively illustrated in the recent reviews (Araujo, Dixon et al. 2011, Oskui, French et al. 2013, Muraleedharan and Jones 2014, Zarotsky, Huang et al. 2014).

These include general population studies and specific disease groups of patients with either induced or acquired hypogonadism and due to androgen deprivation therapy have shown that there is an increase in cardiovascular disease and death in patients with low testosterone.

However it is also known that acute or chronic illness can cause a decrease in testosterone levels. This raises the question whether testosterone is a biomarker for ill health or a contributory factor for morbidity and mortality in these patients.

Testosterone deficiency has also been shown to reduce insulin sensitivity, increase bone turnover and osteoporosis, cause muscle weakness, cognitive impairment, generalised lethargy and fatigue along with sexual dysfunction (Yeap 2009). A large number of epidemiological studies have suggested that many other disease processes - Alzheimer's disease (Moffat, Zonderman et al. 2004), frailty, obesity (Svartberg, Aasebo et al. 2004) hypercholesterolemia (Haffner, Mykkanen et al. 1993, Van Pottelbergh, Braeckman et al. 2003) and hypertension(Phillips, Jing et al. 1993) are associated with low testosterone levels.

Although there is growing evidence that low testosterone is linked to adverse cardio metabolic state, the issue of testosterone replacement is still controversial. The evidence from



smaller controlled trials and larger population studies suggest testosterone replacement could be beneficial in improving the markers of cardiovascular disease, metabolic syndrome and improving mortality. However lack of large randomised trials to assess the efficacy of testosterone replacement in reducing the mortality and morbidity level leaves many important questions unanswered.

## **1.2. Testosterone and mortality**

### **1.2.1 Population or community studies**

Testosterone level is closely linked to morbidity and mortality in men. There are a number of studies in the last decade looking into the effect of testosterone level and mortality. These include population based studies and patients with specific diseases. The major community based studies are summarised in table 1.1.

The bulk of studies looking into the effect of testosterone levels and mortality suggests a link between low testosterone and increased all-cause mortality (Shores, Matsumoto et al. 2006, Khaw, Dowsett et al. 2007, Laughlin, Barrett-Connor et al. 2008, Tivesten, Vandenput et al. 2009, Vikan, Schirmer et al. 2009, Haring, Volzke et al. 2010, Menke, Guallar et al. 2010, Hyde, Norman et al. 2012). However three studies; (Smith, Ben-Shlomo et al. 2005, Araujo, Kupelian et al. 2007, Haring, Teng et al. 2013) did not show any relationship between testosterone levels and mortality. Major studies are described separately and the merits and limitations of each will be discussed.

**Table 1.1 Population/Community based Studies – all cause and cardiovascular mortality**

Author, year (study name)	Country	N	Follow up	Definition of hypogonadism used	Consequence
Yeap et.al 2014(Yeap, Alfonso et al. 2014)	Australia	3690	6.7	T quartiles	Optimal plasma testosterone is associated with reduced all-cause mortality and higher dihydrotestosterone with reduced ischemic heart disease mortality
Pye et al (Pye, Huhtaniemi et al. 2014)	UK ((European multicentre)	2599	4.3 years	T< 8 nmol/l	2-fold higher in Mortality in those with T less than 8 nmol/L and 3-fold higher in those with three sexual symptoms.
Hyde 2012(Hyde, Norman et al. 2012)	Australia	3637	5.1	T, FT, and LH as restricted cubic splines	Lower free testosterone predicted a higher all-cause mortality
Haring et al., 2010(Haring, Volzke et al. 2010) SHIP	Germany	1954	7.2 years	TT < 8.7 nmol/L (250 ng/dL)	Low serum testosterone levels were associated with an increased risk of all-cause mortality
Menke et al., 2010(Menke, Guallar et al. 2010) (NHANES III)	USA	1114	18 years	90 <sup>th</sup> percentile vs. 10 <sup>th</sup> percentile	Men with low free and bioavailable testosterone levels may have a higher risk of mortality
Yeap et al., 2009(Yeap, Hyde et al. 2009)	Australia	3443	3.5 years	TT quartiles;	Lower testosterone levels predict incident stroke and transient ischemic attack in older men
Vikan et al., 2009(Vikan, Schirmer et al. 2009), TROMSO	Norway,	1568	11.2 years	TT and FT quartiles based on population sample.	Men with free testosterone levels in the lowest quartile had a 24% increased risk of all-cause mortality.
Tivesten et al.2009(Tivesten, Vandenput et al. 2009) MrOS	Sweden	3014	4.5 years	TT quartiles	low serum testosterone and oestradiol have increased risk of mortality, and subjects with low values of both testosterone and oestradiol have the highest risk of mortality.
Lehtonen et al. 2008(Lehtonen, Huupponen et al. 2008)	Finland	187	10 years	NR	Independent inverse association between endogenous testosterone concentration in serum and mortality in 70-year-old non-diabetic men followed for 10 years.
Laughlin et al., 2008(Laughlin, Barrett-Connor et al. 2008) Rancho Bernardo Study	USA	794	11.8 years	TT, FT and bioavailable T quartiles.	Testosterone insufficiency in older men is associated with increased risk of death over the following 20 years
Araujo et al., 2007(Araujo, Kupelian et al. 2007) MMAS	USA	1686	15.3 years	TT, highest quartile vs. lowest.	endogenous sex steroid levels have weak associations with mortality
Khaw et al., 2007(Khaw, Dowsett et al. 2007) EPIC-Norfolk	Norway	2314	7 years	TT quartiles.	Endogenous testosterone concentrations are inversely related to mortality due to cardiovascular disease and all causes.
Shores et al., 2006(Shores, Matsumoto et al. 2006)	USA	858	4.3 years	TT< 250 ng/dL (< 8.7 nmol/L) or FT < 0.75 ng/dL (< 0.03 nmol/L)	Low testosterone levels were associated with increased mortality
Smith et al., 2005 (Smith, English et al. 2005)	Wales	2512	16.5 years	TT < 12 .1 nmol/L	No significant association between testosterone levels and mortality
Shores et al., 2004(Basaria and Dobs 2007)	USA	44	6 months	TT ≤3.0 ng/mL or FT ≤9.0 pg/mL	Low testosterone levels were correlated with decreased physical function and increased risk for 6-month mortality

TT: Total Testosterone FT: Free Testosterone BT = bioavailable testosterone; CHD = coronary heart disease; CKD: chronic kidney disease; COPD = chronic obstructive pulmonary disease; CVD = cardiovascular disease; ED = erectile dysfunction; EPIC-Norfolk = European Prospective Investigation into Cancer in Norfolk; HR = hazard ratio; Mets = metabolic syndrome; MMAS = Massachusetts Male Aging Study; MrOS = Swedish Osteoporotic Fractures in Men; NA = not applicable; NHANES = Third National Health and Nutrition Examination Survey Mortality Study; NR = not reported; NS = not significant; SHIP = Study of Health in Pomerania .

The study by Shores and colleagues (Shores, Matsumoto et al. 2006) followed up 585 male veterans over the age of 40 years, followed up for a mean period of 4.3 years. In this group testosterone levels were below normal in approximately 20% of the patients and in another 30% the levels were equivocal. There was increased all-cause mortality in men with low testosterone as compared with those with normal testosterone (35% vs 20%). Furthermore men with low and equivocal testosterone levels had higher mortality than men with normal testosterone levels after adjustment for age and co morbidities.

The Rancho Bernardo study (Laughlin, Barrett-Connor et al. 2008) a prospective population based study in a community- dwelling group of about 800 men with a mean age of 74 years were followed for an average of 11.8 years. During this period 538 deaths occurred with a mortality rate of 57.5 per 1000 person years. Subjects were analysed in quartiles according to the testosterone levels. After adjusting for age, adiposity and lifestyle choices the risk of death was 44% higher for men in the lowest quartile of total testosterone relative to the highest.

The EPIC-Norfolk (Khaw, Dowsett et al. 2007) study was a nested case-control study based on 11606 men aged 40 to 79 years. After exclusion of patients with cancer or cardiovascular disease at baseline, 825 men who subsequently died were compared with a control group of 1489 men still alive, matched for age. Testosterone concentrations were inversely related to mortality due to all-cause, cardiovascular disease and cancer. An increase of 6 nmol/L serum testosterone was associated with a 0.81 multivariate adjusted mortality odds ratio for mortality.

The Pomorania study (Haring, Volzke et al. 2010) reported a prospective population based study of 1954 men followed up for a mean of 7.2 years. They found that men with low testosterone had a significantly greater mortality risk from all causes. After adjusting for co variates including age, BMI and smoking status low testosterone continued to be associated with increased mortality.

The Tromsø study (Vikan, Schirmer et al. 2009), analysed a cohort of 1568 randomly selected men followed up for 10 years. They reported men with free testosterone levels in the lowest quartile had a 24% increased risk of all-cause mortality.

A prospective population based cohort study (Tivesten, Vandenput et al. 2009) in which they studied more than 3000 elderly men with a mean age of over 75years. After a follow up of 4.5 years there was an increase in mortality in the low testosterone group with multivariate adjusted hazard ratio for mortality was 1.65 for the men with low testosterone. In subjects with low levels of both testosterone and oestradiol risk of death nearly doubled (HR, 1.96).

The Third National Health and Nutrition Examination Survey Mortality Study (Menke, Guallar et al. 2010), included 1,114 US men with a mean age of 40 years who had no history of cardiovascular disease or cancer. This was a prospective study of men over 18 years of age. The results showed there was an increased risk of all cause and cardio vascular mortality with low free testosterone and bioavailable testosterone during follow-up between baseline and up to 9 years. However during the further follow up between 9 to 18 years this association were lost.

The Health in Men (HIM) study from Western Australia (Hyde, Norman et al. 2012) reported the results of a cohort of over 3600 men followed up over 5 years. The average age was 77 years. In this study low free testosterone were associated with increased all-cause and

cardiovascular mortality. The total testosterone levels did not show any significant association with mortality. Interestingly high levels of luteinising hormone and sex hormone binding globulin (SHBG) were also associated with increased all-cause and cardiovascular disease mortality. This may potentially due to the detection of true late onset hypogonadal patients who have high LH.

More recently a study published by Yeap et al, reported mortality in a cohort of 3690 community-dwelling men aged 70-89 years, followed up for 6.6 years, in relation to quartiles of plasma testosterone or dihydrotestosterone and oestradiol (Yeap, Alfonso et al. 2014). After adjusting for other risk factors, testosterone and dihydrotestosterone were inversely associated with all-cause mortality they further concluded that an inverse correlation between the levels of dihydrotestosterone was and ischaemic heart disease mortality

### **1.2.2 Studies in specific disease populations**

Apart from the above studies which are from population or community dwelling men there are many studies which shows low testosterone as predictor of mortality in specific disease groups. These studies are summarised in table 2. The major studies are discussed further in detail in the following sections.

**Table 1.2 Disease specific population studies – all cause and cardiovascular disease mortality**

Author, year	Country	Population studied	N	Follow up	Consequence
Tint et al.(Tint, Hoermann et al. 2016)	Australia	Type 2 diabetes patients	531	7.6 years	A decrease in cFT and an increased SHBG increased mortality
Sinclair et al(Sinclair, Grossmann et al. 2016)	Australia	Men with cirrhosis of the liver	145	8.3 months	low testosterone was significantly associated with mortality
Bello AK(Bello, Stenvinkel et al. 2014)	Canada	Hemodialysis	623	6.8 years	Higher serum testosterone levels were associated with decreased unadjusted risk of death
Khurana et al (Khurana, Navaneethan et al. 2014)	USA	CKD stage3- non dialysis dependent	2419	2.3 years	Higher log testosterone was associated with significantly lower mortality.
Wehr (Wehr, Pilz et al. 2011)	Austria	Men referred for angiography	2078	7.7 years	Higher FT associated with decreased CHF mortality
Wang et al., (Wang, Wang et al. 2010)	China	Males with CHF	175	5 years	TT was significantly associated with survival
Lerchbaum et al.(Lerchbaum, Pilz et al. 2012),	Austria	Males referred for coronary angiography	2069	7.7 years	A combined deficiency of FT and 25(OH) D is significantly associated with fatal events in a large cohort of men referred for coronary angiography
Militaru et al., (Militaru, Donoiu et al. 2010)	Romania	Males with MI	126	30 days	Low level of T was independently related to total short-term mortality
Malkin et al (Malkin, Pugh et al. 2010)	UK	Men referred for angiography	930	6.9 years	Excess mortality in the testosterone deficient group compared to normal
Carrero et al.(Carrero, Qureshi et al. 2009)	Sweden	Male HD patients	126	41 months	Men with testosterone values in the lowest tertile had increased all-cause and CVD mortality
Kyriazis et al., (Kyriazis, Tzanakis et al. 2011)	Greece	Male HD patients	111	37 months (median)	Testosterone deficiency in male HD patients is associated with increased CVD and all-cause mortality
Carrero et al.(Carrero, Qureshi et al. 2011)	Sweden	Male patients with ESRD	260	3 years	Testosterone deficiency was independently associated with cardiovascular co-morbidity
Gungor et al., (Gungor, Kircelli et al. 2010)	Turkey	Male HD patients	420	32 (months)	The overall survival rate was significantly lower in patients within the low testosterone tertile compared with those within the high tertile
Haring et al., (Haring, Nauck et al. 2011)	Germany	Males with CKD	1822	9.9 years	Men with kidney dysfunction and low TT concentrations had more than 2-fold increased all-cause mortality risk
Corona et al.(Corona, Monami et al. 2010)	Italy	Males presenting to ED clinic	1687	4.3 years	Low testosterone levels are associated with a higher mortality of MACE
Lin et al.(Lin, Lee et al. 2011),	USA	Men with MetS.	596	15.6 years	The combination of lower bioavailable testosterone and MetS is associated with an increased cardiovascular mortality in the men
Ponikowska et al.(Ponikowska, Jankowska et al. 2010)	Poland	Males with T2DM and stable CAD	153	19 months (median)	In diabetic men with stable CAD, testosterone and DHEAS deficiencies are related to high CV mortality

CHD = coronary heart disease; CKD: chronic kidney disease; COPD = chronic obstructive pulmonary disease; CVD = cardiovascular disease; CV=Cardiovascular; MI= Myocardial infarction; ESRD = end stage renal disease; FT= free testosterone; cFT= Calculated free testosterone; HD = haemodialysis; Mets= metabolic syndrome; TT= total testosterone; MACE= Major adverse cardiovascular events

A study from Third National Health and Nutrition Examination Survey (NHANES III), reported a follow up of 596 men, of which 187 had metabolic syndrome. The results showed that odds for metabolic syndrome were inversely related to the calculated bioavailable testosterone. Similarly the cardiovascular mortality risk was lower in the higher testosterone group. (Lin, Lee et al. 2011).

### **1.3. Relationship of testosterone to cardiovascular mortality and morbidity**

In the studies looking at mortality in low testosterone state, the major cause of death was cardiovascular disease (Khaw, Dowsett et al. 2007, Laughlin, Barrett-Connor et al. 2008, Carrero, Qureshi et al. 2009, Yeap 2009, Corona, Monami et al. 2010, Haring, Volzke et al. 2010, Menke, Guallar et al. 2010, Ponikowska, Jankowska et al. 2010, Haring, Nauck et al. 2011, Kyriazis, Tzanakis et al. 2011, Lin, Lee et al. 2011, Lerchbaum, Pilz et al. 2012, Yeap, Alfonso et al. 2014). There is also a strong link to metabolic syndrome and diabetes as described in a systematic review recently (Zarotsky, Huang et al. 2014). It is well-known that male sex and increasing age are independent risk factors for coronary disease.

Recent studies have provided evidence about the strong link between testosterone and cardiovascular disease as discussed extensively in the reviews (Jones 2010, Kelly and Jones 2013). The link between testosterone and cardiovascular disease has interested researchers for many years. In one of the earliest studies in this field researchers from USA (Hamm 1942, Lesser 1946) reported improvement in angina symptoms after testosterone treatment. In the 1990s researchers raised the possibility of a link between low testosterone and coronary artery disease (Phillips, Jing et al. 1993). Recent studies have supported these earlier findings. The

study from our group has reported that when compared with controls, men with proven coronary artery disease had lower levels of testosterone levels (English, Steeds et al. 2000, Jones, Jones et al. 2003, Malkin, Pugh et al. 2003, Jones, Nettleship et al. 2005). Our research group have further reported that testosterone is a direct vasodilator of vascular smooth muscles (Jones, Pugh et al. 2003, Kelly and Jones 2013). Studies have reported linking testosterone deficiency to cardiac failure (Tappler and Katz 1979, Kontoleon, Anastasiou-Nana et al. 2003).

In the Rancho Bernado study, one of the major epidemiological studies linking testosterone to mortality (Laughlin, Barrett-Connor et al. 2008), the leading cause of death was cardiovascular disease (529 deaths of which 264) (HR 1.38;95%CI 1.02–1.85). This increase in cardiovascular mortality persisted even after excluding death for first 5 years.

In the EPIC-Norfolk study (Khaw, Dowsett et al. 2007) a major European study looking at the testosterone levels and mortality, there were 369 cardiovascular deaths (of a total of 825 deaths). They reported that increased cardiovascular mortality persisted after age and covariate adjustment.

The LUdwigshafen RIsk and Cardiovascular Health (LURIC) study, a prospective study of 2078 men who were referred for angiography between July 1997 and January 2000 (Wehr, Pilz et al. 2011). They were followed up for 7.7 years. The study reported in multivariate-adjusted analyses, a significantly decreased risk of congestive cardiac failure mortality for men in the highest free testosterone quartile compared with men in the lowest free testosterone quartile which persisted after further adjustment for statin and spironolactone. This significance was lost in the fully adjusted model and they concluded that there is an



independent association of low free testosterone levels with increased mortality from congestive cardiac failure. A further report from the same group reported an increased cardiovascular mortality with low free testosterone but not with low total testosterone levels were not associated with increase mortality (Lerchbaum, Pilz et al. 2012).

Our research group reported a 7 year follow up study of over 900 men with coronary artery disease (Malkin, Pugh et al. 2010). The results showed an increased mortality in the low testosterone group. In this study, apart from low bioavailable testosterone, other factors influencing the survival were LV dysfunction,  $\beta$  blocker and aspirin therapy. After left ventricular dysfunction low testosterone was the strongest predictor of mortality. The survival curves confirmed that low bioavailable testosterone was significantly associated with all-cause ( $p < 0.0001$ ) and cardiovascular ( $p = 0.007$ ) mortality. It concluded that low bio available testosterone, a more sensitive marker of androgen action, is associated with cardiovascular mortality.

A small study of 150 men with type 2 diabetes and coronary artery disease, reported results after a follow up period of 19 months (Ponikowska, Jankowska et al. 2010). There were 43 (28%) cardiovascular deaths. They identified testosterone and dehydroepiandrosterone sulphate (DHEAS) deficiencies as independent risk factors for increase in cardiovascular mortality.

A study by Corona et al looked at the major adverse cardiovascular events (MACE) in relation to testosterone levels (Corona, Monami et al. 2010) where over 1600 patient from andrology clinic were studied. They found that low testosterone levels are associated with a higher mortality relating to MACE.

Testosterone levels fall acutely after a myocardial infarction and can take up to two months to recover (Pugh, Channer et al. 2002). Testosterone levels in an acute setting may also be important in adverse cardiac outcomes following a major cardiovascular event. A study of 126 patients with acute myocardial infarction, reported low level of testosterone levels at the time of admission and was significantly related with 30 day mortality (Militaru, Donoiu et al. 2010).

A prospective study of 126 patients undergoing haemodialysis (Carrero, Qureshi et al. 2009) was followed up for 40 months. Men with low testosterone had increased all-cause mortality due to cardiovascular disease. This increase in mortality rate persisted after multi-variate analysis including pre-existing cardiac disease, medication and diabetes albumin and inflammatory markers.

The surrogate markers of cardiovascular disease have been studied extensively in relation to testosterone levels. Many of these studies show worsening surrogate markers for cardiovascular disease with low testosterone levels. This has been extensively discussed in a recent review where the authors concluded that cardiovascular and all-cause mortality is linked with bioavailable testosterone levels with low levels predicting higher mortality (Oskui, French et al. 2013). They further concluded that patients with cardiac disease have a low testosterone levels and the severity of disease correlates with the level of testosterone. Low levels of testosterone has also been linked to the increase in the numbers of coronary arteries with stenosis (Nettlehip, Jones et al. 2009).

Increased vascular stiffness and deterioration in lipid profiles and diabetic control occurs as early as 3 months after initiation of androgen suppression treatment for prostate cancer

(Smith, Bennett et al. 2001). In a group of 22 prostate cancer patients followed up for six months there was an increase in augmentation index after a 3-month period.

Although the majority of studies published so far show a correlation between low testosterone levels and increase in all cause and cardiovascular mortality, one should be cautious about the causal effect of low testosterone and mortality till further interventional data is available. In this context it is worth discussing a similar effect reported in patients with vitamin D deficiency and mortality. In the meta-analysis of the eight prospective cohort studies from Europe and US showed an increase in all cause and cardiovascular mortality with a pooled risk ratio of 1.57 (95% CI 1.36 to 1.81) in vitamin D deficient population. The study further reported increased risk of cancer mortality risk ratio of 1.70 (1.00 to 2.88) in those with a history of cancer (Schöttker, Jorde et al. 2014). Similar to testosterone studies, the long term data regarding the benefit of vitamin D replacement in terms of longevity is still awaited.

#### **1.4 Testosterone and mortality in renal disease**

Many studies report low testosterone levels in CKD linking to increased mortality as noted in the table. One was a large study involving 2,419 men with CKD stages 3-4 (Khurana, Navaneethan et al. 2014). In the multi-variate adjusted model including that for testosterone supplementation higher log testosterone was associated with significantly lower mortality (HR per 1 log unit, 0.70; 95% CI, 0.55-0.89). When compared to the highest quintile, the second lowest quintile of testosterone was associated with higher mortality (HR, 1.53; 95% CI, 1.09-2.16). The authors concluded that low total testosterone level may be associated with higher mortality in men with CKD stages 3-4.

A similar population-based Study of Health in Pomerania followed 1822 men with CKD for 9.9 years (Haring, Nauck et al. 2011). They reported a 2-fold increase in all-cause mortality risk (HR, 2.52; 95% CI, 1.08-5.85) with low testosterone and kidney dysfunction. Similarly increased mortality has been reported in men undergoing dialysis who also have low testosterone compared to those with normal testosterone levels (Gungor, Kircelli et al. 2010, Carrero, Qureshi et al. 2011, Kyriazis, Tzanakis et al. 2011).

## **1.5 Metabolic syndrome, diabetes, cardio-metabolic markers and testosterone**

### **1.5.1. Metabolic syndrome**

Obesity is a major health problem in all parts of the world and the prevalence of obesity, metabolic syndrome and type 2 diabetes continue to rise sharply which poses an international health challenge. Obesity leads to adverse metabolic effects on blood pressure, lipid and glucose metabolism mediated by insulin resistance. Cardiovascular disease is a leading cause of morbidity and mortality worldwide. Men are at a higher risk of cardiovascular morbidity and mortality.

Metabolic syndrome was first described in 1923 as an association between hypertension and gout (Kylin 1923). There were modifications over the years in which new criteria were added (Vague 1947) (Reaven 1988). Reaven described "Syndrome X" as a constellation of insulin resistance, hyperglycaemia, hypertension, low HDL-cholesterol and increased VLDL – triglyceride levels. Metabolic syndrome is associated with an increased risk of myocardial infarction, cerebrovascular disease and cardiovascular death as well as diabetes, fatty liver

and several cancers. Obesity is most commonly defined by body mass index in clinical practice but central obesity as clinically determined by waist circumference or waist-to-hip ratio is a stronger predictor of insulin resistance, diabetes and cardiovascular disease (Larsson, Svardsudd et al. 1984, Larsson, Seidell et al. 1989, Huang, Rodreiguez et al. 1997). Insulin resistance is recognised as a central component in the development of metabolic syndrome and diabetes. The close link between visceral obesity and insulin resistance is based on the high metabolic activity of visceral fat.

Controversy continues as to whether the concept of metabolic syndrome is useful in clinical practice. Some experts believe that once a diagnosis of type 2 diabetes is made this becomes the overarching diagnosis from that point onwards and the concept of metabolic syndrome becomes redundant. A key argument centres on whether the presence of the syndrome predicts cardiovascular risk better than the constituent parts. There are also variations among different populations in the measures used for definition of metabolic syndrome. Even with these shortcomings, the idea of the metabolic syndrome does seem useful in that cardiovascular risk factors often cluster together.

**Table 1.3 Criteria for diagnosing metabolic syndrome**

	<b>WHO (1999)</b>	<b>IDF (2006)</b>	<b>NCEP ATP III (2004)</b>
Essential Feature	Diabetes, impaired glucose tolerance or insulin resistance <sup>a</sup>	Central obesity (men >94cm waist, women >80cm waist)	No essential feature
Diagnosis Requires	Essential feature plus 2 from;	Essential feature plus 2 from;	Diagnosis requires three factors from;
	Hypertension (>140/90)	Hypertension (>130/85)	Hypertension (>130/85)
	Hypertriglyceridemia (>1.7mmol/l)	Hypertriglyceridemia (>1.7mmol/l)	Hypertriglyceridemia (>1.7mmol/l)
		Low HDL cholesterol	Low HDL cholesterol
	Low HDL cholesterol <sup>b</sup>	(<1.03mmol/l)	(<1.03mmol/l)
		Raised fasting glucose	Raised fasting glucose
	Central obesity <sup>c</sup>	(>5.6mmol/l)	(>5.6mmol/l)
	Microalbuminuria <sup>d</sup>		Central obesity <sup>e</sup>

Definition of the metabolic syndrome according to WHO (World Health Organisation), IDF (International Diabetes Federation) and NCEP (National Cholesterol Education Program Expert Panel III) criteria.

<sup>a</sup> = Impaired glucose tolerance= glucose >7.8mmol on 2 hour glucose tolerance test. Insulin resistance= in highest quartile of relevant population. <sup>b</sup> = HDL cholesterol <0.9mmol/l in men, <1.0mmol/l in women. <sup>c</sup> = Waist-hip ratio >0.9 in men, >0.85 in women or BMI greater than 30. <sup>d</sup> = albumin-creatinine ratio >30. <sup>e</sup> = Waist circumference >102cm in men, >88cm in women.

A new joint statement from a number of professional organizations (the International Diabetes Federation (IDF), the National Heart, Lung, and Blood Institute (NHLBI), the World Heart Federation, the International Atherosclerosis Society, and the American Heart Association (AHA) (Alberti, Eckel et al. 2009), has identified specific criteria for the clinical diagnosis of metabolic syndrome. Patients with three of the five criteria — elevated waist circumference,

elevated triglycerides, reduced HDL-cholesterol levels, elevated blood pressure and elevated fasting glucose levels are considered to have the syndrome.

### **1.5.2. Testosterone in the metabolic syndrome diabetes and insulin resistance**

Metabolic syndrome is closely linked to testosterone levels in men. Cohen in 1999 suggested the Hypogonadal -Obesity cycle (Cohen 1999). The lipoprotein lipase (enzyme responsible for triglyceride breakdown and storage in the adipose tissue) activity is inhibited by testosterone. The hypothesis suggests that the aromatase activity in the adipose tissue promotes a cycle in which the testosterone levels are progressively lowered which in turn leads to more adiposity.

The more recently proposed Hypogonadal-Obesity-Adipocytokine hypothesis extends this hypothesis to explain why the body is unable to detect and respond to the low androgen levels. The inflammatory cytokines, leptin and oestradiol inhibits the hypothalamic-pituitary-gonadal (HPG) axis leading to a state of hypogonadotropic hypogonadism - depicted in figure 1 (Kelly and Jones 2015). There is evidence that the negative feedback on the HPG axis in men is mainly mediated via its aromatisation to oestradiol (Hayes, Seminara et al. 2000, Hayes, Decruz et al. 2001). This hypothesis would explain the clinical evidence of low gonadotrophins in diabetes and metabolic syndrome (Dhindsa, Prabhakar et al. 2004). This could further explain the finding of increased FSH and LH secretion in men who were treated with agents which block the aromatase activity (Hill, Arutchelvam et al. 2009). The high aromatase activity of the central adipose tissue leads to the conversion of more testosterone to oestrogen (Vermeulen, Kaufman et al. 2002). This might partly explain the clinical finding

of relatively higher level of oestrogen found in obese men. There is also evidence from studies of pluripotent stem cells that testosterone promotes myocyte development and inhibits the adipocyte formation in pluripotent stem cells (Singh, Artaza et al. 2003). In a testosterone deficient state the inhibitory effect of testosterone on adipocyte is decreased and could contribute to their proliferation causing an increase in fat mass.

Testosterone has also been shown to increase beta adrenergic receptor numbers. This will lead to more adipose tissue catabolism (De Pergola 2000). There is evidence that the decrease in lipolysis is the main cause of obesity in androgen receptor knock out mouse model looking at the metabolic effects testosterone deficiency state (Yanase, Fan et al. 2008).



**Figure 1.3 The hypogonadal–obesity–adipocytokine cycle**

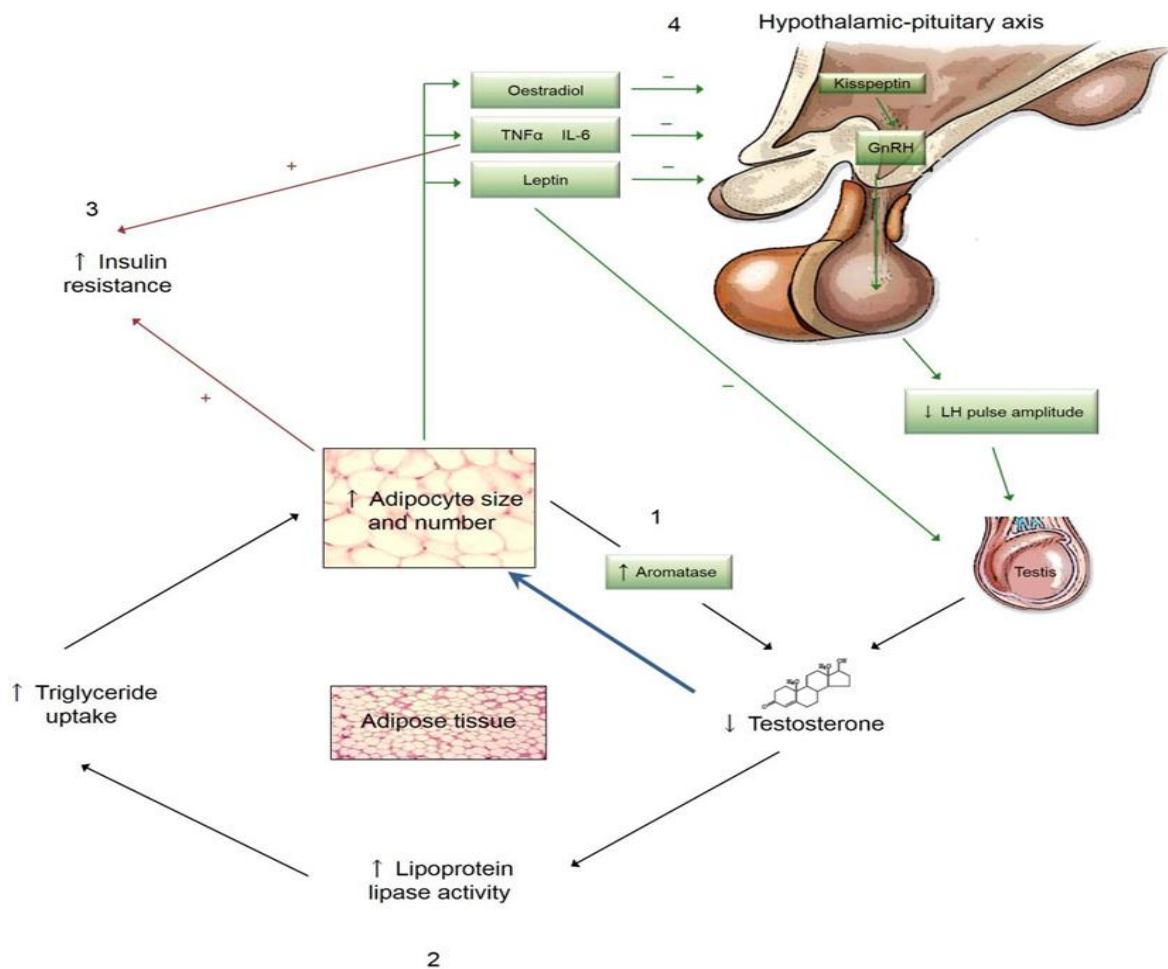


Figure 1.3 the hypogonadal–obesity–adipocytokine hypothesis. High aromatase activity in adipocytes converts testosterone to oestradiol (1). Reduced tissue testosterone facilitates triglyceride storage in adipocytes by allowing increased lipoprotein lipase activity (2) and stimulating pluripotent stem cells to mature into adipocytes (blue arrow). Increased adipocyte mass is associated with greater insulin resistance (3). Oestradiol and adipocytokines tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin 6 (IL6) and leptin (as a result of leptin resistance in human obesity) inhibit the hypothalamic–pituitary–testicular axis response to decreasing androgen levels (4). Kisspeptin neurons are inhibited by oestradiol, inflammation and leptin resistance and thus reduce gonadotropin-releasing hormone (GnRH) stimulation of the pituitary and subsequent luteinizing hormone (LH) release. Reduced LH pulse decreases gonadal stimulation and testosterone release, thus causing a state of hypogonadotropic hypogonadism. Furthermore, leptin also directly inhibits the stimulatory action of gonadotropins on the Leydig cells of the testis to decrease testosterone production. + Positive effect; – negative effect. Adapted from Jones (Jones 2007)

There are several clinical studies showing increased body fat in men with low testosterone levels. A study looking at the body composition in relation to testosterone reported the results

of 57 elderly men (from a sub group study from a larger cohort of 372 men), which showed serum testosterone levels were negatively correlated with percentage body fat, abdominal fat and plasma insulin levels (Vermeulen, Goemaere et al. 1999).

In recent years a number of clinical studies looking into the relationship between testosterone and metabolic syndrome in general population have been published.

One large study of over 1800 non diabetic men reported that in men with metabolic syndrome had a significantly lower total testosterone, free testosterone and SHBG levels compared those without metabolic syndrome (Laaksonen, Niskanen et al. 2003). The authors concluded that low testosterone and SHBG are independently related to metabolic syndrome. The results from a study of 130 men reported that plasma DHEA levels and testosterone levels negatively correlated with risk of metabolic syndrome (Blouin, Despres et al. 2005).

The Baltimore Longitudinal Study of Aging (Rodriguez, Muller et al. 2007) reported the results of a 6 year follow up of over 600 healthy men. The results demonstrated that total testosterone and SHBG were inversely related to the development of metabolic syndrome on long term follow up.

A cross sectional study of 2500 non diabetic men over the age of 70 years reported that low testosterone and SHBG were associated with metabolic syndrome, They further concluded that men with hypogonadotropic hypogonadism had the highest prevalence of metabolic syndrome (Chubb, Hyde et al. 2008).

Similarly low levels of testosterone were found to be associated with hyperinsulinemia, diabetes and obesity (Haffner, Karhapaa et al. 1994, Simon, Charles et al. 1997). In a community-based population study looking at a cohort of 110 men of Japanese American

men, baseline testosterone levels negatively correlated with central adiposity (Tsai, Boyko et al. 2000).

A recent review analysed the available evidence and concluded that there is a strong evidence that in men, low testosterone state is an independent risk factor for development of metabolic syndrome and type 2 diabetes (Kelly and Jones 2015). There are many studies showing a link between body fat and testosterone levels (Kapoor, Malkin et al. 2005) (Zumoff, Strain et al. 1990, Pasquali, Casimirri et al. 1991) (Khaw and Barrett-Connor 1992, Gapstur, Gann et al. 2002). Further studies using CT or MRI, have confirmed the inverse relation between testosterone and body fat deposit (Seidell, Bjorntorp et al. 1990, Haffner, Valdez et al. 1993, Garaulet, Perez-Llamas et al. 2000, Tsai, Fujimoto et al. 2004).

Many of the above studies did not have fasting testosterone levels and the results could have been skewed by the recent evidence that an acute lowering of gonadotropin-stimulated testosterone secretion occurs in both older and younger males with acute oral energy loading (Schwartz, Patel et al. 2015). Similarly Caronia et al reported an acute reduction in both total and free testosterone levels after glucose intake, without compensatory increase in gonadotrophins (Caronia, Dwyer et al. 2013). The study concluded men found to have low testosterone levels should have their testosterone levels re-evaluated on a fasting level.

The hallmark of metabolic syndrome and diabetes is Insulin resistance. This is often associated with higher body mass index. There are a number of studies looking into insulin resistance and testosterone levels. A large meta-analysis by Ding et al., looking into the major studies relating to endogenous sex hormones in men and women, reported that there is a high prevalence of low testosterone levels in men with diabetes and/or metabolic syndrome (Ding, Song et al. 2006). A number of studies suggest low testosterone level increases the risk of

future diabetes or insulin resistant state (Haffner, Shaten et al. 1996, Stellato, Feldman et al. 2000, Oh, Barrett-Connor et al. 2002, Laaksonen, Niskanen et al. 2004, Kupelian, Page et al. 2006, Selvin, Nelson et al. 2007).

The previous study from our research team reported that hypogonadism is present in up to 40% of type 2 diabetic men (Kapoor, Aldred et al. 2007). Importantly direct measurement of bioavailable testosterone removed any effect of SHBG on the outcomes. This finding was supported by another study which reported that 33% of men have low testosterone levels in a cohort of 103 patients with type 2 diabetes (Dhindsa, Prabhakar et al. 2004).

Several longitudinal studies have shown that low testosterone is an independent risk factor for development of diabetes and metabolic syndrome (Stellato, Feldman et al. 2000) (Haffner, Shaten et al. 1996) (Oh, Barrett-Connor et al. 2002) (Laaksonen, Niskanen et al. 2004).

How low testosterone levels lead to the development of metabolic syndrome and type 2 diabetes is still not clearly explained. The increase fat deposition from the low testosterone state may be a one of a several contributory factors towards the development of insulin resistance.

The mechanisms linking testosterone with insulin resistance and type 2 diabetes are still not fully understood. Although testosterone deficiency leads to increased fat deposition which would result in increasing insulin resistance, it does not explain the total action on insulin sensitivity.

### **1.5.3. Effect of androgen suppression on metabolic syndrome, type 2 diabetes and cardiovascular risk profile**

Androgen suppression in men predispose to development of metabolic syndrome. There are many studies showing an increased cardiovascular risk and mortality in these group of patients. This strengthens the view that testosterone deficiency leads to increased metabolic and cardiovascular risks. The important studies looking into the effects of androgen deprivation therapy on components of metabolic syndrome are summarised in table 1.4.

One of the study, a 4.5 year follow up of a large cohort of over 70,000 men with prostate cancer aged above 70 years in which a third received GnRH therapy (Keating, O'Malley et al. 2006). The patients with androgen suppression therapy had worsening cardiovascular risk profile and increased mortality. A further study from the same team reported that treatment with GnRH agonists was associated with statistically significant increased risks of incident diabetes, coronary heart disease, myocardial infarction stroke and mortality (Keating, O'Malley et al. 2010). Similarly another large study looking at 1231 patients with androgen deprivation therapy and 7250 controls, reported an increased incidence of diabetes (RR 1.36 (P = 0.01) in patients receiving androgen deprivation therapy (Lage, Barber et al. 2007). There are a number of smaller studies, again showing an increased cardiovascular risk following androgen deprivation therapy as detailed in the recent review (Bosco, Crawley et al. 2015).

**Table 1.4 Outcome of major studies in patients with androgen deprivation therapy in relation to metabolic syndrome and diabetes**

Author and year	Out come	Number of patients	Findings
Keating et al.(Keating, O'Malley et al. 2010)	Diabetes	14,597 ADT. 22,846 no ADT	No ADT: Ref. GnRH agonists: 1.28 (95% CI: 1.19–1.38) Orchiectomy: 1.16 (95% CI: 0.87–1.54) Combined androgen blockage: 1.17 (95% CI: 0.96–1.42). Oral anti-androgens: 1.02 (95% CI: 0.72–1.45)
Lage et al(Lage, Barber et al. 2007)	Diabetes	1,231 on ADT. 7,250 no ADT	While controlling for other factors, the estimated relative risk of incident diabetes associated with the receipt of ADT was 1.36 (95% CI: 1.07–1.74)
Keating et al(Keating, O'Malley et al. 2006)	Diabetes	26,570 on ADT. 46,626 on ADT	No ADT: Ref. GnRH agonists: 1.44 (95% CI: 1.34–1.55) Orchiectomy: 1.34 (95% CI: 1.20–1.50)
Braga-Basaria et al(Braga-Basaria, Dobs et al. 2006)	Mets <sup>1</sup> Obesity Hyperglycaemia Hypertriglyceridemia Low HDL Hypertension	20 on ADT 18 no ADT	Prevalence of MetS: 55% vs 22% (ADT vs no ADT). Prevalence of obesity: 75% vs 33%. Prevalence of hyperglycaemias: 65% vs 16%. Prevalence of hypertriglyceridemia: 55% vs 44%. Prevalence of low HDL: 35% vs 50%. Prevalence of hypertension: 45% vs 28%
Basaria et(Basaria, Muller et al. 2006)	Hyperglycaemia	18 on ADT 17 no ADT	Men on ADT had significantly higher levels of fasting serum glucose (131.0 mg/dL) compared with men not on ADT (103.0 mg/dL; P: 0.01)
Bo et al(Bo, Zhang et al. 2011)	Metabolic changes <sup>2</sup>	46 orchiectomy/ ADT 37 prostatectomy no ADT. 50 controls	After 3 months ADT group had increased levels of fasting serum insulin and LDL compared to the other 2 groups (P< 0.05) After 12 months ADT group had increased levels of waist circumference, fasting serum insulin and glucose, total cholesterol, HDL and LDL compared to the other 2 groups (P<0.05)
Alibhai et(Alibhai, Duong-Hua et al. 2009)	Diabetes Acute myocardial infarction Sudden death	19,079 on ADT/ Orchiectomy 19,079 men with PCa no ADT	Increased risk of diabetes HR 1.16 (95% CI: 1.11–1.21). No increased risk of MI, HR 0.91 (95% CI: 0.84–1.00) or of sudden death HR 0.96 (95% CI: 0.83–1.10)

Adapted from Bosco et al. (Bosco, Crawley et al. 2015) <sup>1</sup> MetS Definition of the National Cholesterol Education Programme—Adult treatment panel III. <sup>2</sup> MetS Definition of the International Diabetes Federation. ADT – Androgen deprivation therapy. PCa- prostatic cancer

In a study of 1014 patients with hypopituitarism for 8 years the only hormonal deficiency associated with increased mortality was untreated gonadotrophin deficiency. The major causes of death in these patients were from vascular and respiratory disease (Tomlinson,

Holden et al. 2001). Men with Klinefelter's Syndrome have an increased risk of dying from diabetes, cardiovascular disease, respiratory disease and cancer (Bojesen, Juul et al. 2006).

#### **1.5.4. The role of testosterone in dyslipidaemia, hypertension and atherosclerosis**

Dyslipidaemia is an integral part of type 2 diabetes and metabolic syndrome and derangements in the lipid profile relates to atherosclerotic process in these patients. Two of the commonly measured lipids are part of definition of metabolic syndrome, the high density lipoprotein cholesterol (HDL-C) and triglyceride (TG). The HDL C is the reverse transporter of lipids and protective against atherosclerosis whereas triglycerides, and low density lipoprotein cholesterol (LDL-C) increase the risk of cardiovascular events and are pro atherogenic (Simons, McCallum et al. 1998, Maron 2000). There is some evidence to suggest that the effect of HDL-cholesterol is gender specific (Fan and Dwyer 2007), with a lower HDL-C correlates with coronary artery disease more in men compared women (Johnsen, Mathiesen et al. 2005). Some studies also suggested a positive correlation between HDL and testosterone levels (Van Pottelbergh, Braeckman et al. 2003, Stanworth RD 2007).

There is evidence that a high testosterone levels are associated with lower total and LDL cholesterol (Barrettconnor 1992, Haffner, Valdez et al. 1993, Simon, Charles et al. 1997). A meta-analysis looking into the effects of intramuscular testosterone on lipid profile reported a significant improvement in LDL-C in men receiving the therapy. (Whitsel, Boyko et al. 2001).

The TIMES2 study, an international multi-centre study of testosterone gel in hypogonadal men reported significant improvement in total cholesterol, LDL cholesterol and Lipoprotein a (Jones, Arver et al. 2011) .

A number of studies have highlighted association between testosterone and hypertension some studies showed low testosterone association with hypertension (Messerli, Garavaglia et al. 1987, Khaw and Barrettconnor 1988, Svartberg, von Muhlen et al. 2004, Kapoor, Clarke et al. 2007, Bocchi, Carvalho et al. 2008) and other studies reporting increase in risk of hypertension with anabolic steroid abuse (Sullivan, Martinez et al. 1998, Mottram and George 2000, Maravelias, Dona et al. 2005).

Carotid intima media thickness (CIMT) is used as a surrogate marker for cardiovascular atherosclerotic process. There are many studies showing low testosterone levels are associated with increased CIMT. The major studies are summarised in table 1.3

One of the studies involving 195 men, showed those with low testosterone levels were associated with greatest increase in CIMT over a 4-year period follow up. (Muller, van den Beld et al. 2004). Further studies have confirmed these findings in healthy men and in men with type 2 diabetes. (De Pergola, Pannacciulli et al. 2003, Fukui, Kitagawa et al. 2003, van den Beld, Bots et al. 2003). A larger study of 1482 men from the Trosno study reported that total testosterone and sex hormone-binding globulin levels were inversely associated with the age-adjusted carotid intima media thickness (Svartberg, Von Muhlen et al. 2006). More recently a larger cohort study of 2290 men from the same group reported an inverse association between testosterone levels and total carotid atherosclerosis after adjusting for co-variates ( $P < 0.05$ ), (Vikan, Johnsen et al. 2009).



**Table 1.5 Summary of studies showing association between CIMT and testosterone**

Study Name	Sample Size	Sample Age Range	Major Findings
Van den Beld et al (van den Beld, de Jong et al. 2000) (CS)	403 Men	73 to 94 (mean age, 77.8)	<ul style="list-style-type: none"> <li>After adjustment for age, serum total testosterone was inversely related to carotid artery IMT.</li> </ul>
Fukui et al (Fukui, Kitagawa et al. 2003)(CS)	154 Diabetic men	Mean age, 62 (age range not provided)	<ul style="list-style-type: none"> <li>FT was inversely associated with carotid artery IMT.</li> <li>Free testosterone is inversely associated with carotid artery plaque score,</li> <li>Carotid IMT and plaque score were significantly higher in patients with lower levels of FT.</li> </ul>
De Pergola et al (De Pergola, Pannacciulli et al. 2003)(CS)	127 Overweight or obese men	18 to 45 (mean age, 34)	<ul style="list-style-type: none"> <li>After adjustment for age, total body fat, central obesity, and fasting glucose concentration, carotid artery IMT was inversely associated with FT.</li> </ul>
Makinen et al(Makinen, Jarvisalo et al. 2005) (CCS)	96 Nondiabetic men	40 to 70 (mean age, 57)	<ul style="list-style-type: none"> <li>After adjustment for age, BMI, blood pressure, smoking, and total cholesterol, TT was inversely associated with carotid IMT.</li> </ul>
Svartberg et al(Svartberg, Von Muhlen et al. 2006) (CCS)	1482 Men	25 to 84 (mean age, 60)	<ul style="list-style-type: none"> <li>After adjustment for age, smoking, physical activity, blood pressure, and lipid levels, TT were inversely associated with carotid IMT.</li> <li>The association between TT and carotid IMT was not independent of BMI.</li> <li>There was no association between FT and carotid IMT.</li> </ul>
Fu et al(Fu, Gao et al. 2008) (CCS)	106 Men	50 to 70 (mean age, 64)	<ul style="list-style-type: none"> <li>FT was independently inversely associated with carotid IMT.</li> </ul>
Vikan et al(Vikan, Johnsen et al. 2009) (CS)	2290 Men	55 to 74 (mean age, 66)	<ul style="list-style-type: none"> <li>After adjustment for age, systolic BP, smoking, and use of lipid-lowering medications, total testosterone was inversely associated with total carotid plaque area.</li> <li>SHBG was not associated with changes in carotid IMT or plaque area.</li> </ul>
Muller et al(Muller, van den Beld et al. 2004) (CS)	195 Men	73 to 91 (mean age, 77)	<ul style="list-style-type: none"> <li>FT was inversely associated with mean progression of carotid IMT independent of age.</li> <li>FT was inversely associated with mean progression of carotid IMT after adjustment for cardiovascular risk factors.*</li> </ul>

Adapted from Osukui et al (Osukui, French et al. 2013). BMI -body mass index; BP-blood pressure; CCS-case-control study; CS-cross-sectional study; FT- free testosterone; IMT- intima-media thickness; SHBG-sex hormone-binding globulin; TT-total testosterone.

\*Cardiovascular risk factors included body mass index, waist-to-hip-ratio, hypertension, diabetes, smoking, and serum cholesterol levels.

A direct link has recently been made between long-term testosterone treatment and a significant reduction in CIMT in healthy middle-aged men independent of BMI status (Zitzmann, Vorona et al. 2008).

The Rotterdam study (Elisabeth Hak, Witteman et al. 2002) investigated the association of levels of DHEA-S and total and bioavailable testosterone with aortic atherosclerosis among 1,032 non-smoking men and women aged 55 years and over found an independent inverse association between levels of testosterone and aortic atherosclerosis in men.

There is growing evidence to the role of inflammatory cytokines in the process of atherosclerosis (Jones and Saad 2009). Studies have shown that testosterone has immunomodulatory properties (Corrales, Almeida et al. 2006, Jones and Saad 2009) with some studies showing suppressing of pro inflammatory cytokines (Li, Danis et al. 1993, D'Agostino, Milano et al. 1999, Gornstein, Lapp et al. 1999, Hofbauer, Ten et al. 1999, Hatakeyama, Nishizawa et al. 2002) and other studies showing the stimulation of anti-inflammatory cytokines (Liva and Voskuhl 2001). A previous study from our group has reported that serum interleukin 1 $\beta$  (IL1 $\beta$ ) inversely related with serum testosterone levels in men with coronary artery disease (Nettlehip, Pugh et al. 2007). Another study from our group reported an inverse correlation between pro inflammatory cytokines (CRP and IL-6) and testosterone levels in men with type 2 diabetes. (Kapoor, Clarke et al. 2007).

Although these above data shows overwhelmingly beneficial effects of testosterone on cardiovascular risk factors, the long term real life clinical efficacy data in relation to cardiovascular mortality are still lacking. In this context the similarity of HRT in post-menopausal women is worth discussing. Although there were a large number of studies showing cardio protective effects of combined HRT the WHI (women's health initiative) of

an increased risk of CHD with combined HRT in WHI was at variance with observational studies (Writing Group for the Women's Health Initiative 2002).

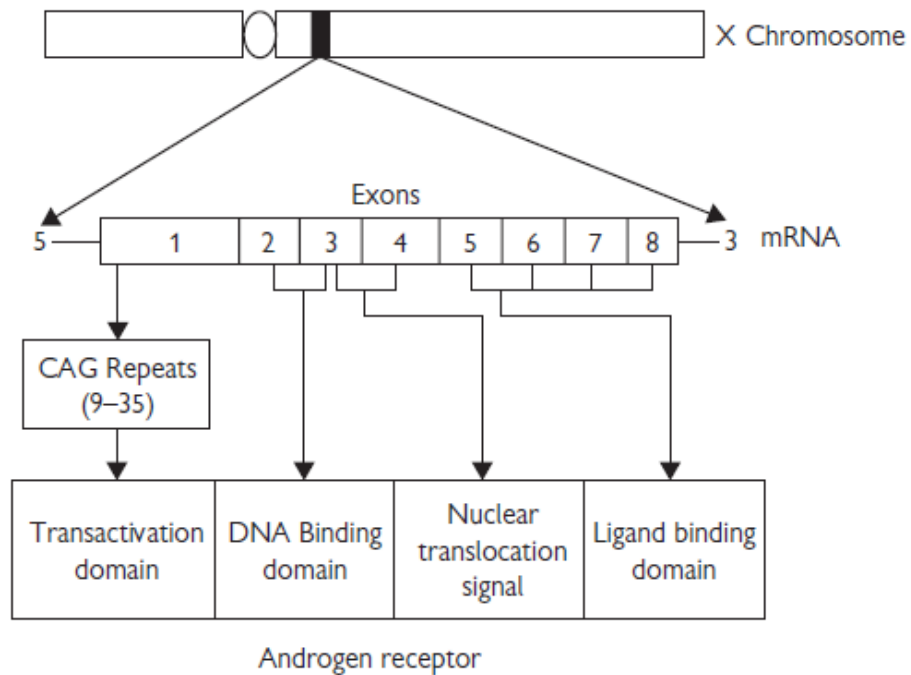
## **1.6. Role of androgen receptor in diabetes, obesity and metabolic syndrome**

### **1.6.1. The androgen receptor and androgen receptor gene**

The androgen receptor is a member of the nuclear receptor superfamily and shares functional and structural homology with other nuclear receptors in having three domains concerned with transactivation, DNA binding and ligand binding respectively (Lubahn, Joseph et al. 1988).

The unstimulated androgen receptor exists in an inactive complex with molecular chaperones such as Heat Shock Proteins (HSP) 90/70/56 which maintain a high affinity ligand binding conformation (Pratt and Toft 1997). After entering the target cell androgens interact with the ligand binding domain thereby inducing a cascade of conformational changes as well as translocation of the stimulated androgen receptor from the cytoplasm to the nucleus. In the nucleus two androgen receptors combine to produce a homodimer and the DNA binding domains interact with androgen response elements, which are specific DNA sequences usually in the promoter or enhancer regions of androgen receptor target genes (Chang, Saltzman et al. 1995). Cofactor interaction is then followed by transcription activation.

**Figure 1.4 Schematic representation of androgen receptor gene**



The androgen receptor gene is sited close to the centromere of the X chromosome (Lubahn, Joseph et al. 1988) which determines that males have one allele whereas females possess two. Its size is approximately 180 kb and it comprises eight exons (figure 1.4). Exon one encodes the transactivation domain, exons two and three the DNA binding domain and exons four-eight the ligand binding domain (Jenster, Vanderkorput et al. 1992).

### **1.6.2. The androgen receptor CAG (AR CAG) repeat polymorphism**

A large number of androgen receptor gene mutations have been shown to affect androgen receptor function which can lead to partial or complete insensitivity. Complete insensitivity manifests as female phenotype. The phenotype of partial androgen insensitivity can present as a spectrum of disorders ranging from gender ambiguity to under-virilisation and/or subfertility with unambiguous male gender (Hiort, Sinnecker et al. 1996).

X linked spinal and bulbar muscular atrophy (SBMA also known as Kennedy's disease) is a lower motor neurone disease first described in 1968 by Kennedy et al (Kennedy, Alter et al. 1968). An association with marked hypo androgenic traits was recognised and subsequent work identified the cause of SBMA to be an abnormal expansion in a polymorphic CAG repeat sequence within exon 1 of the androgen receptor gene encoding a poly glutamine stretch (La Spada, Wilson et al. 1991).

In-vitro studies have shown that transcription activity of the stimulated receptor is affected by this CAG repeat sequence inversely such that transcription is maximal when AR CAG is 0 and is progressively decreased but not abolished as AR CAG expands through and beyond the normal range (Chamberlain, Driver et al. 1994, Kazemiesfarjani, Trifiro et al. 1995).

Experiments have confirmed the functional significance of this finding in a mouse model where animals had CAG repeat sequences of 12, 21 and 48 introduced into the androgen receptor. The animals were grossly normal but seminal vesicle volume was inversely related to AR CAG (Albertelli, Scheller et al. 2006).

### **1.6.3. Clinical correlations of the AR CAG polymorphism in men**

The population distribution of AR CAG is approximately normal. There are racial differences in AR CAG with an average between 21 and 22 in published Caucasian populations as compared to 18-20 in African populations (Kittles, Young et al. 2001)(Kittles, Young et al. 2001) and 22-23 in East Asia (Hsing, Gao et al. 2000). These differences are seen in the increased incidence of prostate cancer in African versus Asian populations (Hsing, Tsao et al. 2000).

Studies have shown that AR CAG polymorphism is associated with adiposity. The report from a study involving 106 healthy young men, showed that AR CAG length was positively correlated with higher leptin levels, percentage of body fat and fasting insulin levels (Zitzmann, Gromoll et al. 2003). A separate study showed raised BMI in the upper quartile of AR CAG compared with other participants (Alevizaki, Cimponeriu et al. 2003). This suggests that greater transcriptional activity of stimulating a receptor with shorter AR CAG may have advantageous effects on anthropometric measures. Further studies have reported a positive correlation between leptin and AR CAG length both in healthy men (Zitzmann and Nieschlag 2003) and in men with type 2 diabetes (Stanworth, Kapoor et al. 2008).

Some studies have shown positive correlation between AR CAG length and insulin levels in healthy men (Zitzmann and Nieschlag 2003). Kennedys syndrome, which is a neurological disease caused by abnormally long AR-CAG (CAG>37 repeats), have high prevalence of type 2 diabetes. AR CAG length also correlates positively with HDL-C levels and endothelium dependant vasodilatation in diabetic men (Zitzmann, Brune et al. 2001).

A study of 131 consecutive male patients undergoing coronary angiography found that men in the upper quartile of AR CAG were more likely to have significant stenosis of at least one coronary artery than men with shorter AR CAG (Alevizaki, Cimponeriu et al. 2003). Some studies however, did not show a correlation between AR CAG length and cardiovascular risk profile (Page, Kupelian et al. 2006).

## **1.7 Testosterone replacement therapy**

Testosterone levels are decreased in a variety of inflammatory conditions due to disruption in the hypothalamic-pituitary-gonadal (HPG) axis. There is inverse correlation between the severity of inflammation and seriousness of the disease with the testosterone levels. .

As described above testosterone has shown to have immunomodulatory properties with studies showing testosterone increases anti-inflammatory cytokines and suppresses pro-inflammatory cytokines. Conversely it is shown that IL-6 administration induced prolonged suppression of testosterone levels in healthy men without affecting the gonadotrophin levels suggesting a more direct action on testosterone production by IL-6 (Tsigos, Papanicolaou et al. 1999).

The above discussion raises the question whether the potential benefits of testosterone action could be transferred into clinical practice to improve the cardiovascular profile in men with low testosterone potentially improving the longevity. Large scale long term prospective interventional studies are needed to answer this question. Unfortunately such studies of testosterone replacement therapy have not been done or adequately powered to detect effects

on clinically significant cardiovascular events (Gruenewald and Matsumoto 2003, Allan and McLachlan 2004, Liu 2004, Kaufman and Vermeulen 2005, Isidori and Lenzi 2007).

**1.7.1. Effect of Testosterone replacement therapy on glycaemic control, insulin resistance and lipid profile**

A number of studies have shown that testosterone therapy has beneficial effects on glycaemic control. These are summarised in Table 1.6.



**Table 1.6 Major studies showing the effects of testosterone replacement therapy on indices of glycemic control**

Study Name	Sample Size	End Points	Main Findings
Corona et al(Corona, Monami et al. 2011) (meta-analysis, n=37 studies)	1822 Diabetic men and 10 009 nondiabetic men (meta-analysis)	HgA1c, fasting plasma glucose, triglycerides	-HgA1c decreased by 0.76% -Fasting plasma glucose decreased by 1.18 mmol/L -TG decreased by 0.67
Jones et al(Jones, Arver et al. 2011) (DBRCT)	220 Hypogonadal men with T2DM and/or MetS	HOMA-IR, HgA1c, body composition	-HOMA-IR decreased by 15.2% after 6 months -HOMA-IR decreased by 16.4% after 12 months -HgA1c decreased by 0.44% after 9 months
Kapoor et al(Kapoor, Goodwin et al. 2006) (DBPCC)	24 Hypogonadal men with T2DM	HOMA-IR, HgA1c, fasting plasma glucose	-HOMA-IR decreased by 1.73 -HgA1c decreased by 0.37% -Fasting plasma glucose decreased by 1.58 mmol/L
Heufelder et al(Heufelder, Saad et al. 2009) (SBRCT)	16 Hypogonadal men with T2DM	HOMA-IR, HgA1c, fasting plasma glucose	-HOMA-IR decreased by 4.2 -HgA1c decreased by 1% after 13 weeks -HgA1c decreased by 1.5% after 52 weeks -Fasting plasma glucose decreased by 1.9
Kalinchenko et al (DBRCT)	113 Hypogonadal men with MetS	HOMA-IR, fasting plasma glucose, BMI, WC, waist-to-hip ratio	-HOMA-IR decreased by 1.49 -No significant change in fasting plasma glucose -Significant reduction in BMI, weight, waist-to-hip ratio, hip circumference, and waist circumference
Boyanov(Boyanov, Boneva et al. 2003) (Open Label)	48 Hypogonadal men	Fasting glucose, BMI, Waist hip ratio, percentage body fat, lipid profile.	-Significant reductions in weight, waist-hip ratio and percentage body fat. -Fasting and post-prandial blood glucose levels were significantly reduced, -HbA1c fell by 1.8%
Malkin et al (Malkin, Jones et al. 2007)(SBPCC)	13 Men with CHF and no T2DM	HOMA-IR, fasting plasma glucose, glucose tolerance, body composition	-HOMA-IR decreased by 1.9 -Fasting plasma glucose decreased by 0.61 mmol/L -Total body mass increased by 1.5 kg -Percent body fat decreased by 0.8%
Dhindsa(Dhindsa, Ghanim et al. 2016)	92 men with type 2 diabetes	HOMA-IR Inflammatory markers Gene expression	There was a decrease in subcutaneous fat mass (-3.3 kg) and increase in lean mass (3.4 kg) -The expression of insulin signaling genes in adipose tissue was significantly lower - significant fall in circulating concentrations of free fatty acids, C-reactive protein, interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , and leptin

Adapted from Osuki et al(Oskui, French et al. 2013)BMI indicates body mass index; CHF, congestive heart failure; DBPCC, double-blind placebo-controlled cross over study; DBRCT, double-blind randomized controlled trial; HgA1c, haemoglobin A1c; HOMA-IR, homeostatic model of insulin resistance; IM, intramuscular; MetS, metabolic syndrome; SBPCC, single-blind placebo-controlled crossover study; SBRCT, single-blind randomized controlled trial; T2DM, type 2 diabetes mellitus; TD, transdermal; TG, triglycerides; , testosterone replacement therapy; WC, waist circumference.

A study in 1991, one of the earliest looking into effect of testosterone on body mass index, showed testosterone replacement caused a reduction in waist hip ratio after 6 weeks of therapy (Rebuffe-Scrive, Marin et al. 1991).

The first double blind placebo controlled study with a cross over design; looking into the effect of testosterone replacement therapy on glycaemic control was from our research team (Kapoor, Goodwin et al. 2006). The results showed after a 3 month therapy with testosterone there was a significant improvement in HOMA-R in insulin naïve patients and reduction in insulin dose in those who were on insulin. The results also showed a reduction in the fasting blood glucose, fasting insulin levels, and HbA1c.

Recently this finding has been confirmed in a larger study of 48 type 2 diabetic men with symptoms of androgen deficiency (Boyanov, Boneva et al. 2003). After 3 months of testosterone undecanoate therapy there were significant reductions in weight, waist-hip ratio percentage body fat and glucose levels were significantly reduced and the HbA1c improved.

Another single blind randomised study involved 32 hypogonadal men with type 2 diabetes and metabolic syndrome who were randomised to either diet and exercise alone or with addition of testosterone gel therapy. The results showed a significant reduction in HbA1c, HOMA -R index, serum triglycerides and an increase in HDL-C after 52 weeks in the testosterone treated group (Heufelder, Saad et al. 2009).

The more recent TIMES2 study (Testosterone replacement in men with Metabolic Syndrome or type 2 diabetes, a multicentre study) studied 220 men with hypogonadism and type 2 diabetes and/or metabolic syndrome who were randomised to testosterone gel or placebo. The

results showed testosterone replacement therapy was associated with improvement in insulin resistance, reductions in percentage body fat, total and LDL cholesterol and lipoprotein a .

The Moscow study was a randomized, placebo-controlled, double-blinded study of 184 men with metabolic syndrome and hypogonadism. After 18 weeks of testosterone undecanoate therapy there were significant decreases in weight, BMI, waist circumference leptin, IL-1 $\beta$ , TNF-alpha and CRP (Kalinchenko, Tishova et al. 2010).

Most recently the study by Dhindsa et al, reported testosterone treatment in men with type 2 diabetes and hypogonadism increases insulin sensitivity (Dhindsa, Ghanim et al. 2016). A total of 94 men with type 2 diabetes were recruited into the study; after 24 weeks of intramuscular testosterone there was a significant improvement in insulin sensitivity, a decrease in subcutaneous fat mass and reduction of expression of insulin signalling genes (IR- $\beta$ , IRS-1, AKT-2, and GLUT4) in adipose tissue. Further the testosterone treatment caused a significant fall in circulating concentrations of free fatty acids, C-reactive protein, interleukin-1 $\beta$ , tumour necrosis factor- $\alpha$  and leptin.

There are studies showing reduction in waist circumference, (Saad, Gooren et al. 2007, Saad, Gooren et al. 2008) and body fat content (Kenny, Gruman et al. 2001, Agledahl, Hansen et al. 2008) resulting from testosterone replacement in hypogonadal men.

The major drawback of these studies is that the testosterone levels were not measured in a fasting state and may not reflect the actual testosterone status.

### **1.7.2. Effect of testosterone replacement therapy on hypertension**

There are some clinical studies reporting an improvement in blood pressure after testosterone replacement therapy. Marin et al (Marin, Holmang et al. 1992) reported a reduction in diastolic blood pressure after 8 months of transdermal testosterone replacement therapy in 23 obese men. Similarly in a cohort of 23 men who had intramuscular testosterone treatment for osteoporosis significant reductions in systolic and diastolic blood pressure was noted (Anderson, Francis et al. 1996). Similar reductions in blood pressure were reported in long-term intra-muscular testosterone therapy, one study involving hypogonadal men (Zitzmann and Nieschlag 2007) and the second in men with metabolic syndrome and hypogonadism (Kalinchenko, Vorslov et al. 2007).

Previous studies show that testosterone acts as a direct vasodilator via calcium channel blockade (Scragg, Jones et al. 2004, Hall, Jones et al. 2006, Scragg, Dallas et al. 2007). This suggests that direct action on blood vessels may be important in the effect of testosterone on blood pressure.

### **1.7.3 Long term effects of testosterone replacement therapy in men with hypogonadism**

The studies discussed above clearly suggest low testosterone level is a risk factor for worsening of cardiovascular risk profile in men. There is also evidence to suggest testosterone improves at least some of these risk factors. There is evidence at the molecular level too; testosterone favourably influences the metabolic pathways of carbohydrate and lipid metabolism. However the clinical benefit of long term testosterone replacement (TRT) in men with late onset hypogonadism (LoH) is still widely debated. There is wide agreement about the need for TRT in men with LoH who has raised FSH, LH. However role TRT in relation to obesity- or ill-health-related hypogonadotropic hypotestosteronaemia is still not clear.

A recent systematic review and meta-analysis reviewed 75 published trials relating to testosterone replacement involving 3016 patients with testosterone replacement and 2448 patients with placebo groups. They concluded, within the available evidence that testosterone replacement was not related to any increase in cardio vascular risk.

There are only a few long term studies in relation to testosterone and mortality. One of the study involving 1031 men age of 40 years reported a reduced mortality in hypogonadal men who had testosterone replacement therapy as compared to those who did not. (Shores, Smith et al. 2012). There was a 10% increase in mortality in the untreated group. After multivariate adjustment, testosterone replacement was associated with decreased risk of mortality (hazard ratio 0.61; 95 % confidence interval 0.42-0.88; P = 0.008) suggesting a beneficial effect of long term testosterone replacement therapy in hypogonadal men.

In the more recent study, Hackett et al. followed up 857 men with T2DM for 4 years. Following baseline testosterone, the patients had been randomised to receive testosterone undecanoate or placebo. Compared with the low testosterone, untreated group, mortality in the normal testosterone (HR: 0.62, CI: 0.41-0.94) or the testosterone replacement (HR: 0.38, CI: 0.16-0.90) groups was significantly reduced (Hackett, Heald et al. 2016). They further analysed the data to see the effect of PDE5 inhibitors on mortality. Mortality in the PDE5I treated patients was significantly reduced compared with the PDE5I untreated group (OR: 0.06, CI: 0.009-0.47). The study concluded that testosterone replacement therapy is independently associated with reduced mortality in men with type 2 diabetes. However a more logical conclusion would seem to be that improved sexual activity is good for men's health, whether this is achieved by PDE5 inhibitors or testosterone.

There are some studies which raised concerns regarding testosterone replacement therapy. A study involving over 200 elderly men of over 74 years reported that testosterone replacement therapy increased the risk of cardiovascular events (Basaria, Coviello et al. 2010). However the study used a higher starting dose of testosterone in frail elderly men and did not follow the usual clinical practice of starting with low dose and up titrating if necessary. This study was unusual and does not reflect common clinical practice. The authors rightly concluded that broader inference was not possible regarding the testosterone replacement therapy in the general population based on the findings owing to the unique population studied.

Another US study, a retrospective study of 8709 men with baseline TT of 10.4 nmol/L or less undergoing coronary angiography involved follow-up for a mean of 840 days (Vigen, O'Donnell et al. 2013). Of 7486 patients not receiving testosterone therapy, 681 died, 420 had MIs and 486 had strokes. Among 1223 patients receiving testosterone therapy, 67 died,

23 had MIs and 33 had strokes. A complex statistical analysis reversed the trend and concluded that there was a greater risk of cardio vascular mortality and adverse outcomes in the testosterone treated group. However the study raised several concerns including the exclusion of 1132 patients experiencing cardio vascular events were excluded because they were prescribed after the event. But these patients should have been included in the low testosterone untreated group. The authors later revised the numbers in the study and conceded that 104 women had wrongly been included in the results.

A database study by Finkle et al. studied prescribing data in men treated with testosterone replacement therapy without any data on the symptoms or blood results reported. It concluded that in older men and in younger men with pre-existing diagnosed heart disease, there is an increased risk of myocardial infarction in the first 3 months of initiation of testosterone prescription (Finkle, Greenland et al. 2014). This study was criticised for many reasons. Benefits of therapy would take much longer to manifest and other studies had excluded the first 3 months from analysis as the events would likely be related to the pre-existing condition. Most importantly fatal cardiovascular events and all-cause mortality data were not collected despite the major impact of testosterone replacement therapy in other studies being seen on mortality and not event numbers. Twelve-month post-treatment data were collected but not presented. The event rates within the groups prior to treatment were strangely identical. They reported a small increase in non-fatal cardiac events in men commenced on testosterone replacement therapy more marked in those with increased risk. Overall events in the study were lower than predicted from comparable research. They failed to report deaths failing to realise that a treatment that reduced mortality was likely to increase non-fatal events (Hackett 2016).

Most of the above studies are retrospective and not able to clearly clarify the long term benefits or otherwise of testosterone replacement therapy. This highlights the need for a large scale randomised study to answer the questions relating to testosterone replacement in men with hypogonadism.

## **1.8 Role of SHBG in cardio-metabolic risk profile**

SHBG is a large glycoprotein which forms homodimers and possesses two testosterone-binding sites. It is synthesised in liver. Traditionally SHBG is considered as a carrier protein and stores testosterone in the circulation. The protein bound testosterone is inactive. SHBG binds to an endocytic receptor (megalin) on the cell membrane and is then internalized into the cell. The gene encoding SHBG is located in the p12–p13 region on the short arm of chromosome 17 (Berube, Seralini et al. 1990). Studies have shown that testosterone has a biphasic effect on SHBG with both low and high concentrations of testosterone reduce SHBG secretion. Some studies shown that exogenous testosterone administration reduce SHBG concentrations (Edmunds, Stubbs et al. 1990). There is emerging evidence that SHBG has additional biological functions to transporting the sex steroids in the blood. Recent findings also support a specific receptor for SHBG (RSHBG) implying a more direct role of the protein in certain intracellular signalling pathways (Rosner, Hryb et al. 1998, Le, Nestler et al. 2012) and the SHBG bound sex steroid molecules have been located inside the cells. Megalin or Low density lipoprotein-related protein 2 an endocytic receptor facilitates the endocytosis of SHBG bound testosterone (Rosner, Hryb et al. 1998).



Many studies have suggested that SHBG levels are linked to insulin resistance, metabolic syndrome and diabetes (Ding, Song et al. 2006, Wallace, McKinley et al. 2013). The studies are summarised in table 1.7.

**Table 1.7 Summary of Prospective Studies of SHBG in development of Type 2 diabetes**

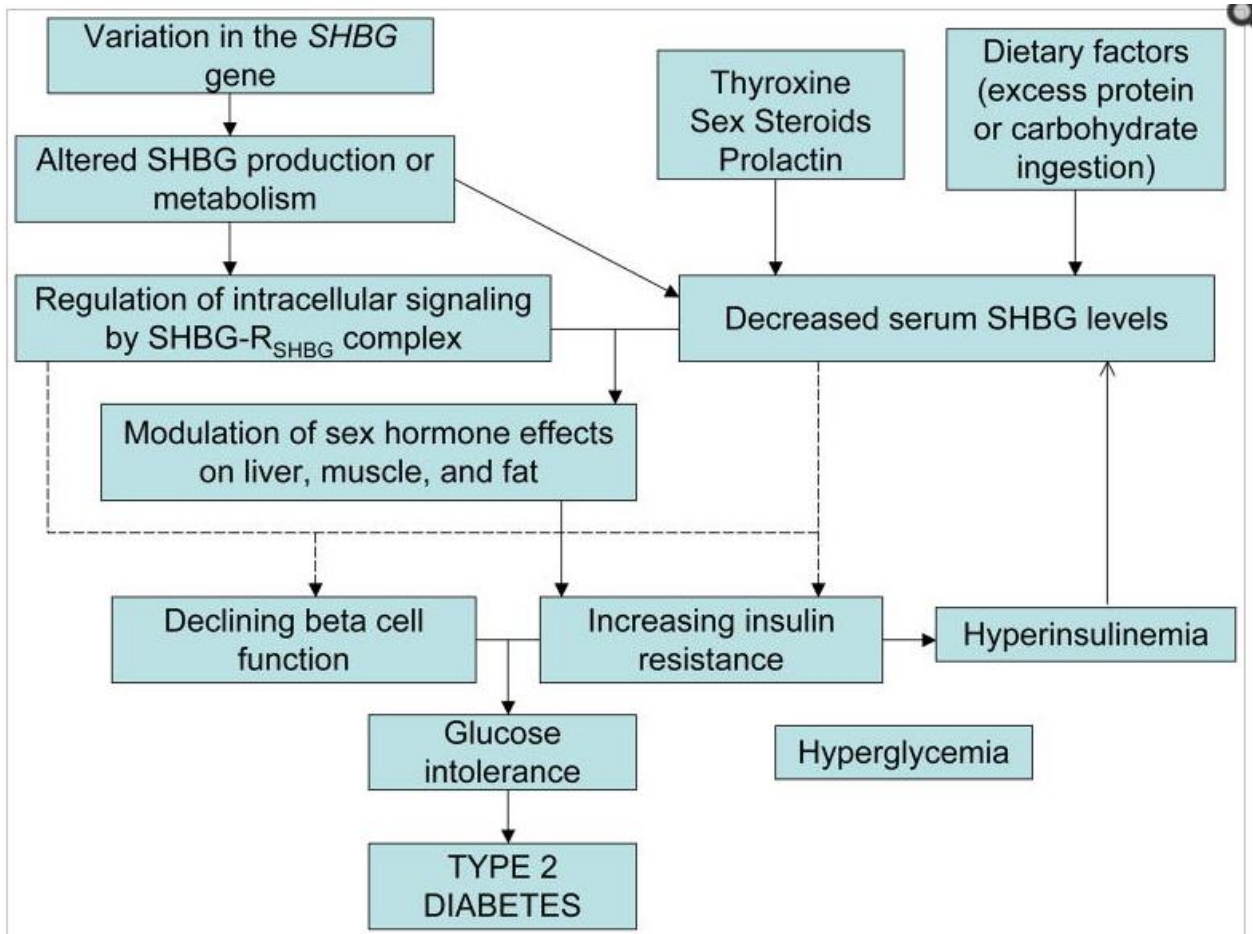
Country	Sex	Duration of f/u yrs.	Number of patients	Cases of T2DM	Mean Age in years	Mean BMI in Kg/m <sup>2</sup>	Mean SHBG in cases- nmol/l	Mean SHBG in controls nmol/l	p=
Sweden	F	12	1484	43	46.8	-	55	88	<0.001
USA	M	5	352	176	44.8	29.4	34	41	0.08
USA	M	13	1128	90	53.7	27	26	32	<0.001
Norway	M	9.1	1454	76	59.4	26	42	53.3	<0.001
USA	F	4.7	1612	116	63.1	28.6	35.7	54.5	<0.001
Finland	M	11	702	57	51.3	26.2	26.2	35.6	<0.01
United States	M	8.9	1030	54	53.9	27.1	24.4	32.3	<0.001
USA	M	3	203	20	68.3	23.7	45.7	45.1	NS
	F	3	280	23	65.4	23	56.5	69.7	<0.05
Sweden	M	13	446	35	67	25.4	39.9	51.5	0.053
USA	F <sup>†</sup>	8	61	19	52.7	29.5	66	78.5	NS
	F <sup>‡</sup>	8	48	19	38.5	25.9	41.6	74.4	<0.001

Adapted from Le et.al(Le, Nestler et al. 2012)Abbreviations: BMI body mass index; F – female; M - male; NR - not reported; NS - not significant; SHBG- sex hormone-binding globulin; T2DM - type 2 diabetes mellitus. Standard deviations for mean age and BMI are shown where available. Standard error for SHBG measurements are shown where available. To convert SHBG (nmol/L) to µg/dl, multiply by 0.025. \*Study included in Ding et al, 2006 meta-analysis. †Postmenopausal. ‡Premenopausal.

A study reported in the cross-sectional analysis in 6475 young adults (mean age 31, 57% men), higher SHBG was linked with a more favourable cardio-metabolic risk profile including associations with lipoprotein subclasses, fatty acid composition, amino acids,

ketone bodies and inflammation-linked glycoproteins (Wang, Kangas et al 2015). Furthermore, they found in a prospective analysis of 1377 young adults with 6-year follow-up indicated that SHBG is also associated with future insulin resistance. In a recent review looking at the effect of SHBG and type 2 diabetes, the authors concluded that there is an association between SHBG levels and the risk of developing type 2 diabetes. There is also evidence that genetic polymorphisms of SHBG gene result in variable risk of developing type 2 diabetes (Le, Nestler et al. 2012). The authors further postulated that multiple factors including nutritional, metabolic and hormonal influence including that of insulin could affect hepatic SHBG production. In addition SHBG levels are determined in part by genetic variation. The proposed pathways of SHBG actions are summarised in figure 1.5. Regardless of aetiology, alterations in SHBG may contribute to alterations in glucose homeostasis through modulation of sex hormone bioavailability.

**Figure 1.5 Potential metabolic pathways by which SHBG influence the carbohydrate metabolism**



Adapted from (Le, Nestler et al. 2012)

There are no interventional studies to see if these associations reflect a causal relationship between SHBG and cardio-metabolic risk profile or whether it merely reflects the altered sex hormone profile which in turn leads to changes in cardiovascular risk profile. There is no long term study examining the effect of SHBG levels in the cardiovascular profile of patients with type 2 diabetes and metabolic syndrome.

## **1.9 Potential metabolic mechanisms of testosterone action**

The biochemical and molecular mechanism of testosterone action linking to metabolic syndrome and type 2 diabetes is poorly understood. There is only a small number of studies which specifically looked into the action of testosterone in carbohydrate and lipid metabolism (Kelly and Jones 2013). In type 2 diabetes and metabolic syndrome there is a defective response of the target tissues to insulin causing inadequate glucose utilisation and increased lipogenesis. This leads to excess fatty acid release from adipose tissue and leads to fat deposition in non-adipose tissue including liver and adipose tissue as a “spill over effect (Yu and Ginsberg 2005). This excess fat deposition in non-adipose tissues in turn contributes to the insulin resistance. Studies have correlated liver and muscle fat with insulin sensitivity in rats (Lim, Son et al. 2009) (Lim et al. 2008). The studies have shown that tissues differ in their insulin sensitivity and resistance (Stumvoll, Jacob et al. 2000). These evidences suggest that the beneficial effect of testosterone reported in various clinical studies are likely due to the tissue specific actions of testosterone on various metabolic pathways involving carbohydrate and lipid metabolism in adipose tissue, liver and muscle.

### **1.9.1 Testosterone action in muscle tissue**

Studies have shown that low testosterone levels are associated with a reduced lean body mass. There is an inverse relation between relative muscle mass and insulin (Srikanthan and Karlamangla 2011). Both catabolic and anabolic effects of testosterone on the muscle would contribute to the insulin resistance seen in low testosterone state. However the exact mechanism of testosterone action leading to the changes in insulin-resistance in skeletal muscle is still not known.

### **1.9.1.1 Testosterone action on carbohydrate metabolism in muscle tissue**

Animal studies have shown that castration leads to decreased muscle glycogen content in the levator ani muscle of rats and testosterone administration reverses this (Leonard 1952, Apostolakis, Matzelt et al. 1963, Bergamini, Bombara et al. 1969), (Ramamani, Aruldhas et al. 1999). Glycogen synthase an important enzyme in the glycogen synthesis in muscle is increased with testosterone administration. This has been implicated in the metabolic abnormality of muscle in castrated rats. Another key enzyme, glycogen phosphorylase, which inhibits glycogenolysis, was reduced in testosterone deficient state and returned to normal on testosterone supplementation. This in turn led to the restoration of increased glucose level.

Glucose transporter type 4 (GLUT4) is an insulin regulated glucose transporter found mainly in adipose tissue and muscle. There are several studies showing that a decreased GLUT4 expression leads to decreased insulin sensitivity in the tissues, and various defects involving IRS1 and GLUT4 has been described in type 2 diabetes patients (Pessin and Saltiel).

Testosterone administration has been shown to increase molecular expression in adipocyte and skeletal muscle cultures. There are further studies showing up regulation of mRNA expression of IRS2 in the muscles of men treated with testosterone (Salehzadeh, Rune et al. 2011). In the androgen receptor deficient testicular feminised mouse (Tfm) mouse GLUT4 expression increased following testosterone replacement. This suggest an androgen independent action of testosterone on the GLUT4 expression (McLaren D 2012).

Testosterone has also shown to modulate key enzymes in the glycolytic pathway.

Phosphofructokinase (PFK) and Hexokinase two key enzymes in the glycolytic pathway were shown to increase by addition of testosterone or dehydroepiandrosterone in cultured

skeletal muscle (Sato, Iemitsu et al. 2008). Study on Tfm mouse also demonstrated a reduction in Hexokinase 2 expression in the skeletal muscle compared with wild type (McLaren D 2012). Hexokinase expression, did not improve after testosterone administration suggesting an androgen receptor dependant action of testosterone in modulating the skeletal muscle expression of Hexokinase 2.

Glucose-6-phosphate dehydrogenase, key enzyme in the pentose phosphate pathway, has been shown to increase in the rat muscle in response to testosterone (Max and Knudsen 1980, Max 1984).

### **1.9.1.2 Testosterone action on lipid metabolism in muscle tissue**

Clinical studies have demonstrated decreased fat oxidation and increased glucose oxidation in androgen deprived states (Reis, Liberman et al. 2009). There is also evidence that in induced androgen deficient states in healthy young men there is a decline in lipid oxidation leading to adiposity (Mauras, Hayes et al. 1998). In men with hypo gonadotropic hypogonadism due to hypopituitarism, short term testosterone treatment increased fat oxidation and reduced fat mass (Birzniece, Meinhardt et al. 2009).

Studies have shown that there is increased myocellular lipid accumulation from increased plasma fatty acid induced by lipid infusion (Itani, Ruderman et al. 2002, Stratford, Hoehn et al. 2004). This in turn led to increase insulin resistance, suggesting lipid accumulation in myocytes might contribute to insulin resistance in the muscle. By its action on lipid metabolism testosterone might exert a beneficial effect in reducing the intracellular lipid accumulation in skeletal muscles and thereby increasing the insulin sensitivity.

## **1.9.2 Testosterone action on Liver**

### **1.9.2.1 Testosterone action on glucose metabolism in the liver**

Liver plays a critical role in carbohydrate and lipid metabolism. This is achieved through multiple metabolic pathways which are regulated by various hormonal and non-hormonal factors. The hormonal regulators of hepatic glucose metabolism include insulin, glucagon and adrenalin. It has been shown that testosterone also influence hepatic glucose production through increasing insulin receptors in human adult liver cell line cultures (Parthasarathy, Renuka et al. 2009). In a separate study researchers found testosterone and dihydrotestosterone induce insulin receptors in human larynx carcinoma cell line. In animal studies it was reported that testosterone administration increased insulin receptor expression in liver tissues in castrated rats (Muthusamy, Murugesan et al. 2011). GLUT 4 transporter which plays an important role glucose metabolism is decreased in many tissues including liver following castration in male rats (Muthusamy, Murugesan et al. 2009). This in turn leads to decreased glucose utilisation in these tissues and elevated glucose in peripheral circulation. Testosterone repletion restored this metabolic defect.

### **1.9.2.2 Testosterone action on lipid metabolism in liver**

Hepatic fat content correlates with other features of metabolic syndrome. It is also known that obesity and type 2 diabetes leads to fatty liver. As liver is the first major clearance source of dietary fat via the portal circulation it is exposed to larger levels of free fatty acid (Mrin and Arver 1998). The high free fatty acid state decrease hepatic insulin binding, increase gluconeogenesis and insulin resistance. Ultimately these effects lead to peripheral hyperinsulinemia. Testosterone, through its effect on adipocytes, could reduce the free fatty acid production leading to reduction in insulin resistance. There are conflicting reports of effect of testosterone on liver fat metabolism in clinical studies. Two studies reporting hepatic steatosis associated with low testosterone (Kley, Nieschlag et al. 1975, Völzke, Aumann et al. 2010) while another study found no such correlation (Kley, Nieschlag et al. 1975). In androgen receptor knockout male mouse it was reported that a high fat diet induced hepatic steatosis (Lin, Yu et al. 2008).

It is postulated that the abnormal lipid metabolism results from the up regulation key enzymes and regulatory receptors in the fatty acid synthesis in the liver in androgen deficient state. These include the key enzymes sterol binding protein 1c, Stearoyl-CoA desaturase 1, Acetyl CoA desaturase 1, Acetyl CoA carboxylase and master regulator Peroxisome proliferator-activated receptor gamma.

In studies on Tfm Mouse which lacks functioning androgen receptor our research team has reported effect of testosterone on lipid metabolism possibly mediated by androgen receptor independent pathway (Kelly DM 2012). In knock out mouse model it has been shown that expression of Stearoyl-CoA desaturase 1 and fatty acid synthase were increased in liver of aromatase null male rats compared to the wild type (Chow, Jones et al. 2011). Fatty acid



synthase expression reduced following oestrogen receptor agonist treatment but not the Stearoyl-CoA desaturase 1 enzyme suggesting the some of the beneficial effect of testosterone on lipid metabolism in liver is mediated by aromatase conversion into oestradiol.

### **1.9.3 Testosterone action on adipose tissue**

Obesity and metabolic syndrome are closely linked to androgen deficient state as discussed in earlier sections. There are many studies reporting a negative correlation between the testosterone levels and body fat content(Phillips , Seidell, Björntorp et al. , Haffner, Mykkanen et al. 1993, Couillard, Gagnon et al. 2000, Kapoor, Malkin et al. 2005). The relationship between body fat distribution and testosterone has been further studied using computed tomography (CT) which showed the correlation is mainly limited to visceral fat deposition (Tsai, Boyko et al. 2000) (Seidell, Björntorp et al. , Couillard, Gagnon et al. 2000, Garaulet, Perex-Llamas et al. 2000). More recently studies using MRI and DEXA scanning for body fat has shown an inverse correlation between bioavailable testosterone and visceral adipose tissue in a younger population (Nielsen, Hagen et al. 2007).

Testosterone seems to have differential action on different adipose tissue sites. This is supported by the finding that testosterone supplementation caused a reduction in catecholamine induced lipolysis and a reduction in the expression of the rate limiting enzyme hormone sensitive lipase in the subcutaneous adipose tissue , but not in the visceral adipose tissue (Dicker, Rydén et al. 2004). This is further supported by the clinical studies which showed truncal subcutaneous adiposity is predictive of low testosterone state than the visceral fat (Abate, Haffner et al. 2002).

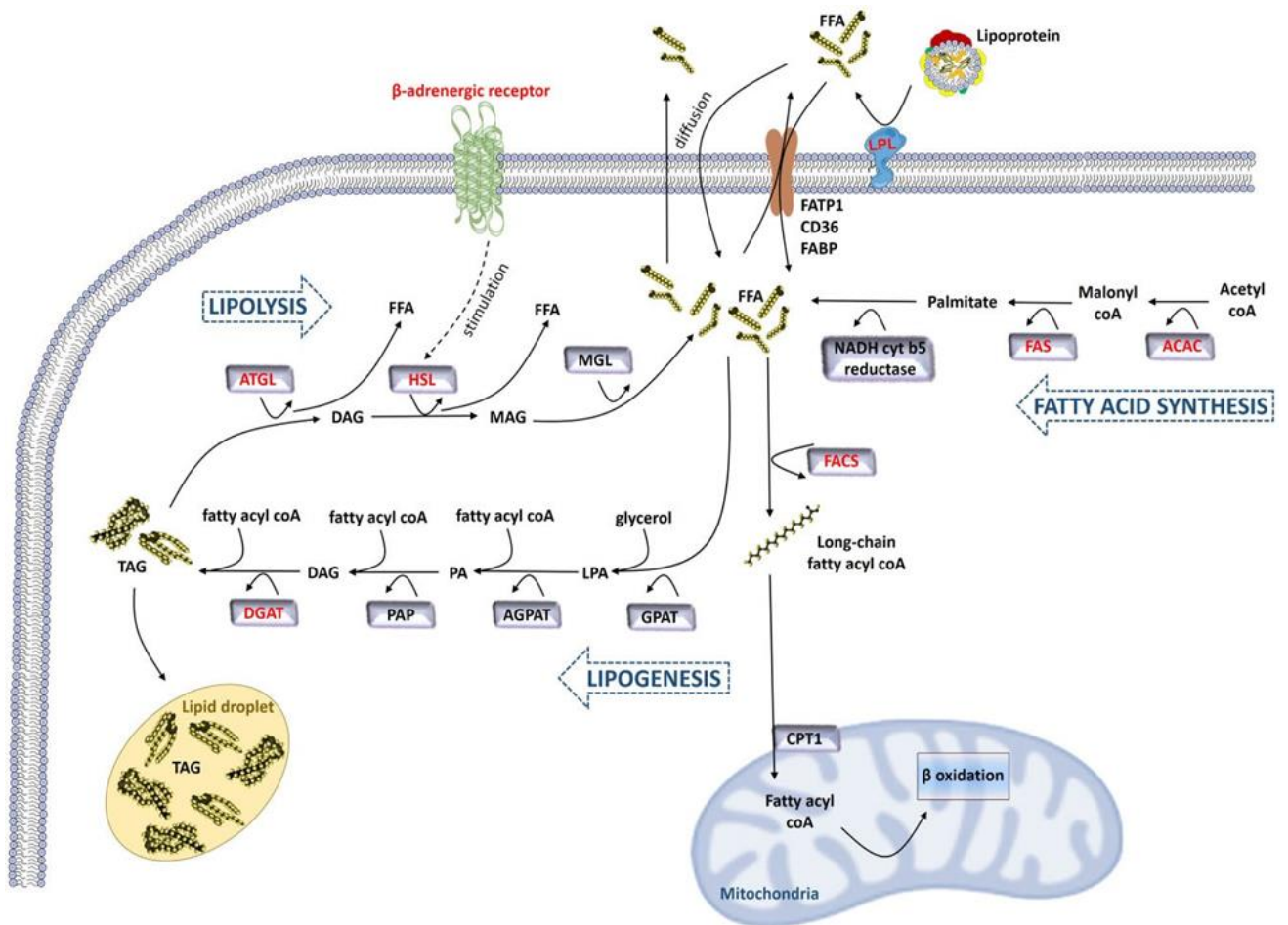
Testosterone affects many key regulators and enzymes of lipid metabolism. Sterol regulatory element-binding proteins (SREBP) are regulatory proteins which play a central role in lipid metabolism controlling the expression of a range of enzymes in the lipid metabolic pathways (Brown and Goldstein 1999). In animal studies there was significant up regulation of lipogenic genes including that of SREBP by androgens (Hall, Hoven et al. , Rosignoli, Nicolas et al. 2003) (Heemers, Maes et al. 2001). However as noted earlier, testosterone has tissue specific actions on metabolic pathways and these studies did not address the androgens receptor independent mechanisms in the metabolic pathways in adipose tissue. Hence the reported stimulatory effect of androgens in non-adipose tissues-may not be applicable in the context of metabolic syndrome and type 2 diabetes where the aberrant metabolic pathways mainly affects adipose tissue liver and muscle.

Testosterone has an inhibitory effect on Lipoprotein lipase, a major enzyme in the lipid metabolism in adipose tissue, hydrolyse triglycerides to free fatty acid which is taken up for storage in the adipose tissues after re esterification (Eckel 1989). This enzyme has been implicated in the pathogenesis of obesity (Gruen, Hietanen et al.). Testosterone has been shown to be inversely correlating with the lipoprotein lipase levels in the visceral adipose tissue (Ramirez, McMurry et al.). In interventional studies it has been shown that testosterone supplementation reduced abdominal adipose tissue lipoprotein lipase (Rebuffescribe, Marin et al. 1991). Further studies also suggested a differential action of testosterone with the lipid uptake reduced more in visceral adipose tissue than in subcutaneous adipose tissue (Marin, Lonn et al. 1996). This led to the conclusion that in testosterone deficient state there is a redirection of triglyceride storage in the visceral adipose tissue.

Acetyl CoA carboxylase and fatty acid synthase two key enzymes in the lipid metabolism, has been shown to be regulated by testosterone. In the androgen deficient mouse there was a significant increase in the mRNA expressions of these two key enzymes in subcutaneous

adipose tissue (Macotela, Boucher et al. 2009). The potential targets of testosterone action on lipid metabolism are shown in figure 1.6.

**Figure 1.6 Potential targets of testosterone action on lipid metabolism**



The potential influence of testosterone on targets of cellular lipid metabolism is indicated in red text. ACAC, acetyl coA carboxylase; AGPAT, acylglycerol-3-phosphate acyltransferase; ATGL, adipose triglyceride lipase; CPT1, carnitine palmitoyltransferase 1; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; FABP, fatty acid binding protein; FACS, fatty acyl-coA synthetase; FAS, fatty acid synthase; FATP1, fatty acid transport protein 1; FFA, free fatty acid; GPAT, glycerol-3-phosphate acyltransferase; HSL, hormone-sensitive lipase; LPA, lysophosphatidic acid; MAG, monoacylglycerol; MGL, monoacylglycerol lipase; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; TAG, triacylglycerides. Adapted from Kelly et al (Kelly and Jones 2015)

From the above discussion it is clear that testosterone has a wider role in the metabolic pathways and the actions are likely to be tissue specific with androgen receptor dependant and independent pathways playing significant role. Through its actions on lipid and carbohydrate metabolism, testosterone reduces fat mass and increases muscle mass, thereby reducing insulin resistance. Further studies are needed to clearly demonstrate the molecular pathways and the tissue specific actions of testosterone via AR dependant and AR independent pathways.

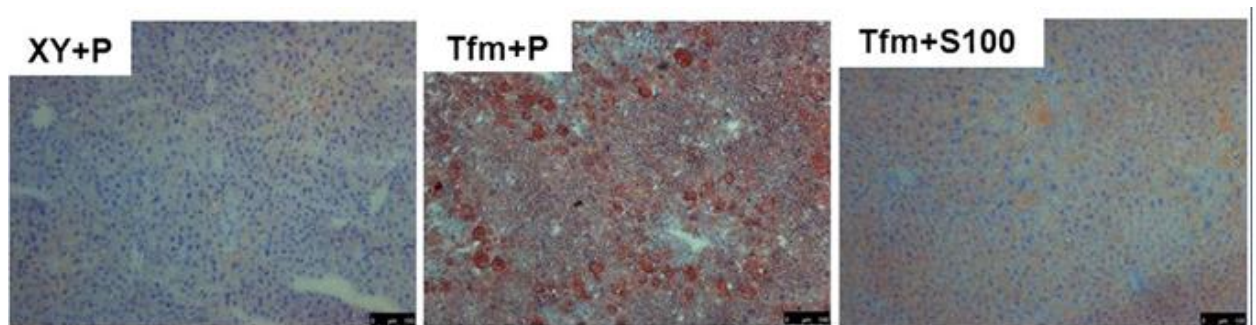
### **1.10 Testicular Feminised (Tfm) mouse**

Tfm mouse has a defective androgen receptor resulting from an x linked single base pair deletion in the gene encoding the classical androgen receptor. The truncated protein resulting from the transcription of the defective gene renders the receptor unable to bind to androgens (He, Kumar et al. 1991). This makes the animal unresponsive to androgen through the receptor and phenotypically female. However unlike the human equivalent of androgen insensitivity syndrome these animals have very low circulating levels of testosterone. This results from a concomitant deficiency of enzyme  $17\alpha$  hydroxylase in the Leydig cells of the testis, a key enzyme in steroidogenesis (Murphy and Oshaughnessy 1991). The serum levels of testosterone are 10 fold lower in the Tfm mouse compared with XY littermates (Jones, Pugh et al. 2003). Due to the absence of negative feedback loop to the pituitary mediated via androgen receptor, the luteinising hormone levels are high. However due to the lack of  $17\alpha$  hydroxylase circulating testosterone remains reduced (Murphy and Oshaughnessy 1991). The unique feature of a non-functioning androgen receptor and the low levels of testosterone make the Tfm mouse ideal for the study of testosterone action which are independent of androgen

receptor. Previous data from our research team which studied the effect of testosterone on atherosclerosis has shown that physiological testosterone replacement significantly reduced fatty streak formation of the aortic root in the Tfm mice (Nettleship, Jones et al. 2007). The high fat fed Tfm mouse demonstrated an increase in aortic root fatty streak formation which is only partly attenuated by oestrogen receptor blockade suggesting an androgen independent mechanism (Nettleship, Jones et al. 2007).

Further studies from our research group has demonstrated that high fat fed Tfm mouse developed significant hepatic steatosis as compared to the XY littermates (Kelly, Nettleship et al. 2014). This was significantly reduced following the administration of physiological levels testosterone figure 1.7. Further experiments demonstrated that supra-physiological level of testosterone caused further reduction in hepatic lipid accumulation and oestrogen receptor blockade only partially reversed the changes. This suggested an androgen receptor independent effect of testosterone on hepatic lipid metabolism.

**Figure 1.7 Hepatic lipid deposition in high fat fed Tfm mouse compared to XY littermates**



XY+P –Placebo treated wild type mouse, Tfm+P placebo treated Tfm Mouse Tfm+S100, testosterone treated Tfm mouse. Fat staining with Oil-red-o with haematoxylin background (Kelly, Nettleship et al. 2014)

## **1.11 Conclusions and prelude to thesis**

From the evidence described above it is clear that there is a correlation between testosterone levels and metabolic syndrome. Low testosterone levels have been shown to be a risk factor for future development of insulin resistance which is the hallmark of metabolic syndrome and type 2 diabetes. The studies further suggest there is also increased risk of mortality in testosterone deficient state.

Although the exact mechanisms are still unclear, it seems testosterone deficiency leads to the state of insulin resistance through its effect on multiple metabolic pathways involving carbohydrate and lipid metabolism. Further evidence presented above suggests testosterone replacement leads to improvement of metabolic abnormalities induced by the low testosterone state through its actions on metabolic pathways. Testosterone influences major enzymatic pathways involved in the carbohydrate and lipid pathways through direct action on the enzymes and more likely and importantly, through the regulatory receptors. It is also clear that testosterone exerts its action in a tissue dependent manner with different metabolic pathways involved in liver, subcutaneous adipose tissue, visceral adipose tissue and muscle. This varied action of testosterone on different tissues highlights the need for looking into tissue specific actions of testosterone when analysing the molecular mechanism and generalisation from single tissue studies could be misleading.

The evidence presented from clinical studies suggests there are clinically significant benefits from testosterone replacement therapy. The beneficial effects in hypogonadal men with insulin resistant states of type 2 diabetes and metabolic syndrome seem to be due to the metabolic profile in carbohydrate and lipid metabolism in liver, muscle and adipose tissues. Some of these effects are seen in the very early stages of the replacement therapy and some are long lasting changes. Many studies also suggest that testosterone replacement is

associated with improvements in cardiovascular risk profile in men with metabolic syndrome and type 2 diabetes.

Further clinical studies have shown that low testosterone is linked with a higher mortality both in general population and in specific disease groups. The effect of testosterone replacement on the longevity of men with hypogonadism is not yet clearly known. This is due to the lack of long term prospective studies looking into the mortality in men treated with testosterone. A few studies discussed above show there may be beneficial effect for testosterone replacement on mortality but other failed to support this. There is no long term data on testosterone and mortality or cardiovascular risk profile in men with type 2 diabetes and low testosterone.

The remainder of the thesis I will be investigating the role of testosterone in type 2 diabetes, metabolic syndrome and other cardiovascular risk and mortality and the effect of testosterone replacement on these parameters. The molecular mechanisms of testosterone action in various tissues looking especially into the carbohydrate and lipid metabolism are also studied in animal model. Chapters 2 and 3 will investigate the effect of testosterone on mortality in type 2 diabetic men and long-term effect of testosterone on glycaemic control and cardiovascular risk profile including the effect of testosterone supplementation on mortality and cardiovascular risk profile. Chapter 4 is a cross-sectional study investigating the effect of testosterone on cardiovascular risk profile, including the surrogate marker CIMT. In chapter 5 I will look into the role of androgen receptor in the cardio metabolic actions of testosterone, specifically looking at a naïve potentially more sensitive indicator of androgen action, the ratio of testosterone to AR CAG (T/AR CAG and BioT/AR CAG) ratio. I also will be looking into the long term relationship of glycaemic control and other cardiovascular risk profile in the diabetic population to the serum SHBG levels independent of testosterone

levels. In Chapter 6 I will be investigating the molecular basis of testosterone action by studying the testicular feminised mouse model. The effect of testosterone on major metabolic pathways of carbohydrate and lipid metabolism in various metabolically active tissues is investigated by analysing the mRNA expression of key molecules in liver, adipose tissue and muscle.





## **Chapter Two**

### **Longitudinal Study: Long term effect of testosterone on mortality in men with type 2 diabetes**

*My contribution to this chapter includes design, majority of patient recruitment for the follow up from initial cohort, data collection for the follow up, calculation of bioavailable and free testosterone, statistical analysis, presentation and publication of results. As this is a follow up study, the baseline data for clinical assessment and biochemical data used for mortality analysis is from the previous researchers of the team.*

#### **2.1 Background and Introduction**

As discussed in detail in chapter one, there are several studies showing a correlation with low testosterone levels and mortality. This has been reviewed in the meta-analysis which reviewed the published papers relating to low testosterone and mortality (Araujo, Dixon et al. 2011). The authors concluded that there is an association between low testosterone levels and all cause and cardiovascular mortality. Many studies have also looked into specific diseases causing increased mortality in testosterone deficient state and reported links with increased respiratory and cancer deaths in men with low testosterone along with the cardiovascular disease (Khaw, Dowsett et al. 2007, Laughlin, Barrett-Connor et al. 2008, Ponikowska, Jankowska et al. 2010). On the other hand in men suffering from chronic disease like coronary artery disease, chronic renal disease were also found to have an increased mortality if they have low testosterone as compared to those who had higher levels of testosterone (Carrero, Qureshi et al. 2009, Malkin, Pugh et al. 2010, Zarotsky, Huang et al. 2014).

Similarly many studies have also linked low testosterone to type 2 diabetes and/ or metabolic syndrome (Ding, Song et al. 2006, Kapoor, Aldred et al. 2007, Wang, Jackson et al. 2011). There is evidence that apart from total testosterone, the free testosterone and bioavailable testosterone were low in men with type 2 diabetes. These findings suggest that SHBG changes (which can affect total testosterone levels) may not fully explain the findings and testosterone itself is major factor, either cause or effect (or a combination of both), in the reported metabolic associations. As discussed in chapter one, previous reports from our research team has demonstrated a high prevalence of hypogonadism up to 40%, in men with type 2 diabetes. (Kapoor, Aldred et al. 2007). There is a large number of studies showing an adverse cardio-metabolic risk from low testosterone levels through its effect on body mass index, adiposity, lipid profile and insulin resistance (Jones 2010, Kelly and Jones 2013) . Type 2 diabetes is a major cause of morbidity and mortality worldwide. The most recent WHO global report on diabetes states that there are 422 million people worldwide living with type 2 diabetes. The report further estimates that type 2 diabetes in a major cause of morbidity and mortality due to blindness, kidney failure, heart attacks, stroke and lower limb amputation. Among this, cardiovascular disease is the major cause of death in men with type 2 diabetes.

As noted above low testosterone has been found to be associated with cardiovascular disease and diabetes (Jones 2010). One of the surrogate markers of cardio vascular disease, the carotid intimal thickness (CIMT), is found to be inversely associated with testosterone levels in many studies (Fukui, Kitagawa et al. 2003, Muller, van den Beld et al. 2004, Svartberg, Von Muhlen et al. 2006). Further studies also suggested a progression of atherosclerosis on follow up of men with hypogonadism (Muller, van den Beld et al. 2004). Inflammatory cytokines which are linked to metabolic syndrome and cardio vascular disease are adversely affected in testosterone deficient state. Some studies have reported improvement in these

adverse inflammatory markers after testosterone replacement therapy (Kapoor, Clarke et al. 2007, Nettleship, Pugh et al. 2007).

There are studies which reported improvement in the glycaemic profile and cardiovascular risk profile after testosterone replacement therapy in the short term (Malkin, Pugh et al. 2004, Agarwal and Oefelein 2005, Kapoor, Goodwin et al. 2006, Heufelder, Saad et al. 2009, Kalinchenko, Tishova et al. 2010, Jones, Arver et al. 2011). This is discussed in detail in chapter 2. The TIMES 2 study showed a significant improvement in cardiovascular risk profile after testosterone replacement therapy (Jones, Arver et al. 2011). There are short term studies which showed an increase in cardiovascular events in men receiving testosterone replacement therapy (Basaria, Coviello et al. 2010).

One of the large population studies which studied over 1000 men over the age of 40 years , looking into the long term effect of testosterone levels and replacement therapy on mortality reported that low testosterone levels were associated with an increased mortality on long term follow up and testosterone replacement therapy seems to have beneficial effect in reducing the mortality among the men with low testosterone (Shores, Smith et al. 2012).

In men with type 2 diabetes, there is only limited evidence from the literature regarding the effect of low testosterone on mortality on the long term. Also there are no studies looking into the effects of testosterone replacement on longevity in these men.

## **2.2 Hypothesis**

I hypothesised that low testosterone increases the mortality in men with type 2 diabetes and in hypogonadal men physiological testosterone replacement therapy would improve the survival compared to those who did not receive the replacement therapy.

## **2.3 Research Design and Methods**

This study is a follow up of the cohort of patients from our previous study (Kapoor, Aldred et al. 2007). This study further included a group of men who had their testosterone levels measured, at Barnsley hospital, during the same period as the original cohort study period of October 2002 and December 2005. Their data were collected from the hospital date base. This gave a large sample of nearly 600 men whose testosterone levels were assessed during the initial screening period. The study was approved by the South Yorkshire Research Ethics Committee. To study the effect of low testosterone on mortality the subjects were divided into groups based on different cut off levels of testosterone and bioavailable testosterone. The groups analysed were 1). Total testosterone cut off at 10.4 nmol/l as per the Endocrine society guidelines (Bhasin, Cunningham et al. 2010) 2). 8nmol/l as used in most UK laboratories 3). Bioavailable Testosterone cut off of 2.6nmol/l as this is one of the accepted levels of bioavailable testosterone below which it is considered as hypogonadal range and 4). Calculated free testosterone level of  $\leq 225$ pmol/l. There were two different assays used for analysis of baseline testosterone. For those from the hospital database assay was performed using competitive chemiluminescence assay (Bayer Advia Centaur- Siemens Medical Solutions Diagnostics) and those from the research database patients, solid phase enzyme

immunoassay (DRG Instruments GmbH, Germany) was used. Both assays are validated methods for assessing Testosterone. 36 patients from the research cohort had a repeat testosterone assay performed using the competitive chemiluminescence assay within a few months of the original screening. The results were comparable by the two methods. The difference between the means of the two groups was 0.33nmol/l (95%CI -1.22 to 1.88; p=0.669). All blood samples were taken between 0800 hours to-1000 hours. SHBG was measured by solid phase enzyme immunoassay (DRG Instruments GmbH, Germany) for the research patients and solid phase two site chemiluminescent enzyme immunometric assay (Siemens Immulite, Siemens Medical Solutions Diagnostics) for the hospital patients.

For assessing the effect of testosterone on the mortality we assigned those patients who received testosterone replacement therapy over 12 months, as a separate group of TRT and the three groups were analysed. Mean duration of follow up was 41.6±20.7 months. 60 patients received testosterone for >12 months, 51 of these having treatment >2 years. All the patients who received testosterone replacement therapy were initiated and monitored through the department of diabetes and endocrinology. The routine clinical practice was to titrate the testosterone supplementation to achieve a 4-6 hour level of upper normal range. Although all the patients from the research cohort with low testosterone levels were offered a choice of testosterone replacement therapy there were some patients who did not have the testosterone replacement therapy. The reasons for this included patient choice, lower cut off for diagnosis of hypogonadism till recent years ( 8nmol/l as compared to 10.4 nmol/l cut-off used in this study) and a concomitant diagnosis of prostate cancer.

Patients were treated with different testosterone replacement preparations including (1% testosterone gel Testogel®/AndroGel® or 2% testosterone gel via a metered pump;

Tostran®/Fortigel®) and intramuscular depot testosterone undecanoate (Nebido®). In the group 55 patients (85.9%) were on testosterone gel; 9 patients (14.1%) on intramuscular testosterone undecanoate during the last 6 months or more of the study period. Of this one patient was started on intramuscular testosterone undecanoate from the start. 3 patients were on buccal testosterone before changing to intramuscular testosterone. Rest of the patients were started on testosterone gel at the start.

The mean peak level achieved during the study period was 22.8(±9.9). 43(67%).Patients achieved a level of 18 nmol/l or more. Patient compliance was good as evidenced by the progressive improvement in the testosterone levels in most of the patients during the follow up visits. As per clinical safety guidelines these patients had their haemoglobin, haematocrit and PSA checked at regular intervals and appropriate dose adjustment or onward referral were made if needed.

The causes of death were obtained from the hospital records and through the local and national registry where the death occurred outside the hospital. To avoid the bias of acute illness causing low testosterone we excluded deaths that occurred in the first 6 months of initiations of testosterone replacement. There were no deaths due venous thromboembolism in the testosterone treated patients.

Baseline data regarding co morbidities, concomitant medications, anthropometric measures and biochemical assessments were available from the original cohort data base. The remaining data was obtained from the hospital records. The baseline factors were then analysed for significance between two groups. These factors were then included in the co-variate analysis using the cox regression model.

Sex hormone binding globulin (SHBG) results were available for 436 patients. Bioavailable testosterone (BT) and free testosterone (FT) were calculated using validated mathematical formulae (Vermeulen, Verdonck et al. 1999, Morris, Malkin et al. 2004).

## **2.4 Clinical and biochemical assessment**

### **2.4.1 Recording of demography, medical history and drug history**

Age, medical history and drug history were specifically recorded at the start of the study. This was confirmed with details in the medical notes where possible and in the event of any doubt the patient's GP was contacted, with their permission, to confirm details. For those patients who were added to the study the above data were collected from the hospital records.

### **2.4.2 Measurement of weight and body composition**

Height was measured after removal of shoes. Participants stood with the backs of their ankles, gluteal region and shoulders touching a wall which had a calibrated scale attached. A horizontal plane was applied to the crown of the head to be read against the scale.

Weight and body composition were assessed using Tanita BF-300 body fat analyser (Tanita Corporation, Japan). 'Standard Male' body type measurement was selected and the participants height and age were entered. After removing outer clothing, shoes and socks participants were asked to step onto the weighing platform so the plantar surface of heels and



forefeet were in firm contact with the posterior and anterior electrodes respectively. The device measures weight directly and measures bioelectrical impedance by applying a 50 kHz, 500mA electrical current between the feet to derive body fat percentage on the principle that fat has lower electrical conductivity compared to other body tissues. This method has been validated against under water weighing for body composition analysis (Cable, Nieman et al. 2001). Body fat mass was calculated by multiplying weight by body fat percentage. Lean body mass was calculated by subtracting body fat mass from weight.

#### **2.4.3 Measurement of waist and hip circumference**

Waist circumference was measured at the point midway between the iliac crest and the costal margin. Hip circumference was measured at the widest point around the buttocks. Waist-hip ratio was calculated by dividing waist circumference by hip circumference.

#### **2.4.4 Measurement of blood pressure**

Blood pressure was measured using Colin Press-Mate BP8800C non-invasive blood pressure monitor (Colin Medical Instruments Corporation, Japan). Measurements were taken by placing the appropriately sized cuff around the upper arm such that the pressure sensor was applied to the skin close to the brachial arterial pulse to derive blood pressure from an automated oscillometric technique.

## **2.4.5 Biochemical measurements**

### **2.4.5.1 Sampling technique**

The blood samples were taken between 0800 and 1000h at a fasting state (8hours minimum).

Smokers were asked not to smoke on the morning of an assessment visit.

Venous blood was drawn by venepuncture from the antecubital fossa. Samples were taken in appropriate bottles and mixed by inverting the bottles three times. After the sample had clotted serum was obtained by centrifugation (10 minutes at 3500 revolutions per minute) and immediately frozen at  $-20^{\circ}\text{C}$  pending further analysis. Patients were asked to provide a first morning urine sample.

### **2.4.5.2 Measurement of samples in Barnsley hospital clinical chemistry laboratory**

Measurement of total testosterone was done using competitive chemiluminescence assay (Bayer Advia Centaur- Siemens Medical Solutions Diagnostics). The SHBG measurement was done by solid phase two site chemiluminescent enzyme immunometric assay (Siemens Immulite, Siemens Medical Solutions Diagnostics).

Glucose and urinary microalbumin were measured by Olympus analysers (Olympus Diagnostics, Germany). HbA<sub>1c</sub> was measured by Menarini Analyser HA8160 (Menarini Diagnostics, Italy). Haemoglobin and haematocrit was measured by Cell-Dyn 4000 analyser (Abbott Laboratories, USA). PSA was measured by chemiluminescent micro particle immunoassay (Abbott Laboratories, USA).

### **2.4.5.3 Measurement of serum bioavailable testosterone- summary**

The principle of this technique is to remove testosterone from a test sample using activated charcoal.  $^3\text{H}$ -labelled testosterone is then added to the serum on the basis that it will equilibrate between binding with SHBG and other proteins or remain unbound in the same proportions as the test sample testosterone. The SHBG bound portion is then precipitated out of the sample and the remaining concentration of  $^3\text{H}$ -testosterone is assessed using a beta-counter. The result is compared to a control sample where the SHBG bound  $^3\text{H}$ -testosterone is not precipitated and this proportion is used to calculate the bioavailable testosterone from the total testosterone which is assessed separately. The technique is adapted from that described by Tremblay and Dube (Tremblay and Dube 1974).

### **2.4.5.4 Measurement of serum bioavailable testosterone- method**

Samples were allowed to come to room temperature. 400  $\mu\text{l}$  of sample was dispensed into each of three Eppendorf tubes comprising duplicate test samples and a single control. 400  $\mu\text{l}$  of activated charcoal was added to each Eppendorf tube and samples incubated at room temperature for 30 minutes. At the completion of incubation samples were spun in a centrifuge at 3500 rpm for 20 minutes at 4°C. 200  $\mu\text{l}$  of the resulting supernatant was dispensed into new Eppendorf tubes and 50  $\mu\text{l}$   $^3\text{H}$ -testosterone solution added. The samples were applied to a vortex to ensure good mixing, incubated for 120 minutes at 37°C and then chilled on ice. 200  $\mu\text{l}$  cold, saturated ammonium sulphate solution was added to the test samples with 200  $\mu\text{l}$  saline added to the control. The samples were mixed with a vortex and then spun in a centrifuge at 3500 rpm for 20 minutes at 4°C. 200  $\mu\text{l}$  of the resulting

supernatant was dispensed into counting vials and 3 ml of OptiPhase Hisafe 2 scintillation fluid (Fisher Scientific, UK) added. Activity of the samples was measured by Beta counter and was proportional to the  $^3\text{H}$ -testosterone content. The percentage of bioavailable testosterone was calculated according to the formula.

$\% \text{ Bioavailable testosterone} = \text{Average of duplicate sample (dpm)} \times 100 / \text{control (dpm)}$ .

Total testosterone was measured by ELISA (see section 2.3.2.1) and the concentration of bioavailable testosterone was then calculated as follows;

$\text{Bioavailable testosterone} = \% \text{ Bioavailable testosterone} \times \text{Total Testosterone} / 100$

#### **2.4.5.5 Calculation of free and bioavailable testosterone**

Due to the considerable technical challenge concerning accurate measurement of free testosterone and oestradiol formulae have been developed which aim to estimate free sex hormone levels based on total serum levels of testosterone, oestradiol and SHBG. In our studies we used the formula of Vermeulen to calculate free testosterone (Vermeulen, Stoica et al. 1971) and free oestradiol was calculated from total oestradiol, SHBG and TT by the formula of Sodergard et al (Sodergard, Backstrom et al. 1982).

Formula for free testosterone (Vermeulen, Stoica et al. 1971);

$$\text{Free testosterone} = (-b + \sqrt{b^2 + 4a [\text{Total Testosterone}]}) / 2a$$

$$A = K_{at} + K_{st} + (K_{at} \times K_{st})([\text{SHBG}] + [\text{Albumin}] - [\text{Total Testosterone}])$$

$$B = 1 + K_{st}[\text{SHBG}] + K_{at}[\text{Albumin}] - (K_{at} + K_{st})[\text{Total Testosterone}]$$

$K_{at}$  = Affinity constant for testosterone with albumin =  $3.6 \times 10^4$  in this equation  $K_{st}$  = Affinity constant for testosterone with SHBG =  $1 \times 10^9$  in this equation.

The equation used to calculate bioavailable testosterone was from Morris et al (Morris, Malkin et al. 2004)

$$\text{Ln Bioavailable Testosterone} = -0.266 + (0.995 \times \text{Ln Total Testosterone}) - (0.228 \times \text{LnSHBG})$$

Ln=log Normal.

Measurements using different formulae for calculation of the total and free testosterone revealed similar results, although the absolute values were different as noted in studies (De Ronde, Van der Schouw et al. 2006).

## 2.5 Statistical analysis

Baseline data were summarized as mean +/- standard deviation. The statistical analysis was done using SPSS software, data was analysed for homogeneity using Q-Q plots and adjusted by transforming to log when appropriate. Alternatively non parametric tests were used to analyse correlation between variables which are not normally distributed.

For the mortality analysis, after excluding deaths which occurred in first 6 months (n=4) data were then analysed using SPSS 15 software with support from a University of Sheffield statistician. Kaplan-Meier curves were compared and significance was tested using log rank method. Cox regression (forward conditional) model was used for multi-variate survival analysis. Means, standard deviation (SD) hazard ratios (HR; 95% confidence interval CI) for survival and p values for significance were calculated. Survival curves were plotted for age adjusted and co-variate adjusted models with two groups of total testosterone as categorical values (1  $\leq$ T 10.4nmol/L 2  $>$ TT 10.4nmol/L). Graphs were obtained using SPSS. Similar analyses were done for different cut off levels for testosterone, bio available testosterone and calculated free testosterone levels. For the analysis of effect of testosterone replacement after exclusion of the patients who were treated less than 12 months, the data was divided into three groups 0 – low TT without treatment, 1- low TT with, 2- normal TT. The data were similarly analysed using Cox Regression model in SPSS.

## **2.6 Results**

A total of 591 patients with type 2 diabetes who had testosterone levels performed between the years 2002 and 2005 were identified from the databases. We excluded deaths occurring in the first 6 months (n=4) and those patients with a normal testosterone level at screening but subsequently developed hypogonadism and received treatment (n=6). However the results for mortality were similar when analysed with the excluded patients. There were no deaths due to venous thromboembolism in the testosterone treated group. The remaining 581 subjects were followed up for a mean period of 5.8 +1.7 years. Mean age was 59.5 years (+10.8; range 31-88years). 238 (40.96%) had low TT (<10.4nmol/l) and 343 (59.03%) with TT>10.4nmol/l. Baseline features are summarised in table 2.1

**Table 2.1 Baseline characteristics of low and normal testosterone groups**

Variables	Whole population	Numbers with Missing values	Normal T group(T>10.4 nmol/l)	Low T group (T<10.4nmol/l)	p=
Number	581		343	238	
T levels nmol/l Mean(SD)	12.4(5.5)	0	15.7(4.5)	7.5(2)	<0.001*
Age in years Mean(SD)	59.5(10.8)	0	58.9(10.4)	60.3(11.5)	0.15*
HbA1c Mean(SD)	7.3(1.4)	8(1.4%)	7.2(1.4)	7.5(1.3)	0.002*
Weight in kg Mean(SD)	98(20)	63(10.8%)	95.2(18.5)	102.1(21.4)	<0.001*
Height in cm Mean(SD)	174.3(6.8)	74(12.7%)	174.3(6.8)	174.3(6.8)	0.94*
BMI Mean(SD)	32.4(5.8)	89(15.3%)	31.3(5.3)	33.6(6.1)	<0.001*
Current smokers n(%)	104 (9.3%)	43(7.4%)	67(20.9%)	37(17%)	0.089 <sup>†</sup>
Statin therapy n(%)	260(48.1%)	40(6.9%)	161(50%)	99(45%)	0.29 <sup>†</sup>
ACEI/ARB therapy. n(%)	260(48.1%)	30(5.2%)	183(56%)	125(55.8%)	1 <sup>†</sup>
Pre-existing CV disease n(%)	213(39.2%)	38(6.5%)	120(37.2%)	93(42.3%)	0.25 <sub>†</sub>

\* Analysis by t-test, † by chi (χ<sup>2</sup>)- squared, n(%) - number with percentage within the groups, SD- standard deviation

There were 353 patients from the research cohort and 228 patients from the hospital database fulfilling the inclusion criteria and were included in the final analysis. The hospital data base patients were older and more likely to have pre-existing CV disease and on Statin treatment.

The research cohorts were more likely to be current smokers. There were no difference in weight, height, BMI, glycaemic control, ACE/ARB treatment, testosterone and SHBG levels between the groups.

The Mean T level was 15.7±4.5nmol/l in the normal T group as compared with 7.5±2nmol/l in low T group (p=<0.001). The weight (102.1±21.4 vs. 95.2±18.5 kg, p<0.001) and BMI

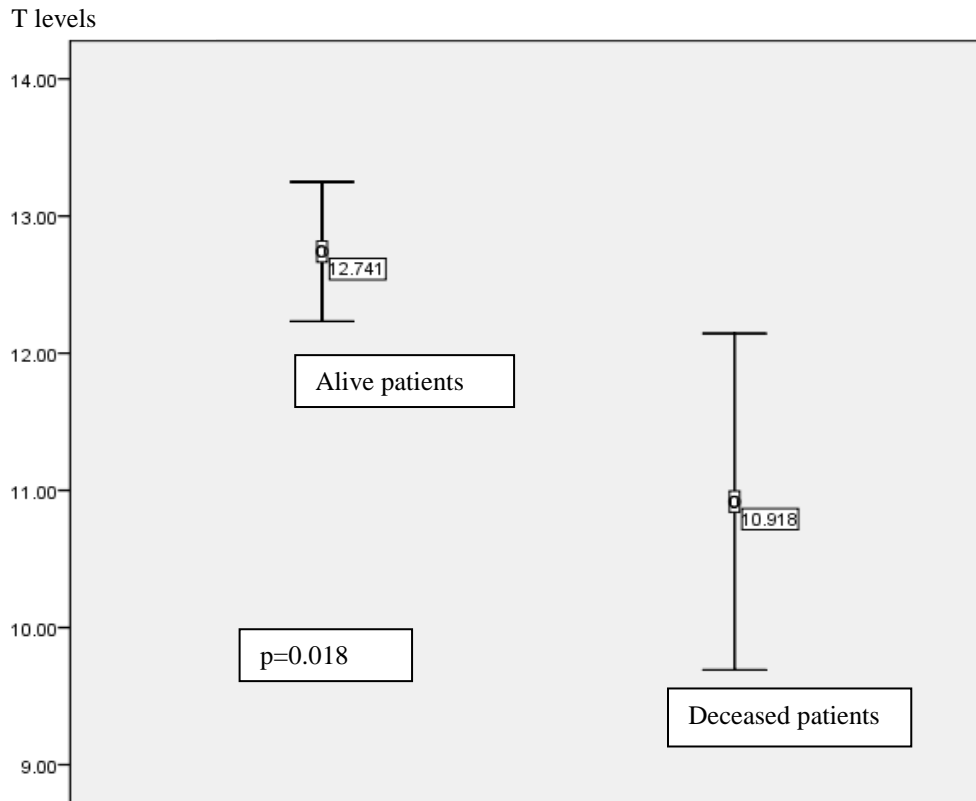
( $33.6 \pm 6.1$  vs.  $31.2 \pm 5.3 \text{ kg/m}^2$ ,  $p < 0.001$ ) were high in the low T group and they were more likely to have poor diabetes control than normal T group (HbA1c  $7.5 \pm 1.3$  vs.  $7.2 \pm 1.4$ ,  $p = 0.002$ ). Both groups were matched for age, smoking status, pre-existing cardiovascular disease, statin and ACE inhibitors or ARB therapy.

### **2.6.1 All-cause mortality in low and normal testosterone groups**

There were 72 deaths after the initial six month period. Individual causes of deaths were - 34 cardiovascular deaths ( including coronary artery disease, cerebrovascular disease, peripheral vascular disease and pulmonary embolism), 13 respiratory disease deaths, 17 cancer deaths, 8 other causes which included gastrointestinal haemorrhage(3), renal failure(1), sepsis(3) and suicide(1). Death due to pulmonary embolism was in the normal testosterone group. Mean testosterone levels were significantly low in patients who died ( $10.9 \pm 5.2$  SD) when compared with those are alive ( $12.6 \pm 5.5$ ;  $p = 0.018$  (Figure 2.1)



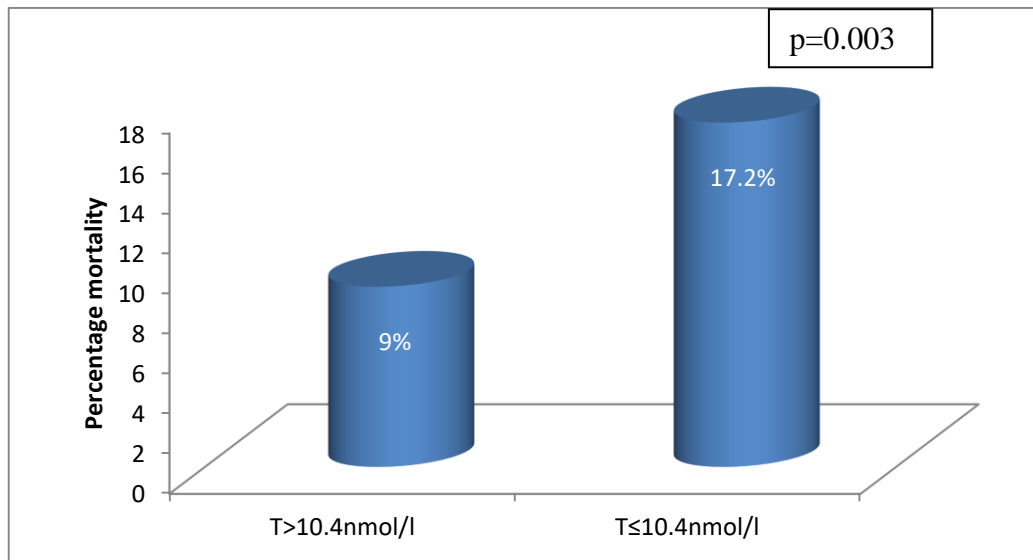
**Figure 2.1** Mean baseline testosterone levels in patients who are deceased and those who are alive



Testosterone levels in nmol/l represented in boxes

The crude mortality rate was 17.2 % (41/ 238) in the low T group as compared to 9% (31/ 343 p=0.003) in the normal T group Figure 2.2

**Figure 2.2 Crude mortality rates in low and normal testosterone group**



The Kaplan-Meier survival curves showed a significant decrease in survival in the low T group compared to normal T group (p=0.002 Log rank). In the Cox regression model (forward conditional) after adjusting for co-variates (Age, pre-existing cardiovascular disease, weight, height, BMI, HbA1c, smoking, statin and ARB/ACEi therapy) at baseline, the hazard ratio for decreased survival was 2.02 (p= 0.009, 95%CI 1.2-3.4, figure 2.3)

The survival curves demonstrated evidence of divergence after 12 months of follow up. In this analysis, apart from testosterone other factors which significantly affected survival were age (HR1.07, 95%CI 1.05-1.11 p<0.001), pre-existing CV disease (2.1 95%CI 1.2-3.6) p=0.008) and HbA1c (1.3, 95%CI 1.1-1.4) p=0.008).

**Figure 2.3** Multi-variate adjusted survival curves for all-cause mortality comparing low and normal testosterone groups with cut off at 10.4 nmol/l for total testosterone

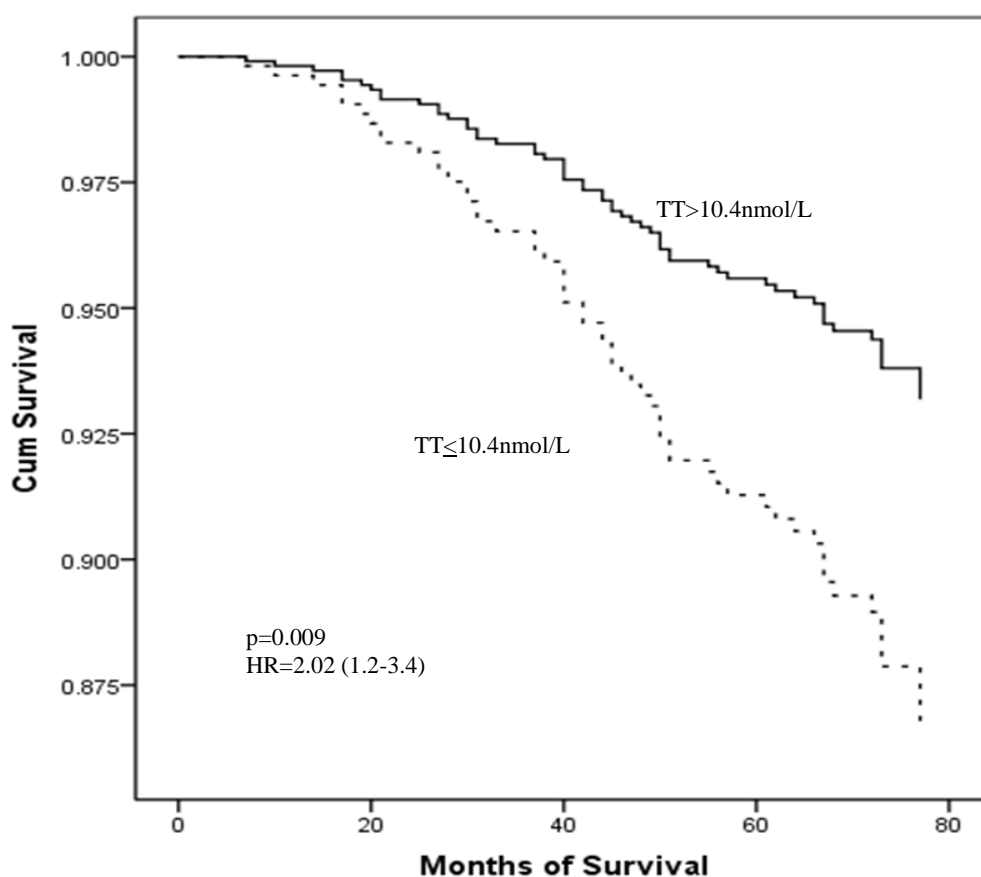


Figure 2.3 - Multi variate adjusted survival curves using cox regression model for all cause mortality based on total testosterone (TT). The solid line represents male subjects with a baseline TT>10.4nmol/L and the broken line TT≤ 10.4nmol/L. HR= hazard ratio for decreased survival after adjusting for BMI, HbA1c, Pre-existing cardiovascular disease, smoking, statin and ACEI/ARB therapy.

Further analysis of patients with a cBT cut off levels of  $< 2.6\text{nmol/l}$  (below the normal range) showed survival curves similar to that of total testosterone (2.4 95% CI 1.3- 4.6  $p=0.006$ ) (Figure 2.4).

**Figure 2.4 Multi-variate adjusted survival curves for all-cause mortality comparing low and normal testosterone groups with cut off at  $2.6\text{nmol/l}$  for bioavailable testosterone**

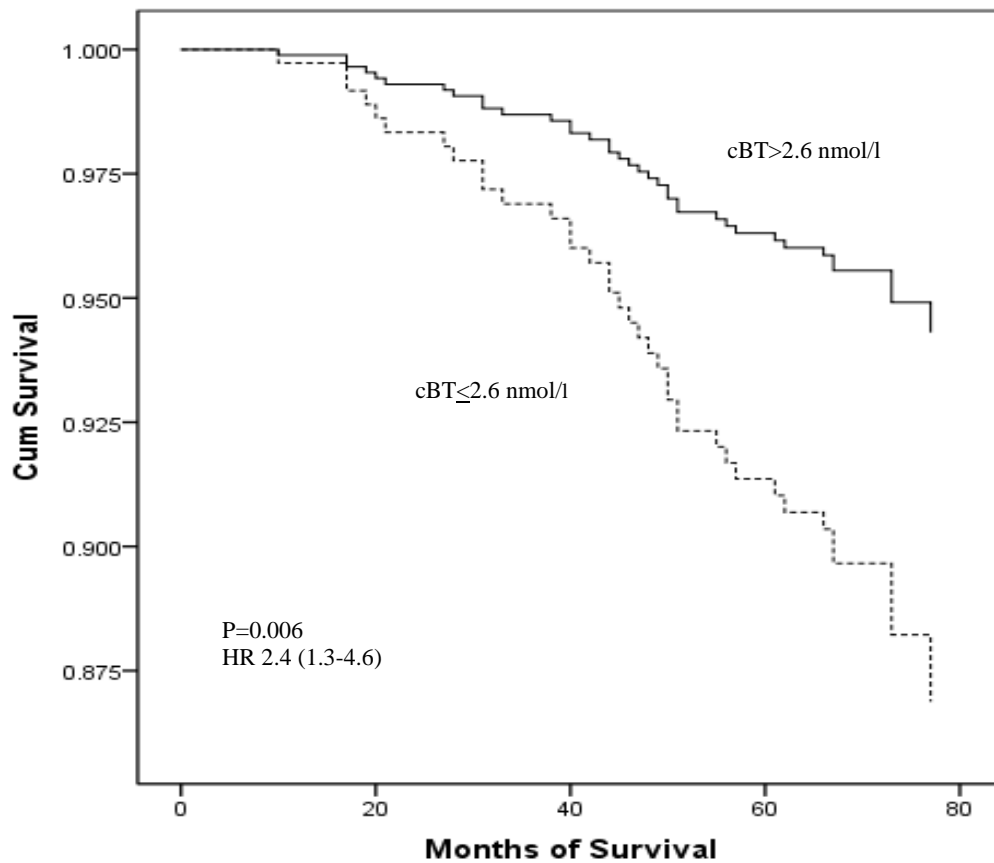


Figure 2.4 - Multi variate adjusted survival curves using cox regression model for all cause mortality based on calculated Bioavailable T(cBT). The solid line represents male subjects with a baseline  $cBT > 2.6\text{nmol/L}$  and the broken line  $TT \leq 2.6\text{nmol/L}$ . HR= hazard ratio for decreased survival after adjusting for BMI, HbA1c, Pre-existing cardiovascular disease, smoking, SHBG, statin and ACEI/ARB therapy.

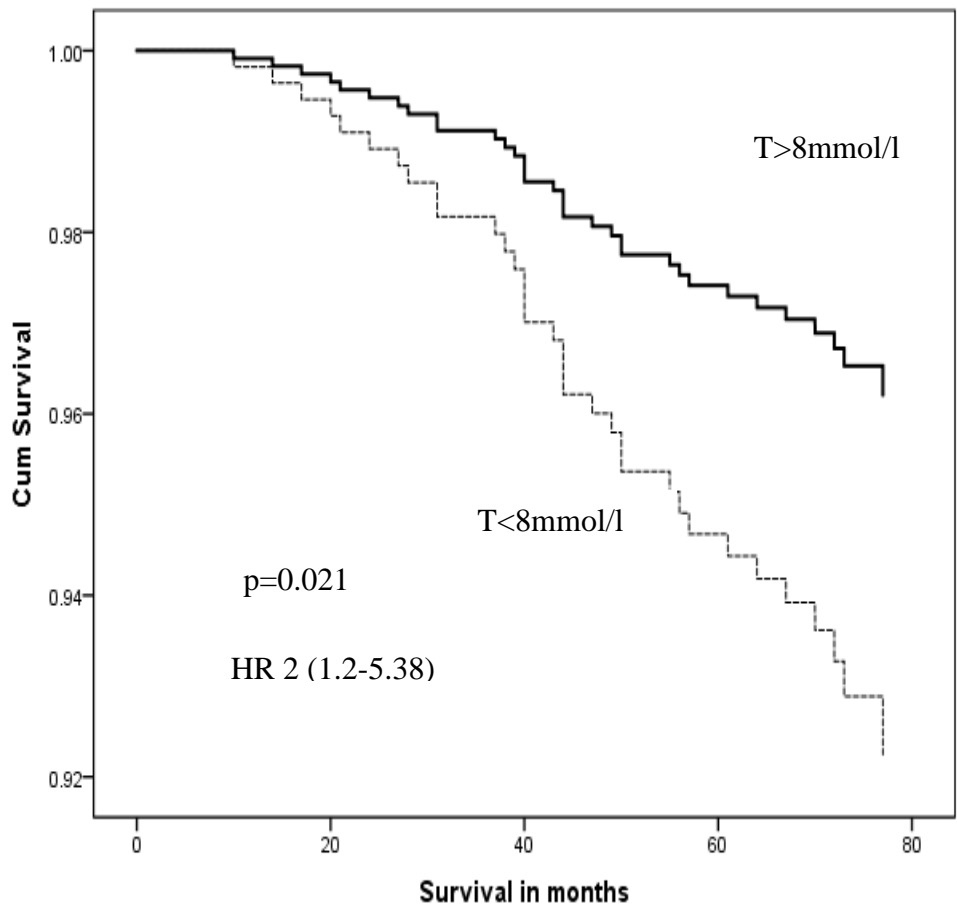
When the data were further analysed for calculated free testosterone using a cut off at 225pmol/l there was no significant difference in mortality between two groups (p=0.19). There was no significant differences in mortality were noted when analysed at different cut off values for calculated free testosterone.

### **2.6.2 Cardiovascular mortality in low and normal testosterone groups**

There were 17 cardiovascular deaths in each group. There were no significant differences between the two groups in cardiovascular and cancer mortality. However in a sub-analysis using a lower total testosterone cut off level of 8.4 nmol/l for hypogonadism (the cut off level used at the local laboratory for diagnosing hypogonadism) there was a significant increase in cardiovascular mortality in low testosterone patients when compared with that of above 8.4nmol/l with a multivariate adjusted hazard ratio was 2.5 (p=0.021 95% CI 1.2-5.38).

The higher mortality persisted after adjusting for SHBG (HR 2.2 95% CI 1.2-4 p=0.008). However when age adjustment was done along with SHBG the significance in mortality was lost but approached significance with a p value of 0.064. These results suggest an influence of age related SHBG change on the cardiovascular mortality outcome. In the co-variate analysis using the 8nmol/l cut-off for low testosterone the other factors which predicted a decreased survival were pre-existing CVD smoking and age.

**Figure 2.5 Multi-variate adjusted survival curves for cardiovascular mortality comparing low and normal testosterone groups with cut off at 8nmol/l for total testosterone**



Multi variate adjusted survival curves using cox regression model for all cause mortality based on total testosterone (TT) cut off level at 8nmol/l. HR= hazard ratio for decreased survival after adjusting for BMI, HbA1c, Pre-existing cardiovascular disease, smoking, statin and ACEI/ARB therapy.

### **2.6.3 Effect of testosterone replacement therapy on survival in men with low testosterone**

As described previously of total of 591 patients with type 2 diabetes who had testosterone levels performed between the years 2002 and 2005 were identified from the databases. We excluded deaths occurring in the first 6 months (n=4). Of these 4 patients none received testosterone replacement therapy. A further 6 patients with a normal testosterone level at screening but subsequently developed hypogonadism and received treatment (n=6) were also excluded from further. There were no deaths due pulmonary embolism reported in patients who received testosterone replacement therapy.

Three groups were analysed to assess the effect on mortality. The normal testosterone group; the hypogonadal group was further divided in to those who received testosterone treatment (the treated group) and those who did not (the untreated group). Of the 238 men with low T 64 patients (27%) received and 174 did not. Mean duration of treatment was  $41.6 \pm 20.7$  (SD) months. 60 patients received it for 12 months or more and 51 had treatment for 2 years or more. As shown in table 3 both groups were equally matched for age, weight, height, BMI, HbA1c, pre-existing CV disease, smoking status, statin, and ACEi and ARB therapy (Table 2.2).

**Table 2.2 Baseline characteristics of treated and untreated groups in low testosterone patients**

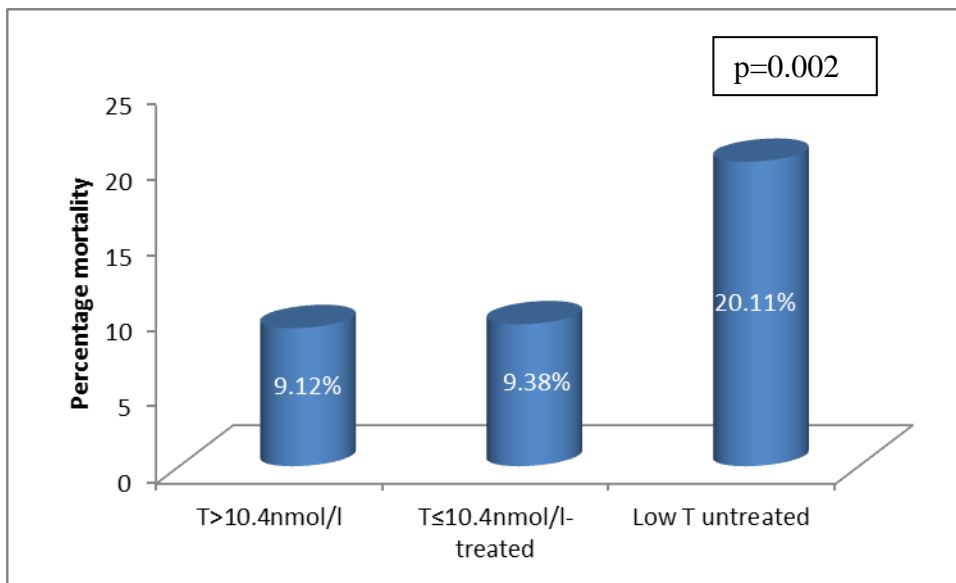
Groups Variables	Untreated	Treated	Missing numbers (% of total)	Significance p=
Number	174	64		
Mean T levels mmol/l (SD)	7.8(1.9)	6.8(2.3)	0	0.003*
Mean Age(SD)	60.9(11.8)	58.5(SD)	0	0.16*
Mean Weight(SD)	100.8(21.8)	105.4(20.1)	30(12.9%)	0.14*
Mean Height (SD)	174.3(7.2)	174.5(5.5)	29(12.5%)	0.99*
Mean BMI (SD)	33.3(6.3)	34.3(5.5)	37(15.9%)	0.31*
Mean HbA1c(SD)	7.4(1.3)	7.7(1.3)	4(1.7%)	0.21*
Statin therapy n(%) )	87(54.4%)	33(56%)	18(7.6%)	0.88†
ACEI/ARB therapy n(%) )	89(54.6%)	36(59%)	14(5.9%)	0.65†
Pre-existing CV disease n(%) )	71(44.3%)	22(36.7%)	19(8%)	0.34†
Current smokers n(%) )	27(16.8%)	10(17.2%)	17(7.3%)	0.39†

\* Analysis by t-test, † by chi ( $\chi^2$ )- squared, n(%) - number with percentage within the groups, SD- standard deviation

There was a significant increase in mortality rate (p=0.002) 20.11% (35/174) in the untreated group in comparison to 9.38% (6/64) in the treated group and 9.12% (31/340) in the normal group) figure2.6.



**Figure 2.6** Crude mortality rates in three groups of patients analysed



Kaplan-Meier curves showed a significant decrease in survival in the untreated when compared with treated and normal groups (Log rank 0.001). In the Cox regression model multivariate analysis was performed including the covariates of age, weight, height, BMI, HbA1c, pre-existing CVD, smoking status, statin, ACEi and ARB treatment. The hazard ratio for decreased survival in the untreated group (2.3, 95% CI 1.3 to 3.9, p=0.004). The survival curve for treated group followed a similar course to that of normal population (Figure 2.7). In this analysis the difference in mortality persisted after adjustment for SHBG along with age. This may be due to the change in SHBG with testosterone replacement therapy which might have an effect on long term survival.

**Figure 2.7 Multi-variate adjusted Survival curves for treated and untreated groups compared to normal with cut off at 10.4 nmol/l for total testosterone**

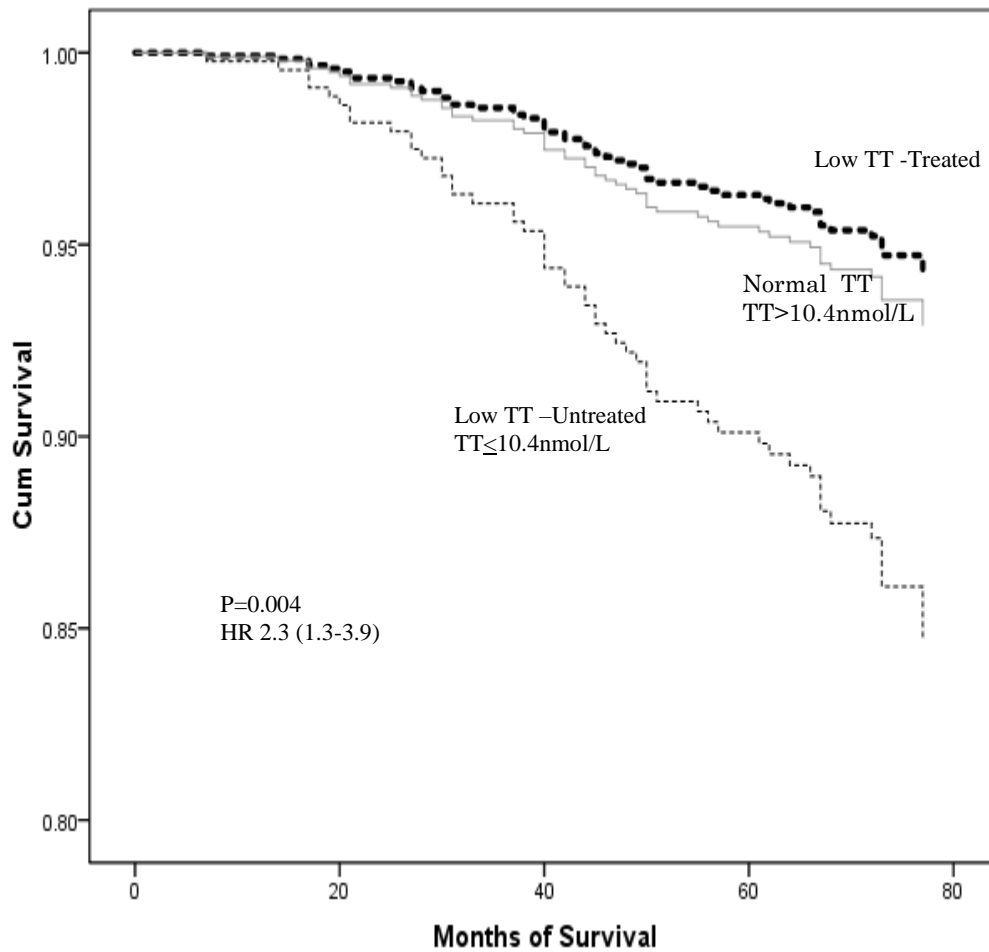


Figure 2.7- Multi variate adjusted survival curves using cox regression model for treated and untreated groups compared to those with normal testosterone. The lighter broken line represents male subjects with total testosterone (TT) levels < 10.4 nmol/l without treatment, the continuous line represent those with TT levels >10.4 and the darker broken line represent those with TT levels < 10.4 and received physiological testosterone replacement

## 2.7 Discussion and conclusion

This is the first, and so far the only one, study till now which reported the long term effect of low testosterone on survival in men with type 2 diabetes. This study demonstrated there is a decreased survival in type 2 diabetic men with concomitant low testosterone. This study further suggests a potential beneficial effect of testosterone replacement therapy on long-term survival in those with low testosterone. Importantly the increase in mortality was found to be independent of age, glycaemic control, BMI, pre-existing cardiovascular disease, current smoking status and treatment with either statins, ACEI or ARB at baseline. Apart from testosterone levels other factors which predicted increased mortality in this analysis were age, baseline HbA1c, and pre-existing CV disease. As expected, HbA1c and BMI were significantly higher in the low testosterone group. The major single cause of mortality in the study was CV disease with acute coronary artery disease accounting for the majority of cases. However, possibly due to the small numbers, increase in CV mortality in the low testosterone group was not statistically significant when a higher cut off for testosterone were used. When a lower cut off was used (8nmol/l) there was a significant increase in cardiovascular death in the low testosterone group. This suggests that a lower testosterone might increase cardiovascular death.

There was also a trend towards an increase respiratory disease mortality in the low testosterone group but significance was lost when adjusted for co-variates. This might also be due to the small number of events in that group. Although several population studies have reported an association of increased mortality with low testosterone the effect of testosterone replacement therapy has not been studied in patients with type 2 diabetes. When we reviewed those patients who received testosterone replacement therapy for one year or longer we found

a beneficial effect improving survival in men with hypogonadism. The data showed that the survival curve followed a similar course to that of the normal testosterone group whereas the untreated group had a worse prognosis. It is important to note that all patients treated with testosterone had careful adjustment of testosterone to achieve levels within the mid to upper normal physiological range for healthy men. This is the first time any study in men with type 2 diabetes has shown that testosterone replacement therapy may improve long-term survival outcome. This is especially important in men with type 2 diabetes who have a considerably reduced life expectancy.

In the recent study involving of over a 1000 male veterans over the age of 40 showed similar results of that of our study in the general population(Shores, Smith et al. 2012) . This study compared survival in treated versus untreated men with hypogonadism with a mortality of 10.3% in the treated group and 20.7% in the untreated group. There was similar rate of mortality in our study with 9.1% in the treated group compared to 20.1% in those who were not treated. This study differed from the veterans study in that we had the advantage of a control group of testosterone replete men with type 2 diabetes. In addition our study included only men with type 2 diabetes whereas the veterans study was a general population with either none or mixed co-morbidities (38% diabetes, 36% sexual dysfunction, 21 coronary heart disease). Nevertheless there are now two studies including this which concur.

## 2.8 Limitations

It is appreciated that a limitation of this study in regard to the effect of testosterone replacement therapy is that the data was collected retrospectively and that the patients were not randomised for treatment. In addition the patients treated were those who were overtly hypogonadal with symptoms and lower testosterone levels (mean baseline T untreated  $7.8 \pm 1.9 \text{ nmol/l}$  v  $6.8 \pm 2.3 \text{ nmol/l}$  treated;  $p=0.003$ ) resulting in a treatment bias. However, the fact that the hazard ratio for mortality deteriorates when the treated patients (which include those with lower testosterone) are excluded suggests that this is not the case. It is important to recognise that these findings do need to be confirmed in an appropriately designed prospective placebo-controlled trial. The findings presented here are the first to suggest a favourable outcome of testosterone replacement therapy on survival in hypogonadal men with type 2 diabetes.

It is possible that, those patients who opted for the testosterone replacement therapy perceived sexual activity more important, and hence had more sexual activity following the testosterone replacement. This could have resulted in a positive cardiovascular health benefit.

Excluding the data for the first 6 months could have resulted in missing the early adverse events from testosterone replacement. However there was no deaths related to venous thromboembolism in any of the testosterone replacement patients, even when the deaths occurred in the first 6 months are included. Statistical analysis was similar even after including those deaths occurred in the first 6 months.

There were two groups of patients in the cohort - one screened for hypogonadism as part of research and the rest were identified from hospital laboratory records. Case notes were used to

obtain baseline data at the time of screening. The cause of death from hospital records and the national registry may not always reflect the exact cause of death. However a large cohort makes this less likely to be a confounding factor. We did not find any significant difference between numbers of pre-existing CV disease in the two groups and deaths in the first 6 months were excluded from analysis. The numbers of events in the whole groups were small and we are unable to predict the median survival.



## **Chapter three**

### **Longitudinal study: Long term effect of testosterone levels on cardiovascular risk profile and cardiovascular events**

*My contribution to this chapter includes design, majority of patient recruitment, all of clinical assessment and blood sampling for the follow up patients, calculation of bioavailable and free testosterone, statistical analysis and presentation of results. As this is a follow up study, the baseline data for clinical assessment and biochemical data used for analysis is from the study by the previous researchers of the team.*

#### **3.1 Background and Introduction**

Insulin resistance and hyperglycaemia are the key characteristics of type 2 diabetes which are often associated with obesity. Testosterone deficiency in men with type 2 diabetes is associated with an adverse cardiovascular risk profile and increased cardiovascular and all-cause mortality as discussed in the recent review (Muraleedharan and Jones 2014). There is now growing evidence that low testosterone is closely linked to diabetes.

The first study to link high prevalence of hypogonadism with type 2 diabetes was from our team which reported up to 40% of patients with type 2 diabetes have low testosterone levels (Kapoor, Aldred et al. 2007). In a study of 1292 healthy non-diabetic men there was an inverse correlation between total testosterone and insulin levels independent of age and obesity (Simon, Preziosi et al. 1992). In the San Antonio Heart Study the researchers found an inverse relation between total and free testosterone with insulin levels (Haffner, Karhapaa et al. 1994).



A large reported data from nearly 4000 men confirmed that there is a high prevalence of low testosterone levels in men with diabetes and/or metabolic syndrome (Ding, Song et al. 2006). There are also studies which suggest that low testosterone may be a precursor of development of diabetes or insulin resistance (Haffner, Shaten et al. 1996, Stellato, Feldman et al. 2000, Oh, Barrett-Connor et al. 2002, Laaksonen, Niskanen et al. 2004, Kupelian, Page et al. 2006). The third National Health and Nutrition Survey (NHANESIII) found that men in the lowest tertile of free or bioavailable testosterone are more likely to have diabetes when compared to the upper tertile after adjustment for age and obesity (Selvin, Nelson et al. 2007).

Testosterone replacement therapy has been shown to improve insulin sensitivity and glycaemic control. The first double-blind placebo controlled study was published by Kapoor et al (Kapoor, Goodwin et al. 2006). In this study testosterone treatment resulted in a significant improvement in the HOMA-IR, fasting blood sugar, fasting insulin and HbA1c, waist circumference and total cholesterol. Another important study is by Heufelder et al (Heufelder, Saad et al. 2009) investigating the effects of testosterone replacement therapy in men with type 2 diabetes. The results showed after 52 weeks of testosterone treatment resulted in a statistically significant improvement in HbA1c and a significant reduction in the HOMA-IR index. There was also reduction in waist circumference, serum levels of triglycerides with a significant increase in serum levels of HDL-C.

The TIMES2 (Testosterone replacement in men with Metabolic Syndrome or type 2 diabetes) was a large multi-centre, randomised, double-blind, placebo-controlled study of over 200 men with type 2 diabetes and/or metabolic syndrome (Jones, Arver et al. 2011). The results showed insulin resistance, as assessed by HOMA-IR, improved after 6 and 12 month therapy. There was also improvement in the sexual function, reductions in percentage body fat, total

and LDL cholesterol and lipoprotein a after 6 months. Although these studies showed short term improvement in glycaemic control and components of metabolic syndrome, there are no studies investigating the long term effect of testosterone on diabetes control and metabolic syndrome.

Sex Hormone Binding globulin (SHBG) is specific binding protein which binds to testosterone and oestradiol regulating their availability in the circulation. Traditionally it is considered as a carrier protein. But recent studies have highlighted a potential biological role of its own with potential therapeutic implications (Wang, Kangas et al. 2015). There are many studies linking lower circulating concentrations of SHBG and higher insulin resistance, presence of metabolic syndrome and higher risk for type 2 diabetes (Ding, Song et al. 2006, Chubb, Hyde et al. 2008, Wallace, McKinley et al. 2013). A more recent study has shown that a lower SHBG strongly predict insulin resistance and diabetes, and a higher circulating SHBG is favourably linked with a wide range of cardio-metabolic risk factors, including lipoprotein lipids and subclasses, fatty acids, amino acids and inflammation-linked glycoproteins (Wang, Kangas et al. 2015). There are no interventional studies to see if these associations reflect a causal relationship between SHBG and the cardio-metabolic risk profile or if it merely reflects the altered sex hormone profile which in turn leads to the changes in cardiovascular risk profile. Also there is no long term study looking into the effect of SHBG levels in the cardiovascular profile of patients with type 2 diabetes.

### **3.2 Hypothesis**

In this study I hypothesise that low testosterone levels have a long term adverse effect on cardiovascular risk profile. The secondary hypothesis is that low SHBG is associated with adverse cardiovascular risk profile in the long term.

### **3.3 Research Design and Methods**

This study is a follow up of the cohort of patients from our previous study (Kapoor, Aldred et al. 2007). The initial cross-sectional study of 355 men over the age of 30 with type 2 diabetes was conducted at Barnsley Hospital NHS Foundation Trust, Barnsley, UK. The study was approved by the South Yorkshire Research Ethics Committee. Subjects for the original cohort were recruited from their attendance at the district wide Retinopathy Screening Service as well as hospital clinic appointments at the Centre for Diabetes and Endocrinology and gave informed consent. The study population contained patients with diabetes usually managed in primary as well as secondary care. One man was of Arabian ethnicity; all the others were white Caucasian.

The cardiovascular events and risk profile study is 7-year follow up (mean 84+11 months) of 203 patients from the cohort described in the previous chapter used for the mortality study. . The remaining subjects died, lost to follow up, moved out of area or declined the invitation for review. The study was approved by the South Yorkshire Research Ethics Committee. The data were analysed for different cut off levels for testosterone as in the mortality study. The groups analysed were,

1. Total testosterone cut off at 10.4 nmol/l as per the Endocrine society guidelines (Bhasin, Cunningham et al. 2010)
2. Total testosterone cut off at 8nmol/l as used in most UK laboratories
3. Bioavailable Testosterone cut off of 2.6nmol/l as this is one of the accepted levels of bio available testosterone below which it is considered as hypogonadal range and
4. Calculated free testosterone level of  $\leq 225$ pmol/l.

All blood samples were taken between 0800-1000h. Similarly SHBG was measured by solid phase enzyme immunoassay (DRG Instruments GmbH, Germany) for the research patients and solid phase two site chemiluminescent enzyme immunometric assay (Siemens Immulite, Siemens Medical Solutions Diagnostics).

All the patients who received were initiated and monitored through the department of diabetes and endocrinology. All patients from the initial research cohort who had hypogonadism were offered testosterone replacement therapy if there were no contraindications. The reasons for not having testosterone replacement therapy included patient choice, lower cut off for diagnosis of hypogonadism till recent years 8nmol/l as compared to 10.4 nmol/l cut-off used in this study) a concomitant diagnosis of prostate cancer.

Patients were treated with either testosterone gel preparations (1% testosterone gel Testogel®/AndroGel® or 2% testosterone gel via a metered pump; Tostran®/Fortigel®) or intramuscular depot testosterone undecanoate (Nebido®). Dose adjusted to give testosterone levels within the mid to upper normal range.

Patient compliance was good as evidenced by the progressive improvement in the testosterone levels in most of the patients during the follow up visits. Patients received routine clinical

management for their glycaemic, lipid and blood pressure control in both primary and secondary care.

Baseline data for co morbidities, concomitant medication, anthropometric measurements and biochemical results were available from the research database. The remaining data was obtained from the hospital records. Bioavailable testosterone (BT) and free testosterone (FT) were calculated using validated mathematical formulae (Vermeulen, Verdonck et al. 1999, Morris, Malkin et al. 2004).

### **3.4 Clinical and biochemical assessment**

#### **3.4.1 Recording of demography, medical history and drug history**

Age, medical history and drug history were specifically recorded at the start of the study. This was confirmed with details in the medical notes where possible and in the event of any doubt the patient's GP was contacted, with their permission, to confirm details. For those patients who were added to the study the above data were collected from the hospital records.

#### **3.4.2 Measurement of weight and body composition**

Height was measured after removal of shoes. Participants stood with the backs of their ankles, gluteal region and shoulders touching a wall which had a calibrated scale attached. A horizontal plane was applied to the crown of the head to be read against the scale.

Weight and body composition were assessed using Tanita BF-300 body fat analyser (Tanita Corporation, Japan). 'Standard Male' body type measurement was selected and the participants height and age were entered. After removing outer clothing, shoes and socks participants were asked to step onto the weighing platform so the plantar surface of heels and forefeet were in firm contact with the posterior and anterior electrodes respectively. The device measures weight directly and measures bioelectrical impedance by applying a 50 kHz, 500mA electrical current between the feet to derive body fat percentage on the principle that fat has lower electrical conductivity compared to other body tissues. This method has been validated against under water weighing for body composition analysis (Cable, Nieman et al. 2001). Body fat mass was calculated by multiplying weight by body fat percentage. Lean body mass was calculated by subtracting body fat mass from weight.

### **3.4.3 Measurement of waist and hip circumference**

Waist circumference was measured at the point midway between the iliac crest and the costal margin. Hip circumference was measured at the widest point around the buttocks. Waist-hip ratio was calculated by dividing waist circumference by hip circumference.

### **3.4.4 Measurement of blood pressure**

Blood pressure was measured using Colin Press-Mate BP8800C non-invasive blood pressure monitor (Colin Medical Instruments Corporation, Japan). Measurements were taken by placing the appropriately sized cuff around the upper arm such that the pressure sensor was applied to the skin close to the brachial arterial pulse to derive blood pressure from an automated oscillometric technique.

## **3.5 Biochemical measurements**

### **3.5.1 Sampling technique**

In the cross-sectional study samples were taken between 0800 and 1000h at a fasting state (8hours minimum). Smokers were asked not to smoke on the morning of an assessment visit. Venous blood was drawn by venepuncture from the antecubital fossa. Samples were taken in appropriate bottles and mixed by inverting the bottles three times. After the sample had clotted serum was obtained by centrifugation (10 minutes at 3500 revolutions per minute) and immediately frozen at  $-20^{\circ}\text{C}$  pending further analysis. Patients were asked to provide a first morning urine sample.

### **3.5.2 Measurement of samples in Barnsley hospital clinical chemistry laboratory**

Measurement of total testosterone was done using competitive chemiluminescence assay (Bayer Advia Centaur- Siemens Medical Solutions Diagnostics). The SHBG measurement was done by solid phase two site chemiluminescent enzyme immunometric assay (Siemens Immulite, Siemens Medical Solutions Diagnostics).

Measurement of total cholesterol, HDL cholesterol and triglycerides was by Olympus AU640 Analyser (Olympus Diagnostics, Germany). LDL cholesterol was calculated using the Friedewald equation (Friedewald, Levy et al. 1972);  $\text{LDL cholesterol} = \text{Total cholesterol} - (\text{HDL cholesterol} + \text{triglycerides}/2.19) \text{ mmol/l}$ .

Glucose and urinary microalbumin were measured by Olympus analysers (Olympus Diagnostics, Germany). HbA<sub>1c</sub> was measured by Menarini Analyser HA8160 (Menarini

Diagnostics, Italy). Haemoglobin and haematocrit was measured by Cell-Dyn 4000 analyser (Abbott Laboratories, USA). PSA was measured by chemiluminescent micro particle immunoassay (Abbott Laboratories, USA).

### **3.5.3 Measurement of serum bioavailable testosterone- summary**

The principle of this technique is to remove testosterone from a test sample using activated charcoal.  $^3\text{H}$ -labelled testosterone is then added to the serum on the basis that it will equilibrate between binding with SHBG and other proteins or remain unbound in the same proportions as the test sample testosterone. The SHBG bound portion is then precipitated out of the sample and the remaining concentration of  $^3\text{H}$ -testosterone is assessed using a beta-counter. The result is compared to a control sample where the SHBG bound  $^3\text{H}$ -testosterone is not precipitated and this proportion is used to calculate the bioavailable testosterone from the total testosterone which is assessed separately. The technique is adapted from that described by Tremblay and Dube (Tremblay and Dube 1974).

### **3.5.4 Measurement of serum bioavailable testosterone- method**

Samples were allowed to come to room temperature. 400  $\mu\text{l}$  of sample was dispensed into each of three Eppendorf tubes comprising duplicate test samples and a single control. 400  $\mu\text{l}$  of activated charcoal was added to each Eppendorf tube and samples incubated at room temperature for 30 minutes. At the completion of incubation samples were spun in a centrifuge at 3500 rpm for 20 minutes at 4°C. 200  $\mu\text{l}$  of the resulting supernatant was



dispensed into new Eppendorf tubes and 50 µl <sup>3</sup>H-testosterone solution added. The samples were applied to a vortex to ensure good mixing, incubated for 120 minutes at 37°C and then chilled on ice. 200 µl cold, saturated ammonium sulphate solution was added to the test samples with 200 µl saline added to the control. The samples were mixed with a vortex and then spun in a centrifuge at 3500 rpm for 20 minutes at 4°C. 200 µl of the resulting supernatant was dispensed into counting vials and 3 ml of OptiPhase Hisafe 2 scintillation fluid (Fisher Scientific, UK) added. Activity of the samples was measured by Beta counter and was proportional to the <sup>3</sup>H-testosterone content. The percentage of bioavailable testosterone was calculated according to the formula.

% Bioavailable testosterone = Average of duplicate sample (dpm) x 100/ control (dpm).

Total testosterone was measured by ELISA (see section 2.3.2.1) and the concentration of bioavailable testosterone was then calculated as follows;

Bioavailable testosterone = % Bioavailable testosterone x Total Testosterone/100

### 3.5.5 Calculation of free and bio available testosterone

Due to the considerable technical challenge concerning accurate measurement of free testosterone formulae have been developed which aim to estimate free sex hormone levels based on total serum levels of testosterone, and SHBG. In our studies we used the formula of Vermeulen to calculate free testosterone (Vermeulen, Stoica et al. 1971) was calculated from SHBG and TT by the formula of Sodergard et al (Sodergard, Backstrom et al. 1982).

Formula for free testosterone (Vermeulen, Stoica et al. 1971);

Free testosterone =  $(-b + \sqrt{b^2 + 4a [\text{Total Testosterone}]}) / 2a$

$A = K_{at} + K_{st} + (K_{at} \times K_{st})([\text{SHBG}] + [\text{Albumin}] - [\text{Total Testosterone}])$

$$B = 1 + K_{st}[SHBG] + K_{at}[Albumin] - (K_{at} + K_{st})[Total\ Testosterone]$$

$K_{at}$  = Affinity constant for testosterone with albumin =  $3.6 \times 10^4$  in this equation  $K_{st}$  =

Affinity constant for testosterone with SHBG =  $1 \times 10^9$  in this equation.

The equation used to calculate bioavailable testosterone was from Morris et al (Morris, Malkin et al. 2004)

$$\ln \text{ Bioavailable Testosterone} = -0.266 + (0.995 \times \ln \text{ Total Testosterone} ) - (0.228 \times \ln SHBG)$$

$\ln = \log$  Normal.

Measurements using different formulae for calculation of the total and free testosterone revealed similar results, although the absolute values were different as noted in studies (De Ronde, Van der Schouw et al. 2006).

### **3.6 Statistical analysis**

We examined the cohort in three groups based on the testosterone levels at different cut off levels as mentioned above. Group 1 is low testosterone patients, group 2 with normal testosterone levels and group 3 those who had testosterone replacement therapy since the initial screening period. To see the effect of low testosterone without the confounding effect of testosterone treatment we further analysed the data excluding those who had testosterone replacement therapy during the follow up (n=45).

To see the effect of SHBG on the cardiovascular risk profile a correlation analysis was done with baseline SHBG and follow up HbA1c. Further the SHBG was divided into quartiles and analysed for associations with the different cardiovascular risk profile.

Baseline data were summarized as mean  $\pm$  standard deviation. The statistical analysis was done using SPSS software, general linear model and repeated measures. Data was analysed for homogeneity using Q-Q plots and adjusted by transforming to log when appropriate. Alternatively non parametric tests were used to analyse correlation between variables which are not normally distributed.

### **3.7 Results**

This cohort has 203 patients who we were able to follow up. Mean follow up period was  $84 \pm 11$  months. Mean age at baseline  $56.6 \pm 8.7$  years (range 34-75). At baseline TT was significantly correlated with the HbA1c ( $r = -0.16$ ;  $p = 0.04$  (Kapoor, Aldred et al. 2007)). Mean baseline TT  $12.4 \pm 5.1$  nmol/l; follow up  $12. \pm 6$  nmol/l ( $p = \text{NS}$ ). SHBG increased ( $30.9$  nmol/l  $+17.8$  to  $34.7$  nmol/l  $+17.1$   $p = 0.001$ ).

In the whole cohort there was a significant deterioration in glycaemic control. The HbA1c deteriorated from  $7.3 \pm 1.2\%$  to  $7.7 \pm 1.5\%$  ( $p < 0.001$ ). There were significant improvements in diastolic ( $82.9 \pm 11$  mmHg to  $77.2 \pm 11.7$  mmHg  $p = 0.000$ ) blood pressure. Lipid profile, weight, body mass index, waist and hip circumference and systolic blood pressure did not differ significantly when adjusted for age and medications. These results are summarised in table 3.1.

**Table 3.1 Baseline and follow up measurements of cardiovascular risk profile**

Factor	Whole Cohort n=203	Multivariate adjusted significance p=
HbA1c(%)- Initial	7.3(±1.2)	0.000
Follow up	7.7(1.5)*	
BMI (kg/m <sup>2</sup> )- Initial	32.4(±5.7)	0.15
Follow up	32.2(±5.8)	
Weight(kg) - Initial	99.5(±21.2)	0.224
Follow up	97.6(19.2%)	
Waist/Hip- Initial	1.01(±0.07)	0.904
Follow up	1.02(±0.08)	
WC(cm) - Initial	109.7(±13.4%)	0.944
Follow up	113(±14.6%)	
TC(mmol/l) - Initial	5(±1)	0.576
Follow up	3.9(±0.9)	
LDL-C(mmol/l) - Initial	2.7(±0.8)	0.321
Follow up	1.9(±0.6)	
HDL-C(mmol/l) - Initial	1.1(±0.3)	0.622
Follow up	1.1(±0.3)	
TG(mmol/l) - Initial	2.8(±2.3)	0.827
Follow up	2.1(±2.7)	
SBP(mmHg) - Initial	143.4(±19)	0.727
Follow up	136.9(±17)	
DBP(mmHg) - Initial	82.9(±11.4)	0.023
Follow up	77.4(±11.7)*	

BMI Body Mass Index; Waist/ Hip- Waist hip ratio; WC- waist circumference; TC- Total Cholesterol; LDL-c- Low density lipoprotein Cholesterol; HDLC- High density Lipoprotein Cholesterol; TG- Triglycerides; SBP- Systolic Blood pressure; DBP-Diastolic Blood pressure. \*Significant at P<0.05, after adjusting for medications and age.

### 3.7.1 Cardiovascular Medications

In the whole cohort 156 patients (77.2%) were on metformin, 44 (21.8%) on sulphonyl urea, 89 (44.1%) on insulin, 39 (19.5%) on gliptins, 179 (88.6%) on statins, 132 (65.3%) on aspirin, 151 (74.8%) on ACE/ ARB, and 69 (34.2%) on calcium channel blockers.

The normal T group were more likely to be on insulin (60%) as compared to the low T group (42.1%) and normal T group (37%), (  $p=0.056$ ). Conversely this group were less likely to be on Sulphonylureas (8.9% vs. 21% and 30% respectively  $pp=0.055$ ) although both were not statistically significant. There were no significant differences in the use of Metformin, Statins, Aspirin, ACE, ARB, Thiazolidinediones, and Calcium Channel Blockers. The results are summarised in table 3.2.

**Table 3.2 Cardiovascular medications in the three groups ( low T, normal T and TRT)**

Medications	Whole cohort	Group1 (T<8nmol/l) n=19	Group2 (T>8nmol/l) n=139	Group3(TRT) 45	p=
Metformin	156(77.2%)	14(73.7%)	103(74.1%)	39(86.7%)	0.125
Sulphonyl urea	44(21.8%)	6(30%)	34(24.5%)	4(8.9%)	0.055
Glitazones	39(19.5%)	4(21.1%)	26(18.7%)	9(20.9%)	0.912
Insulin	89(44.1%)	6(31.6%)	57(41%)	27(60%)	0.056
Statins	179(88.6%)	17(89.5%)	122(87.8%)	41(91.1%)	0.843
Aspirin	132(65.3%)	11(57.9%)	88(63.3%)	33(73.3%)	0.282
ACE/ARB	151(74.8%)	12(63.2%)	99(71.2%)*	40(88.9%)*	0.015
CCB	69(34.2%)	6(31.6%)	45(32.4%)	19(42.2%)	0.084

CCB - Calcium Channel Blockers; ACE- Angiotensin converting Enzyme Inhibitors; ARB - Angiotensin Receptor Blockers. \*significant at  $p < 0.05$  between groups

### 3.7.2 Hospital admission and cardiovascular events

47.8% of the patients had one or more hospital admissions during the follow up. 14.4% had a myocardial infarction and a further 23.5% had a diagnosis of ischaemic heart disease. There was no significant difference between the groups in the number of hospital admissions or the cardiovascular events. Table 3.3 summarises the events and hospital admissions.

**Table 3.3 Hospital admissions and cardiovascular events**

Event	Whole Cohort n=203	Group1(T<8nmol/l) n=19	Group2(T>8nmol/l) n=139	Group3 () n=45	p=
Hospital Admissions	96(47.8%)	8(42.1%)	68(49.3%)	20(45.5%)	0.793
MI	29(14.4%)	3(15.8%)	22(15.9%)	4(8.9%)	0.522
Other IHD	47(23.5%)	6(31.6%)	29(21.2%)	12(26%)	0.484
CVA	14(6.7%)	1(5.3%)	10(7.1%)	3(6.7%)	0.995
PVD	13(6.4%)	0	7(5%)	6(13.3%)	0.062
Any CVD	77(37.5%)	8(42.1%)	48(34.3%)	21(47.7%)	0.256

Numbers with percentage in brackets, MI – Myocardial Infarction; IHD – Ischaemic heart disease; CVA – Cerebro Vascular Accident; PVD – Peripheral Vascular disease; Any CVD- Patients who had ant CV events. None of the events were significantly different between groups

### 3.7.3 Effect of testosterone levels on cardiovascular risk profile during follow up

The results based on the cut of level for low testosterone at 8 nmol/l are summarised in table

3.4

**Table 3.4 Cardiovascular risk profile comparing initial and follow up data in the three groups based on total testosterone adjusted for age and SHBG**

Factor	Group1(T<8nmol/l) n=19	Group2(T>8nmol/l) n=139	Group3 ( )	p=
HbA1c(%)- Initial	6.9(±1)	7.3(±1.3)	7.5(±1.2)	0.061
	8.1(±1.7)	7.6(±1.5)	7.7(±1.4)	
BMI(kg/m <sup>2</sup> )- Initial	33.1(±5.2)	31.8(±5.9)	34.2(±4.7)	0.29
	33.5(±6.5)	31.3(±5.5)	34.7(±5.8)	
Weight(kg) - Initial	101.1(±15)	97.8(±20.4)	104.9(±15.6)	0.331
	101.1(±17.6)	95.1(±19.5)	105(±17)	
Waist/Hip- Initial	1.03(±0.08)	1(±0.07)	1.05(±0.08)	0.739
	1.02(±0.11)	1.01(±0.07)	1.06(±0.07)	
WC(cm) - Initial	112.5(±14.9)	108(±13.1)	114.7(±12.4)	0.331
	114.6(±13.7)	110.9(±14.7)	120(±12.6)	
TC(mmol/l) - Initial	5.1(±0.8)	4.9(±1)	5(±1.1)	0.705
	4.2(±1.4)	3.9(±0.7)	4(±1)	
LDL-C(mmol/l)- Initial	2.8(±0.8)	2.7(±0.7)	2.8(±1)	0.531
	2.1(±0.6)	1.9(±0.6)	1.8(±0.5)	
HDL-C(mmol/l) -Initial	1.1± (0.2)	1.1(±0.3)	1(±0.3)	0.364
	1 (±0.2)	1.2(±0.3)	1.1(±0.2)	
TG - Initial	3.2(±2.6)	2.6(±2.5)	2.9(±1.2)	0.132
	3.8(±2.5)	1.8(±1.6)	2.2(±1.7)	
SBP - Initial	139.1(±18.9)	142(±19.3)	146.2(±18.1)	0.643
	136.1(±14.2)	136.6(±18)	137.4(±16.8)	
DBP - Initial	79.2(±8.9)	83.1(±12.2)	84.2(±9.2)	0.045
	78.5(±17.5)	78(±10.7)	74.3(±11.4)*	

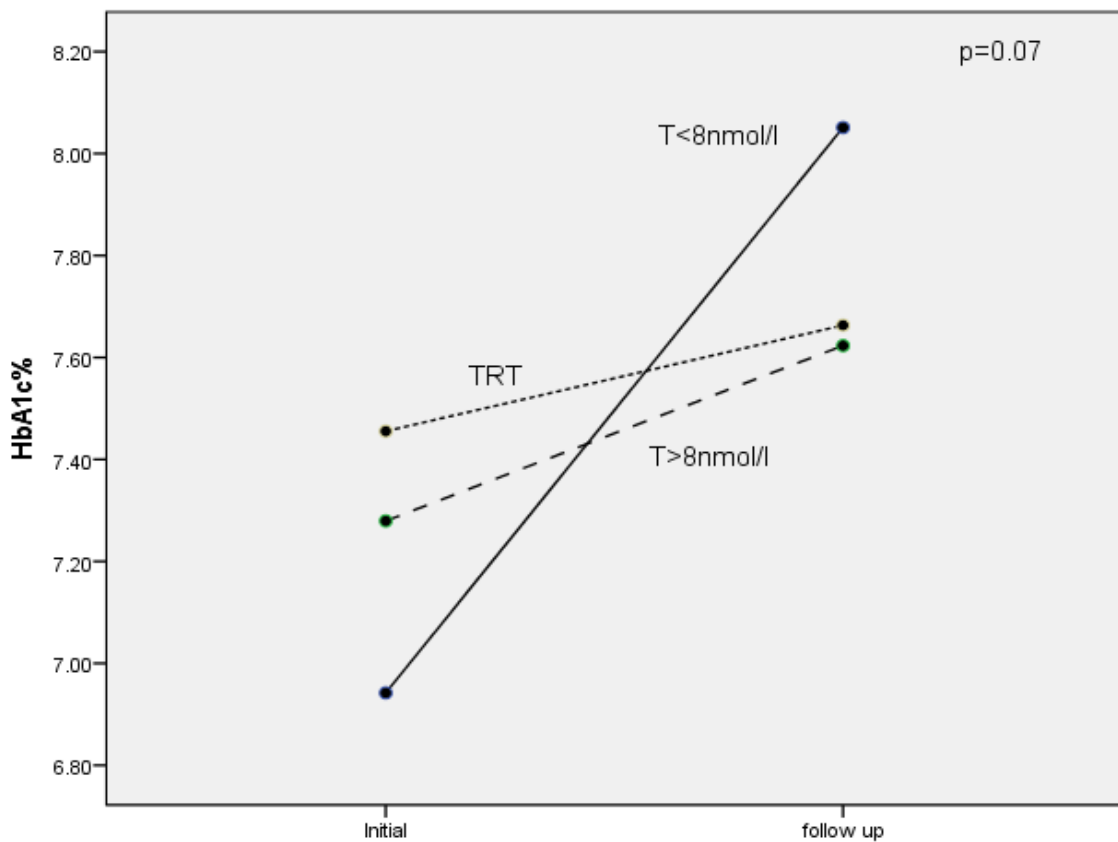
BMI- Body Mass Index; Waist/ Hip- Waist hip ratio; WC- waist circumference; TC- Total Cholesterol; LDL-c- Low density lipoprotein Cholesterol; HDLC- High density Lipoprotein Cholesterol; TG- Triglycerides; SBP- Systolic Blood pressure; DBP-Diastolic Blood pressure. \*Significant at P<0.05, after age and SHBG



### 3.7.3.1 Glycaemic control

There was a trend towards greater deterioration of glycaemic control in the low T group ( $p=0.07$  Figure 3.1) compared to the other two groups. In the low T group HbA1c deteriorated from  $6.9\pm 1.2\%$  at the base line to  $8.7\pm 1.7\%$  at the follow up as compared to  $7.3\pm 1.3\%$  to  $7.6\pm 1.5\%$  in the normal T group and  $7.5\pm 1.2\%$  to  $7.7\pm 1.5\%$  in the group after adjusting for age and SHBG. However this trend was lost when adjusted for concomitant medications.

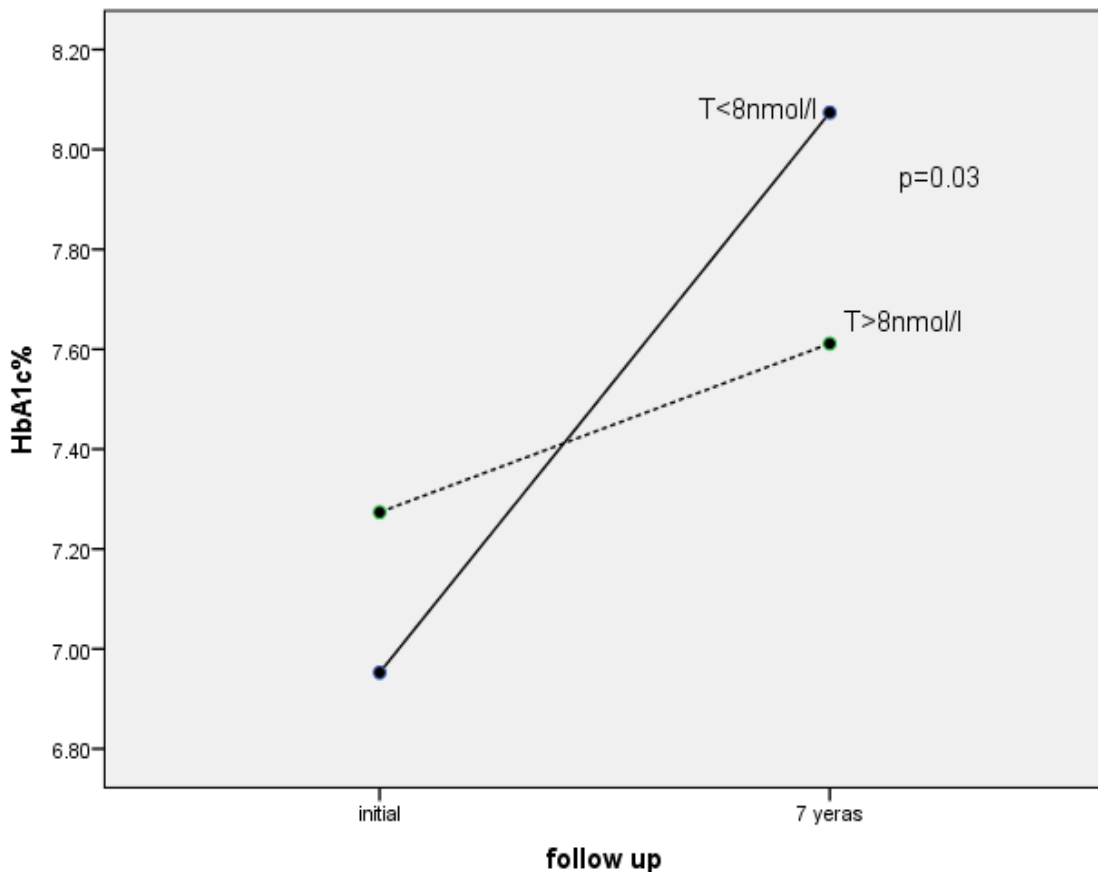
**Figure 3.1 HbA1c change during follow up in three groups**



Age adjusted changes in HbA1c during follow up in low T ( $T \leq 8\text{nmol/l}$   $n=19$ ), Normal T ( $T > 8\text{nmol/l}$   $n=139$ ) and TRT groups ( $n=45$ ) groups adjusting for age

When analysed after excluding the testosterone replacement group there was a significant negative correlation between baseline testosterone and the follow up HbA1c (correlation coefficient = -0.17, p= 0.035) and also the HbA1c difference negatively correlated with the Testosterone difference (correlation coefficient = -0.162, p= 0.046). The HbA1c deterioration was significantly worse in the low T group (6.9±1.2% to 8.7±1.7%) compared that of normal T group (7.3±1.3% to 7.6±1.5%), figure 3.2.

**Figure 3.2 HbA1c change during follow up in low and normal testosterone group at cut off 8nmol/l**



HbA1c changes for low testosterone (  $T \leq 8 \text{ nmol/l}$ ) and normal ( $> 8 \text{ nmol/l}$ ) groups, excluding those who had testosterone replacement adjusting for age, SHBG, and medications

### **3.7.3.2 Body composition**

Weight, BMI, waste circumference, waist hip ratio did not show any significant differences between the groups during follow up.

### **3.7.3.3 Lipid profile and blood pressure**

There were no significant differences between the three groups in the lipid profile. The diastolic blood pressure showed a significant improvement in the group (Low T 80.7( $\pm$ 8.8) to 73.8( $\pm$ 9.2); Normal T group 81.9( $\pm$ 9.3) to 78.3( $\pm$ 10.5); 84.2( $\pm$ 9.2) to 74.3( $\pm$ 11.4)  $p= 0.045$ ) compared to the other two groups in the age and SHBG adjusted model during the follow up. However this significance is lost when adjusted for concomitant medications.

To see the effect of different levels of total testosterone we analysed the data using the cut off of 10.4nmol/l for total testosterone. No significant difference was noted between the three groups during the follow up.

Further the data were analysed at cut off of 2.6 nmol/l for bio available testosterone (table 3.5) and 225 pmol/l for free testosterone (table 3.6). There were no significant differences in the cardiovascular risk profile in the different groups.

**Table 3.5 Cardiovascular risk profile comparing initial and follow up data within three separate groups based on the bioavailable testosterone**

Factor	Group1(BT≤2.6nmol/l) n=20	Group2(BT>2.6nmol/l)n=132	Group3 (TRT) n=45	Age adjusted significance p=
HbA1c(%)- Initial	7.3(±1.2)	7.2(±1.3)	7.5(±1.2)	0.314
Follow up	8.1(±1.8)	7.6(±1.5)	7.7(±1.4)	
BMI(kg/m <sup>2</sup> )- Initial	33.4(±6.3)	31.8(±5.8)	34(±4.8)	0.448
Follow up	33.3(±6)	31.4(±5.6)	34.5(±5.8)	
Weight(kg) - Initial	103.4(±17.5)	97.5(±20)	104.5(±15.4)	0.55
Follow up	101.3(±16.1)	95.2(±19.7)	104.6(±17)	
Waist/Hip- Initial	1.1(±0.07)	1(±0.07)	1.04(±0.08)	0.77
Follow up	1.1(±0.08)	1(±0.08)	1.06(±0.07)	
WC(cm) - Initial	113.5(±13.6)	107.9(±13.2)	114.2(±12.6)	0.376
Follow up	117.3(±12.1)	110.6(±14.7)	119.3(±13)	
TC (mmol/l)- Initial	5(±0.8)	4.9(±1)	5(±1.1)	0.278
Follow up	4.4(±1.3)	3.9(±0.8)	3.9(±1)	
LDL-C(mmol/l) - Initial	3.1(±0.9)	2.7(±0.7)	2.8(±1)	0.447
Follow up	2.3(±0.7)	1.9(±0.5)	1.8(±0.5)	
HDLC(mmol/l) - Initial	1.08(±0.2)	1.19(±0.5)	1.1(±0.2)	0.909
Follow up	1.03(±0.29)	1.11(±0.31)	1.1(±0.2)	
TG(mmol/l) - Initial	3.1(±3)	2.7(±2.5)	2.9(±2.3)	0.076
Follow up	4(±7.1)	1.9(±1.7)	2.2(±2.7)	
SBP(mmHg) - Initial	143.6(±19)	142.4(±19.4)	146(±18)	0.609
Follow up	133.6(±13.7)	136.9(±18.1)	137.7(±16.2)	
DBP(mmHg) - Initial	80.7(±8.8)	82.9(±12.3)	84.2(±9.2)	0.083
Follow up	73.8(±9.2)	78.7(±12)	74.3(±11.4)	

BMI- Body Mass Index; Waist/ Hip- Waist hip ratio; WC- waist circumference; TC- Total Cholesterol; LDL-c- Low density lipoprotein Cholesterol; HDLC- High density Lipoprotein Cholesterol; TG- Triglycerides; SBP- Systolic Blood pressure; DBP-Diastolic Blood pressure

**Table 3.6 Cardiovascular risk profile comparing initial and follow up data in the three groups based on calculated free testosterone adjusted for age**

Factor	Group1 (T≤225pmol/l) n=20	Group 2>225pmol/l) n=132	Group3 (TRT) n=45	Age adjusted significance p=
HbA1c(%)- Initial	7.2(±1.2)	7.2(±1.3)	7.5(±1.2)	0.092
Follow up	7.9(±1.6)	7.5(±1.5)	7.7(±1.4)	
BMI(kg/m <sup>2</sup> )- Initial	32.7(±5.7)	31.7(±6)	34(±4.8)	0.339
Follow up	32.7(±6.5)	31.1(±5.1)	34.5(±5.8)	
Weight(cm) - Initial	101.7(±19.6)	96.4(±19.9)	104.5(±15.4)	0.43
Follow up	100.3(±22.7)	93.6(±17)	104.6(±17)	
Waist/Hip- Initial	1.03(±0.08)	1(±0.06)	1.04(±0.08)	0.345
Follow up	1.02(±0.09)	1(±0.07)	1.06(±0.07)	
WC(cm) - Initial	111.9(±15)	106.9(±12.2)	114.2(±12.6)	0.351
Follow up	114(±16.6)	110.1(±13.2)	119.3(±13)	
TC(mmol/l) - Initial	5(±0.9)	4.9(±1)	5(±1.1)	0.803
Follow up	4.1(±1)	3.9(±0.76)	3.9(±1)	
LDL-C(mmol/l)-initial	2.8(±0.8)	2.7(±0.7)	2.8(±1)	0.449
Follow up	2.1(±0.6)	1.9(±0.6)	1.8(±0.5)	
HDLC(mmol/l) - Initial	1.1(±0.1)	1.2(±0.3)	1.1(±0.2)	0.232
Follow up	1.1(±0.3)	1.1(±0.3)	1.1(±0.2)	
TG(mmol/l) - Initial	2.8(±2.4)	2.6(±2.7)	2.9(±2.3)	0.672
Follow up	2.5(±4.4)	2(±1.8)	2.2(±2.7)	
SBP(mmHg) - Initial	145(±20.3)	141.2(±18.7)	146(±18)	0.321
Follow up	135.2(±17.2)	137(±17.8)	137.7(±16.2)	
DBP(mmHg) - Initial	84(±15.7)	81.9(±9.3)	84.2(±9.2)	0.071
Follow up	77.6(±13.8)	78.3(±10.5)	74.3(±11.4)	

BMI- Body Mass Index; Waist/ Hip- Waist hip ratio; WC- waist circumference; TC- Total Cholesterol; LDL-c- Low density lipoprotein Cholesterol; HDLC- High density Lipoprotein Cholesterol; TG- Triglycerides; SBP- Systolic Blood pressure; DBP-Diastolic Blood pressure

### **3.7.4 Effect of baseline SHBG on the cardiovascular risk profile during follow up**

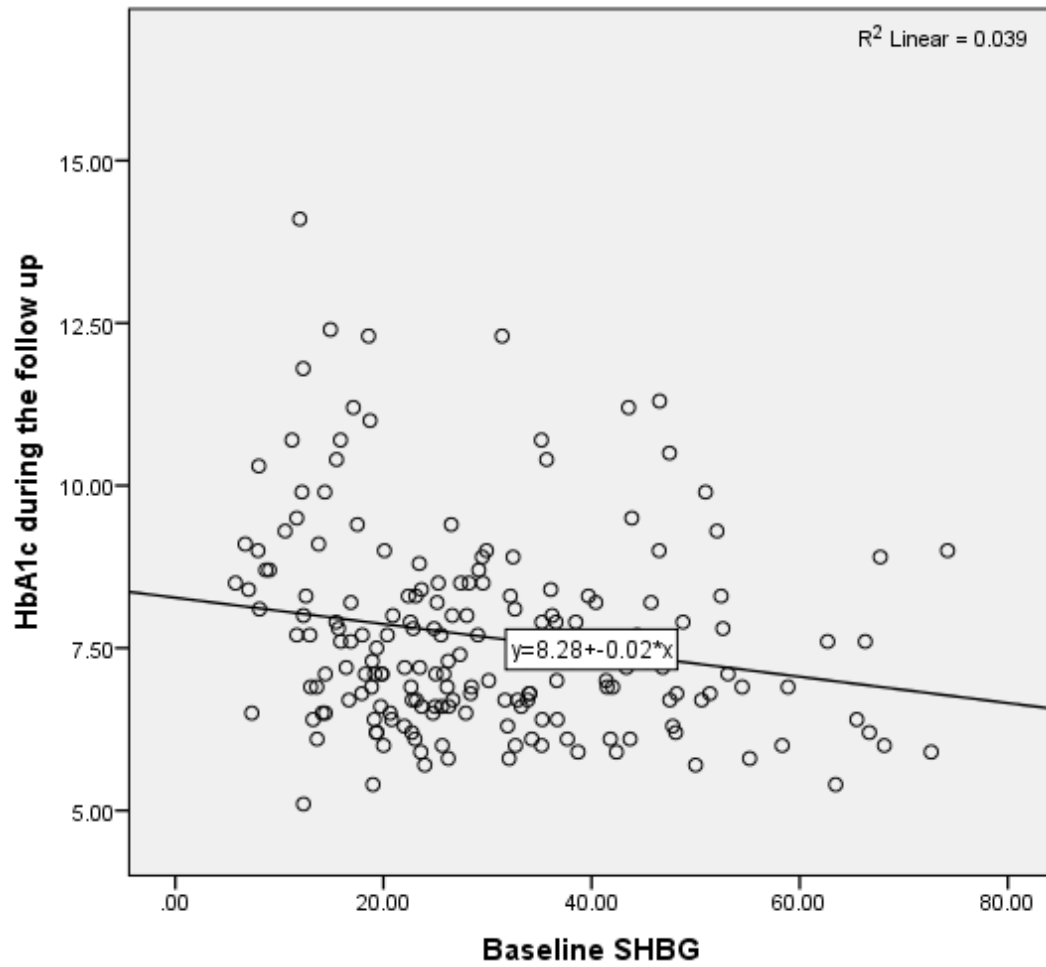
#### **3.7.4.1 Cardiovascular events**

There was no significant difference between cardiovascular events or hospital admissions between the quartiles of SHBG during the follow up.

#### **3.7.4.2 Cardiovascular risk profile in relation to SHBG levels**

There was significant negative correlation between SHBG and the follow up HbA1c (correlation coefficient = -0.194,  $p=0.007$  Figure 3.3). The data was analysed in quartiles of SHBG adjusting for co variates. These results are summarised in table 3.7.

**Figure 3.3** Correlation between baselines SHBG and follow up HbA1c



**Table 3.7 Cardiovascular risk profile in quartiles of SHBG adjusted for age and testosterone levels**

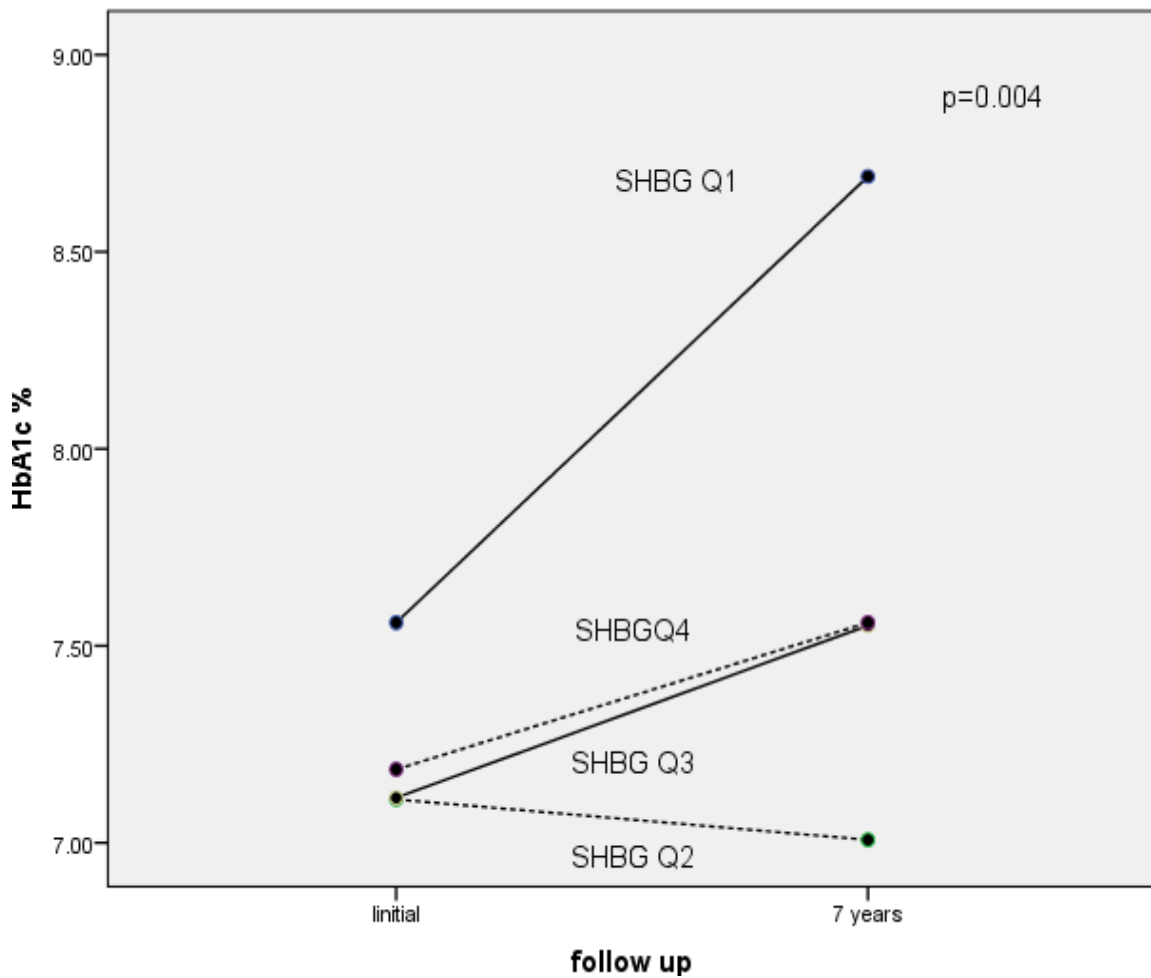
Factor	Quartile 1	Quartile 2	Quartile 3	Quartile 4	Age adjusted significance p=
HbA1c %- Initial	7.6(±1.2)	7.1(±1.1)	7.2(±1.4)	7.3(±1.2)	0.042
Follow up	8.6(±1.9)	7(±0.9)	7.6(±1.3)	7.5(±1.4)	
BMI(kg/m <sup>2</sup> )-	35.5(±7)	31(±4.1)	32.1(±4.9)	30.9(±5.3)	0.442
Initial	34.6(±6.4)	31.3(±4.9)	32(±5)	31(±6.2)	
Follow up					
Weight(kg) - Initial	107(22.8)	95(±13.5)	98.5(±18)	95(±18.4)	0.492
Follow up	105.4(±22.7)	94.8(±17)	96.6(±17.7)	93.5(±20.5)	
Waist/Hip- initial	1.04(±0.07)	1.02(±0.07)	1.02(±0.08)	1(±0.08)	0.78
Follow up	1.04(±0.1)	1.03(±0.06)	1.02(±0.08)	1(±0.08)	
WC(cm)- Initial	114.2(±15)	108.4(±10.9)	109.2(±13.1)	107.2(±13.8)	0.7
Follow up	117(±15.2)	111.8(±12.2)	112.5(±13.4)	110.9(±10.9)	
TC(mmol/l)- -	5.2(±0.9)	4.9(±1)	4.7(±1)	5.1(±0.9)	0.738
Initial	4.1(±1.2)	3.8(±0.6)	3.9(±0.9)	4(±0.9)	
Follow up					
LDL-C(mmol/l)- -	2.8(±0.9)	2.7(±0.2)	2.6(±0.8)	2.9(±0.7)	0.371
Initial	1.8(±0.7)	1.9(±0.4)	2(±0.6)	2(±0.5)	
Follow up					
HDLC(mmol/l)-	1.07(±0.3)	1.16(±0.3)	1.13(±0.3)	1.22(±0.26)	0.461
Initial	1.02(±0.34)	1.05(±0.27)	1.04(±0.23)	1.2(±0.3)	
Follow up					
TG(mmol/l)- -	3.3(±2)	3.2(±2.6)	2.1(±1)	2.4(±1.9)	0.119
Initial	3.4(±4.9)	2(±1.2)	1.6(±0.9)	1.6(±1.9)	
Follow up					
SBP(mmHg)- Initial	145.4(±17.6)	141.2(±18)	141.3(±18)	145.9(±21.9)	0.04
Follow up	136.7(±13.9)	139.6(±16)	136.8(±17.3)	134.6(±20.3)	
DBP(mmHg) -	85.2(±9)	80.5(±1)	81.7(±8.6)	84.4(±16.3)	0.293
Initial	79.2(±12.9)	76.6(±10.4)	76.5(±11.7)	77.3(±11.6)	
Follow up					

Quartile ranges: up to 18.9, 19 to 26.5, 26.6 to 40.1, and 40.2 and above. BMI- Body Mass Index; Waist/ Hip- Waist hip ratio; WC- waist circumference; TC- Total Cholesterol; LDL-c- Low density lipoprotein Cholesterol; HDLC- High density Lipoprotein Cholesterol; TG- Triglycerides; SBP- Systolic Blood pressure; DBP-Diastolic Blood pressure



When analysed with SHBG quartiles (quartile ranges: up to 18.9, 19 to 26.5, 26.6 to 40.1, 40.2 and above) the HbA1c deterioration was significantly worse in the lower SHBG quartile (Q1=7.6(±1.2) to 8.6(±1.9); Q2 =7.1(±1.1) to 7(±0.9); Q3=7.2(±1.4) to 7.6(±1.3); Q4=7.3(±1.2) to 7.5(±1.4) p=0.042, Figure 3.4) as compared to the other 3 quartiles. The significance persisted after adjusting for age, testosterone levels and concomitant medications.

**Figure 3.4 HbA1c Change during the follow up in groups based on SHBG quartiles**



SHBG quartile ranges: Q1= up to 18.9, Q2=19 to 26.5, Q3= 26.6 to 40.1, Q4= 40.2 and above

Systolic blood pressure reduction was also significantly lower (Q1= 145.4( $\pm$ 17.6) to 136.7( $\pm$ 13.9); Q2=141.2( $\pm$ 18) to 139.6( $\pm$ 16); Q3=141.3( $\pm$ 18) to 136.8( $\pm$ 17.3); Q4= 145.9( $\pm$ 21.9) to 134.6( $\pm$ 20.3)  $p= 0.04$ ) in the highest quartile as compared to the lowest quartile. This significance persisted after adjustment for concomitant medications.

There were no significant changes in body composition, Lipid profile and diastolic blood pressure in the different quartiles.

### **3.8 Discussion**

In the study looking at the effect of testosterone on cardiovascular risk factors there was no significant difference in the testosterone levels in the whole population. But there was a significant reduction in testosterone in the testosterone naïve population during the follow-up. A further 18% patients developed testosterone levels less than 8nmol/l in the testosterone naïve group. It is likely that there are more patients who developed hypogonadism in the whole cohort but the number is difficult to ascertain as some of them were diagnosed in the follow up period with different cut-off criteria which changed during the follow up (8nmol/l at the time of screening, and changed variously to 10.4 and 12 nmol/l during the follow up period) and were offered treatment. In spite of this there was a significant number of patients developed hypogonadism during the follow up. This illustrates that duration of diabetes does have an effect on development of hypogonadism. In the whole cohort, as expected from the natural history of type 2 diabetes, there was a significant deterioration in glycaemic control. When analysed after dividing into different groups there was significant deterioration in glycaemic control in the low Testosterone group compared to the normal testosterone group. The significance only became apparent when the testosterone treated cohort was removed. This and the fact that there were no significant difference on the glycaemic control changes

in the normal T group and the testosterone treated group suggest a beneficial effect of testosterone replacement on the long term glycaemic control. As this effect seems to be independent of changes in antidiabetic medications and the weight change this suggests a direct effect of testosterone in the glycaemic control.

Lipid profile, weight, body mass index, waist and hip circumference and blood pressure did not differ significantly among the different cohorts.

This study shows SHBG has an independent effect on glycaemic control and blood pressure. Low SHBG predicted a higher HbA1c and a higher systolic blood pressure during follow up. These changes were persistent after adjusting for age, testosterone levels and concomitant medications. This suggests that SHBG is an independent risk factor for cardio-metabolic risk profile in type 2 diabetes patients on the long term follow up.

Evidence is accumulating that low testosterone is a risk factor for reduced survival in particular in relation to cardiovascular disease. This evidence is supported by long-term follow-up studies in men treated for prostate cancer with androgen deprivation therapy (ADT) compared to those treated conservatively (Keating, O'Malley et al. 2006, Levine, D'Amico et al. 2010). The majority but not all studies found an increase in cardiovascular disease, myocardial infarction, incident diabetes, life threatening arrhythmias and sudden cardiovascular death. Key cardiovascular risk factors can be adversely affected within three months of initiation of ADT (Levine, D'Amico et al. 2010). A science advisory from the American Heart Association, American Cancer Society and the American Urological Association has recommended that all patients receiving ADT should have periodic follow up

for assessment of cardiovascular risk factors and those with co-existing cardiovascular disease should have their treatment for secondary prevention optimised.

It is a common and generally accepted perception that low testosterone levels are a biomarker of ill health. However the role of testosterone deficiency in accelerating disease progression and that of replacing testosterone is not fully understood. Testosterone replacement therapy has been used to treat male hypogonadism since the late 1930's. Preparations of testosterone administration have improved over the last 15 years to allow testosterone replacement to within the normal physiological range. All patients in this study were treated by dose titration in the clinic to achieve serum levels in the mid to upper normal range. Several meta-analyses of trials of testosterone replacement therapy have not found any increase in adverse cardiovascular events in hypogonadal men (Calof, Singh et al. 2005, Haddad, Kennedy et al. 2007, Merce Fernandez-Balsells, Murad et al. 2010, Carson and Rosano 2012). Furthermore endogenous testosterone levels are not associated with cardiovascular events (Carson and Rosano 2012). One study which did report an increase in cardiovascular-related symptoms or events used twice the standard routine clinical initiation dose in frail elderly men with a high number of co-morbidities (Basaria, Coviello et al. 2010). A similar study using standard testosterone dose replacement did not report any increase in cardiovascular problems (Srinivas-Shankar, Roberts et al. 2010). Testosterone replacement to normal testosterone levels has been used in a number of studies involving men with significant cardiac disorders including chronic stable angina and moderate chronic cardiac failure for up to 12 months with no evidence of adverse effects importantly including mortality (English, Steeds et al. 2000, Malkin, Pugh et al. 2006, Caminiti, Volterrani et al. 2009, Mathur, Malkin et al. 2009). The improvement in survival in this type 2 diabetes population supports the evidence that

testosterone has a beneficial effect on health. However it is recognised that until a long-term randomised placebo controlled trial reports results a definitive answer cannot be given.

There is emerging evidence that SHBG is also a bio marker of poor cardio-metabolic risk profile. As reported in a recent study SHBG (Wang, Kangas et al. 2015) was strongly associated with multiple circulating lipids and metabolites reflecting the degree of adiposity and insulin resistance and prospectively associated with the development of insulin resistance in early adulthood. These associations remained robust after adjustment for baseline adiposity, insulin and testosterone levels. Several other studies have also linked low SHBG to development of diabetes (Lakshman, Bhasin et al. 2010, Chen, Brennan et al. 2012).

In conclusion, this longitudinal study shows long term untreated testosterone deficiency has an adverse effect on glycaemic control in type 2 diabetic patients. Although studies have linked development of type 2 diabetes and metabolic syndrome to low testosterone state, as far as we know this is the first study to show a long term adverse effect of low testosterone on glycaemic control of type 2 diabetic men on long term follow up. As there was no significant difference in the glycaemic control between the treated low testosterone patient and the normal testosterone group we also conclude that physiological testosterone replacement has a beneficial effect on long term glycaemic control.

Further a low baseline SHBG found to be predictor of poor glycaemic control on long term follow up. Again although the studies linked low SHBG to development of diabetes this is the first study to report a long-term adverse effect of low SHBG levels on the glycaemic control of type 2 diabetic men. However whether there is a causal relation between the SHBG and the glycaemic control is unknown. Further prospective studies are needed to clarify this and the potential mechanism by which SHBG influence the metabolic pathways.

### **3.9 Limitations**

As this is an observational study there is no causal relationship that could be established.

There is also possibility of confounding factors including the unavailability of the data from a number of patients who died. This group of patient are more likely to be high risk cardiovascular patients and their exclusion could have meant a significant number of events were missed. In the low testosterone group with testosterone levels below 8 nmol/l, there were only 19 patients who did not receive the replacement therapy during the follow up making the analysis and interpretation of the data regarding events and risk profile difficult.



## **Chapter four**

### **Cross-sectional Study: Effect of Testosterone on cardiovascular risk profile in men with type 2 diabetes**

*My contribution to this chapter includes design, majority of patient recruitment for reassessment from the previous cohort, all of clinical assessment, blood sampling, carotid ultrasound and measurement of carotid intima media thickness, calculation of stiffness index  $\beta$ , calculation of free and bioavailable testosterone statistical analysis and presentation of results.*

#### **4.1 Introduction**

As discussed in detail in recent reviews, low testosterone levels is associated with increased cardiovascular morbidity and mortality (Oskui, French et al. 2013, Muraleedharan and Jones 2014) and is emerging as a cardiovascular risk factor in men. It is also becoming clear that low testosterone levels are an independent risk factor for future development of type 2 diabetes and metabolic syndrome. Many recent reviews have highlighted the link between low testosterone, and insulin resistant states of metabolic syndrome and type 2 diabetes (Corona, Mannucci et al. 2009, Grossmann, Gianatti et al. 2010, Jones 2010). The inverse relation between body fat and the testosterone levels have been highlighted in many studies (Kapoor, Malkin et al. 2005). The relationship between abdominal obesity and low testosterone have been highlighted in both cross-sectional (Pasquali, Casimirri et al. 1991) and longitudinal studies (Khaw and Barrett-Connor 1992, Gapstur, Gann et al. 2002).



Furthermore studies have shown that SHBG levels are negatively correlated with waist circumference and waist hip ratio (Khaw and Barrett-Connor 1992, Gapstur, Gann et al. 2002).

Pharmacologically induced androgen deficient states in the treatment of prostatic cancer in associated with an increased incidence of diabetes (Keating, O'Malley et al. 2006), worsening glycaemic control in men with diabetes at baseline (Derweesh, DiBlasio et al. 2007), increased arterial stiffness (Smith, Bennett et al. 2001). There are a number of studies which showed a negative correlation with testosterone levels and atherosclerosis (Jones and Saad 2009, Jones 2010).

Carotid intima media thickness (CIMT) is used as a surrogate marker for cardiovascular atherosclerotic process. Plaque score, which is a measure of atherosclerosis in the carotid artery, and carotid intima media thickness are negatively correlated with testosterone levels (Demirbag, Yilmaz et al. 2005, Svartberg, Von Muhlen et al. 2006). One of the earliest evidence linking low testosterone levels and CIMT was provided by Muller et al. (Muller, van den Beld et al. 2004) CIMT of middle-aged men was documented over a 4-year period and it was found that individuals in the lowest tertile of serum testosterone concentration exhibited the greatest increase in CIMT. Subsequent studies have also described a direct inverse relationship between plasma testosterone levels and CIMT in healthy men and in men with type 2 diabetes.

Increase in LDL cholesterol is associated with an increase cardio vascular risk. HDL cholesterol, on the other hand, is protective. There are studies which showed a negative correlation between LDL-C and testosterone levels (Haffner, Mykkanen et al. 1993, Simon,

Charles et al. 1997) and positive correlation with HDL-C levels (Van Pottelbergh, Braeckman et al. 2003, Stanworth RD 2007).

SHBG has been shown to be independently associated with cardiovascular risk profile in many recent studies. This is discussed in detail earlier in chapter 3.

## **4.2 Hypothesis**

The evidence suggests a strong link between the testosterone levels and cardiovascular risk factors. This led to the hypothesis that testosterone deficiency worsens metabolic syndrome and cardiovascular risk profile in men and physiological replacement of testosterone has beneficial effect on the cardiovascular risk profile in those with low testosterone. I further hypothesised that SHBG is an independent predictor of cardiovascular risk profile in men with type 2 diabetes.

## **4.3 Research Design and Methods**

An initial cross-sectional study of 355 men over the age of 30 with type 2 diabetes was conducted at Barnsley Hospital NHS Foundation Trust, Barnsley, UK (Kapoor, Aldred et al. 2007). This is a cross sectional study of 203 patients from the cohort described in the initial study described above who attended for further review. The point data from the second visit used for the cross sectional study. . The study was approved by the South Yorkshire Research Ethics Committee. Subjects for the original cohort were recruited from their attendance at the district wide Retinopathy Screening Service as well as hospital clinic appointments at the Centre for Diabetes and Endocrinology and gave informed consent. The

study population contained patients with diabetes usually managed in primary as well as secondary care. One man was of Arabian ethnicity; all the others were white Caucasian. The data were analysed for different cut off levels for testosterone as in the mortality study. The groups analysed were

- 1) Total testosterone cut off at 10.4 nmol/l as per the Endocrine society guidelines (Bhasin, Cunningham et al. 2010)
- 2) Total testosterone cut off at 8nmol/l as used in most UK laboratories
- 3) Bioavailable Testosterone cut off of 2.6nmol/l as this is one of the accepted levels of bio available testosterone below which it is considered as hypogonadal range and 4). Calculated free testosterone level of  $\leq 225$ pmol/l.

All blood samples were taken between 0800-1000h. Similarly SHBG was measured by solid phase enzyme immunoassay (DRG Instruments GmbH, Germany) for the research patients and solid phase two site chemiluminescent enzyme immunometric assay (Siemens Immulite, Siemens Medical Solutions Diagnostics).

All the patients who received were initiated and monitored through the department of diabetes and endocrinology. All patients from the initial research cohort who had hypogonadism were offered testosterone replacement therapy if there were no contraindications. The reasons for not having testosterone replacement therapy included patient choice, lower cut off for diagnosis of hypogonadism till recent years 8nmol/l as compared to 10.4 nmol/l cut-off used in this study) a concomitant diagnosis of prostate cancer.

Patients were treated with either testosterone gel preparations (1% testosterone gel Testogel®/AndroGel® or 2% testosterone gel via a metered pump; Tostran®/Fortigel®) or

intramuscular depot testosterone undecanoate (Nebido®). Dose adjusted to give testosterone levels within the mid to upper normal range.

Patient compliance was good as evidenced by the progressive improvement in the testosterone levels in most of the patients during the follow up visits. Patients received routine clinical management for their glycaemic, lipid and blood pressure control in both primary and secondary care.

Baseline data for co morbidities, concomitant medication, anthropometric measurements and biochemical results were available from the research database. The remaining data was obtained from the hospital records. Bioavailable testosterone (BT) and free testosterone (FT) were calculated using validated mathematical formulae (Vermeulen, Verdonck et al. 1999, Morris, Malkin et al. 2004)

#### **4.4 Clinical assessment**

##### **4.4.1 Recording of demography, medical history and drug history**

Age, medical history and drug history were specifically recorded at the start of the study. This was confirmed with details in the medical notes where possible and in the event of any doubt the patient's GP was contacted, with their permission, to confirm details. For those patients who were added to the study the above data were collected from the hospital records.

#### **4.4.2 Measurement of weight and body composition**

Height was measured after removal of shoes. Participants stood with the backs of their ankles, gluteal region and shoulders touching a wall which had a calibrated scale attached. A horizontal plane was applied to the crown of the head to be read against the scale.

Weight and body composition were assessed using Tanita BF-300 body fat analyser (Tanita Corporation, Japan). 'Standard Male' body type measurement was selected and the participants height and age were entered. After removing outer clothing, shoes and socks participants were asked to step onto the weighing platform so the plantar surface of heels and forefeet were in firm contact with the posterior and anterior electrodes respectively. The device measures weight directly and measures bioelectrical impedance by applying a 50 kHz, 500mA electrical current between the feet to derive body fat percentage on the principle that fat has lower electrical conductivity compared to other body tissues. This method has been validated against under water weighing for body composition analysis (Cable, Nieman et al. 2001). Body fat mass was calculated by multiplying weight by body fat percentage. Lean body mass was calculated by subtracting body fat mass from weight.

#### **4.4.3 Measurement of waist and hip circumference**

Waist circumference was measured at the point midway between the iliac crest and the costal margin. Hip circumference was measured at the widest point around the buttocks. Waist-hip ratio was calculated by dividing waist circumference by hip circumference.

#### **4.4.4 Measurement of blood pressure**

Blood pressure was measured using Colin Press-Mate BP8800C non-invasive blood pressure monitor (Colin Medical Instruments Corporation, Japan). Measurements were taken by placing the appropriately sized cuff around the upper arm such that the pressure sensor was applied to the skin close to the brachial arterial pulse to derive blood pressure from an automated oscillometric technique.

#### **4.5 Biochemical measurements**

##### **4.5.1 Sampling technique**

In the cross-sectional study samples were taken between 0800 and 1000h at a fasting state (8hours minimum). Smokers were asked not to smoke on the morning of an assessment visit. Venous blood was drawn by venepuncture from the antecubital fossa. Samples were taken in appropriate bottles and mixed by inverting the bottles three times. After the sample had clotted serum was obtained by centrifugation (10 minutes at 3500 revolutions per minute) and immediately frozen at  $-20^{\circ}\text{C}$  pending further analysis. Patients were asked to provide a first morning urine sample.

##### **4.5.2 Measurement of samples in Barnsley hospital clinical chemistry laboratory**

Measurement of total testosterone was done using competitive chemiluminescence assay (Bayer Advia Centaur- Siemens Medical Solutions Diagnostics). The SHBG measurement was done by solid phase two site chemiluminescent enzyme immunometric assay (Siemens Immulite, Siemens Medical Solutions Diagnostics).

Measurement of total cholesterol, HDL cholesterol and triglycerides was by Olympus AU640 Analyser (Olympus Diagnostics, Germany). LDL cholesterol was calculated using the Friedewald equation (Friedewald, Levy et al. 1972);  $\text{LDL cholesterol} = \text{Total cholesterol} - (\text{HDL cholesterol} + \text{triglycerides}/2.19)$  mmol/l.

Glucose and urinary microalbumin were measured by Olympus analysers (Olympus Diagnostics, Germany). HbA<sub>1c</sub> was measured by Menarini Analyser HA8160 (Menarini Diagnostics, Italy). Haemoglobin and haematocrit was measured by Cell-Dyn 4000 analyser (Abbott Laboratories, USA). PSA was measured by chemiluminescent micro particle immunoassay (Abbott Laboratories, USA).

#### **4.5.3 Measurement of serum bioavailable testosterone- summary**

The principle of this technique is to remove testosterone from a test sample using activated charcoal. <sup>3</sup>H-labelled testosterone is then added to the serum on the basis that it will equilibrate between binding with SHBG and other proteins or remain unbound in the same proportions as the test sample testosterone. The SHBG bound portion is then precipitated out of the sample and the remaining concentration of <sup>3</sup>H-testosterone is assessed using a beta-counter. The result is compared to a control sample where the SHBG bound <sup>3</sup>H-testosterone is not precipitated and this proportion is used to calculate the bioavailable testosterone from the total testosterone which is assessed separately. The technique is adapted from that described by Tremblay and Dube (Tremblay and Dube 1974).

#### 4.5.4 Measurement of serum bioavailable testosterone- method

Samples were allowed to come to room temperature. 400 µl of sample was dispensed into each of three Eppendorf tubes comprising duplicate test samples and a single control. 400 µl of activated charcoal was added to each Eppendorf tube and samples incubated at room temperature for 30 minutes. At the completion of incubation samples were spun in a centrifuge at 3500 rpm for 20 minutes at 4°C. 200 µl of the resulting supernatant was dispensed into new Eppendorf tubes and 50 µl <sup>3</sup>H-testosterone solution added. The samples were applied to a vortex to ensure good mixing, incubated for 120 minutes at 37°C and then chilled on ice. 200 µl cold, saturated ammonium sulphate solution was added to the test samples with 200 µl saline added to the control. The samples were mixed with a vortex and then spun in a centrifuge at 3500 rpm for 20 minutes at 4°C. 200 µl of the resulting supernatant was dispensed into counting vials and 3 ml of OptiPhase Hisafe 2 scintillation fluid (Fisher Scientific, UK) added. Activity of the samples was measured by Beta counter and was proportional to the <sup>3</sup>H-testosterone content. The percentage of bioavailable testosterone was calculated according to the formula.

$\% \text{ Bioavailable testosterone} = \text{Average of duplicate sample (dpm)} \times 100 / \text{control (dpm)}$ .

Total testosterone was measured by ELISA (see section 2.3.2.1) and the concentration of bioavailable testosterone was then calculated as follows;

$\text{Bioavailable testosterone} = \% \text{ Bioavailable testosterone} \times \text{Total Testosterone} / 100$



#### 4.5.5 Calculation of free and bioavailable testosterone

Due to the considerable technical challenge concerning accurate measurement of free testosterone formulae have been developed which aim to estimate free sex hormone levels based on total serum levels of testosterone, and SHBG. In our studies we used the formula of Vermeulen to calculate free testosterone (Vermeulen, Stoica et al. 1971) was calculated from SHBG and TT by the formula of Sodergard et al (Sodergard, Backstrom et al. 1982).

Formula for free testosterone (Vermeulen, Stoica et al. 1971);

$$\text{Free testosterone} = (-b + \sqrt{b^2 + 4a [\text{Total Testosterone}]}) / 2a$$

$$A = K_{at} + K_{st} + (K_{at} \times K_{st})([\text{SHBG}] + [\text{Albumin}] - [\text{Total Testosterone}])$$

$$B = 1 + K_{st}[\text{SHBG}] + K_{at}[\text{Albumin}] - (K_{at} + K_{st})[\text{Total Testosterone}]$$

$K_{at}$  = Affinity constant for testosterone with albumin =  $3.6 \times 10^4$  in this equation  $K_{st}$  =

Affinity constant for testosterone with SHBG=  $1 \times 10^9$  in this equation.

The equation used to calculate bioavailable testosterone was from Morris et al (Morris, Malkin et al. 2004)

$$\text{Ln Bioavailable Testosterone} = -0.266 + (0.995 \times \text{Ln Total Testosterone}) - (0.228 \times \text{LnSHBG})$$

Ln=log Normal.

#### 4.6 Assessment of arterial wall parameters-carotid artery ultrasound

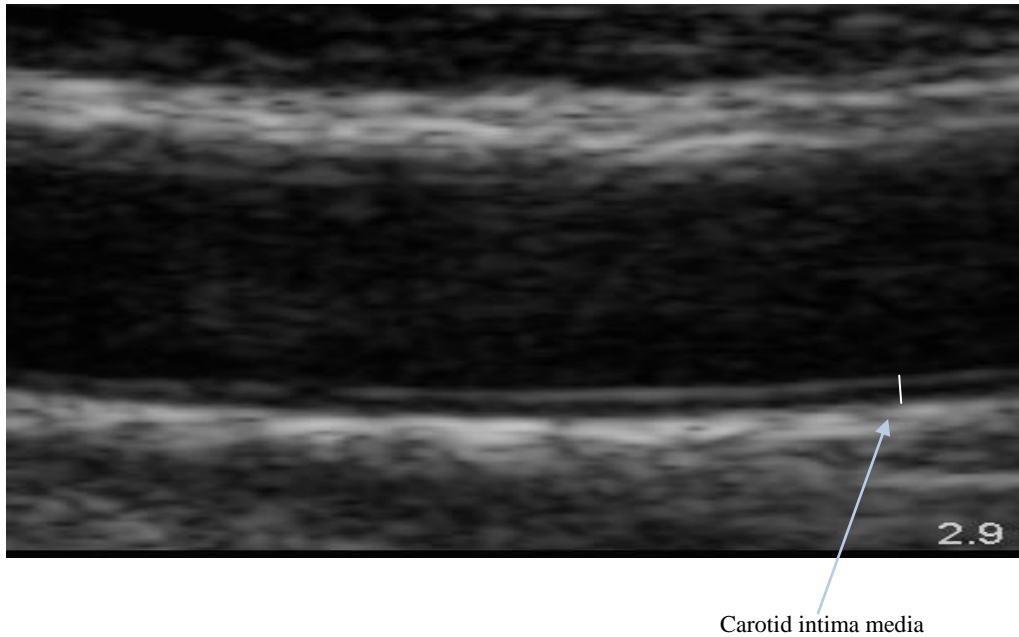
Local arterial stiffness and intima-media thickness of the common carotid arteries was assessed in the clinical studies for the longitudinal and the cross sectional studies.

Participants were examined in the supine position. With the neck slightly extended views were obtained of the common carotid artery immediately before the artery dilates to form the

carotid bulb using the Sonosite Titan High Resolution Portable Ultrasound System (Sonosite Inc., USA) and L38 Vascular/Small Parts Transducer (Sonosite Inc., USA).

Recordings of this section of artery were taken and images selected with the artery at minimal (end-diastolic) and maximal (systolic) diameter. The minimal and maximal diameters of the artery were measured and recorded. The diastolic image was uploaded into a specialised IMT measurement programme (SonoCalc IMT Version 3.0, Sonosite Inc., and USA) and a part of artery not showing evidence of overt atherosclerotic plaque was analysed for IMT. Three sets of vessel diameter and IMT measurements were taken from each common carotid artery. A typical picture of IMT scan is shown in figure 4.1

**Figure 4.1** Carotid ultrasound pictures showing the intima media



The ultrasound probe was removed from the patient between each set of images. Blood pressure was checked at the brachial artery on three consecutive occasions in the dominant arm when the ultrasound measurements were complete and the average of the three measurements was calculated.

#### **4.6.1 Carotid ultrasound Measurement for carotid Intima media thickness**

The patients were examined in the supine position with the neck slightly extended to allow optimal ultrasound views. Images were taken from the common carotid artery immediately before the artery dilates to form the carotid bulb, using the Sonosite Titan High Resolution Portable Ultrasound System (Sonosite Inc., USA) and L38 Vascular/Small Parts Transducer (Sonosite Inc., USA).

Recordings of this section of artery were taken and images selected with the artery at minimal (end-diastolic) and maximal (systolic) diameter. The minimal and maximal diameters of the artery were measured and recorded. The diastolic image was uploaded into a specialised IMT measurement programme (SonoCalc IMT Version 3.0, Sonosite Inc., and USA) and a part of artery not showing evidence of overt atherosclerotic plaque was analysed for IMT. Three sets of vessel diameter and IMT measurements were taken from each carotid artery and the ultrasound probe was removed from the patient between each set of images.

#### **4.6.2 Calculation of Carotid Stiffness index $\beta$**

Blood pressure was checked on three consecutive occasions when the ultrasound measurements were complete and average measurements was calculated. Stiffness index  $\beta$  was then calculated from the diastolic carotid artery diameter (Dd), systolic carotid artery diameter (Ds), diastolic blood pressure (BPd) and systolic blood pressure (BPs) using the formula;

Stiffness index  $\beta = (\ln(Ps/Pd)) \times Dd / (Ds - Dd)$ .

#### **4.7 Statistical analysis**

The results were analysed by dividing the patients into three groups. Group 1 - testosterone levels below 10.4nmol/l and had no testosterone replacement; Group 2 - those with testosterone levels more than 10.4nmol/l and Group 3 - had 3 or more months of testosterone replacement. To see the effect of SHBG, the correlation coefficient was estimated for the cardiovascular risk profile followed by analysis of variance using the general linear model in the quartiles of SHBG, similar to the analysis for testosterone groups. Data were analysed for

homogeneity using Q-Q plots and adjusted by transforming to log when appropriate.

Alternatively non parametric tests (Kruskal-Wallis; Mann-Whitney U) were used to test the null hypothesis. Data was analysed using SPSS statistical package (IBM SPSS version 19).

Baseline data were summarized as mean +/- standard deviation. A p value of  $\leq 0.05$  was considered significant.

## **4.8 Results**

### **4.8.1 Baseline characteristics**

There were 203 patients in the study, the average age was 63.7( $\pm 7.8$ ) years. They were divided into three groups as described above. Group 1 (Testosterone  $< 10.4$  nmol/l) had 77 patients (37.9%); Group 2 (Testosterone levels  $> 10.4$  nmol/l) 81 (39.9%) patients and Group 3 (Those who had testosterone replacement) 45 (22.2%) patients. The baseline characteristics are summarised in Table 4.1. The co-morbidities and the medications were mostly comparable in all the three groups. The normal T group had lower incidence of diagnosed peripheral neuropathy compared to the other groups ( $p = 0.01$ ). The low T group were more likely to be on insulin ( $p = 0.041$ ) and less likely to be on sulphonyl urea (0.022) compared to other two groups. The group were more likely to be on an ACE or ARB ( $p = 0.033$ ).

**Table 4.1 Co morbidities and medications with three groups (Low T, Normal T and TRT) compared**

Variables	Group1 (T<10.4nmol/l) n=77	Group2 (T>10.4nmol/l) n=81	Group3 (Those who had TRT )=45	Whole Cohort N=203	Significance 95% CI p=
Age	64.9±8.6	62.6±9.5	63.4±7.7	63.7±7.7	0.271 <sup>‡</sup>
Myocardial Infarction	14.6(18.8%)	11(13.8%)	4 (8.9%)	29(14.4)	0.345
Atrial fibrillation	7(9.1%)	4(4.9%)	2(4.4%)	13(6.4%)	0.471
Cerebrovascular Disease	3(3.9%)	8(9.9%)	3(6.7%)	14(6.9%)	0.332
Peripheral Vascular Disease	4(5.3%)	3(3.7)	6 (13.3%)	13(6.4%)	0.094
COPD	7(9.1%)	3(3.7%)	5(11.4%)	15(7.4%)	0.23
OSA	3(3.9%)	3(3.7%)	3(6.8%)	9(4.8%)	0.695
Nephropathy	16(20.8%)	10(12.3%)	9(20%)	35(17.2%)	0.32
Retinopathy	18(23.4%)	19(23.5%)	18(40%)	55(27%)	0.087
Peripheral Neuropathy	15(19.5%)	21(25.9%)	20(44.4%)	56(27.6%)	0.01
Hospital Admission	41(53.9%)	35(43.8%)	20(44.4%)	96(47.8%)	0.391
Metformin	57(75%)	60(74%)	39(86.7%)	156(77.2%)	0.229
Sulphonyl urea	23(30.3%)	17(21%)	4(8.9%)	44(21.8%)	0.022* (G1&G3)
Insulin	32(42.1%)	30(37%)	27(60%)	89(44.1%)	0.041* (G1&G3)
Statins	67(88.2%)	71(87.7%)	41(91.1%)	179(88.6%)	0.832
Aspirin	46(65.4%)	53(65.4%)	33(73.3%)	132(65.3%)	0.359
ACE/ARB	56(73.7%)	55(67.9%)	40(88.9%)	151(74.8%)	0.033 (G1&G3)

\*Significant at p=<0.05 Chi-square, <sup>‡</sup>T test, ± standard deviation, COPD-chronic Obstructive pulmonary disease; OSA-Obstructive sleep apnoea; ACE-Angiotensin converting enzyme inhibitors; ARB-Angiotensin receptor blockers;. T – Testosterone; TRT- testosterone replacement therapy.

## 4.8.2 Cardiovascular risk profile

### 4.8.2.1 Effect of Testosterone on Cardiovascular risk profile

The results are summarised in Table 4.2. In the multivariate adjusted model (adjusted for medications, smoking and co morbidities) HbA1c (Group1 =7.9±1.7%; Group2 =7.3±1.2%; Group3 =7.5±1.2%) was significantly higher in low testosterone group compared to the high T group (Figure 4.1). There were no significant differences between treated and normal T groups. There was an inverse relationship between the testosterone levels and HbA1c as illustrated in Figure 4.2 ( $R^2$  linear =0.065).

Hip circumference (Group1=114.1±14; Group2=107±9cm; Group3=112.8±10.3cm), waist Circumference (Group1=114.1±15.9cm; Group2=108.3±12.6cm; Group3 119.1±13.6cm), weight (Group1=98.2±20.Group2=91.8±4.7kg; Group3=103.9±16.5) Waist Hip ratio (Group1=1.03±0.08; Group2=1±0.06; Group3=1.06±0.08) and BMI (Group1=32.5±6; Group2=30.2±4.8; Group3=34.4±5.6) were significantly higher in the low T group. However unlike HbA1c the low T group and TRT groups were similarly higher measures. The percentage body fat was higher in the Low T group and group compared to the normal T group (Group 1=31.9±7.6%; Group2=30±6.8%; Group3=33.5±9.5% p=0.037) when adjusted for age and SHBG but the significance was lost (p=0.073) when multivariate adjustment with medication and co morbidities.

**Table 4.2 Cardiovascular risk profile among the groups three groups (Low T, Normal T and TRT) compared**

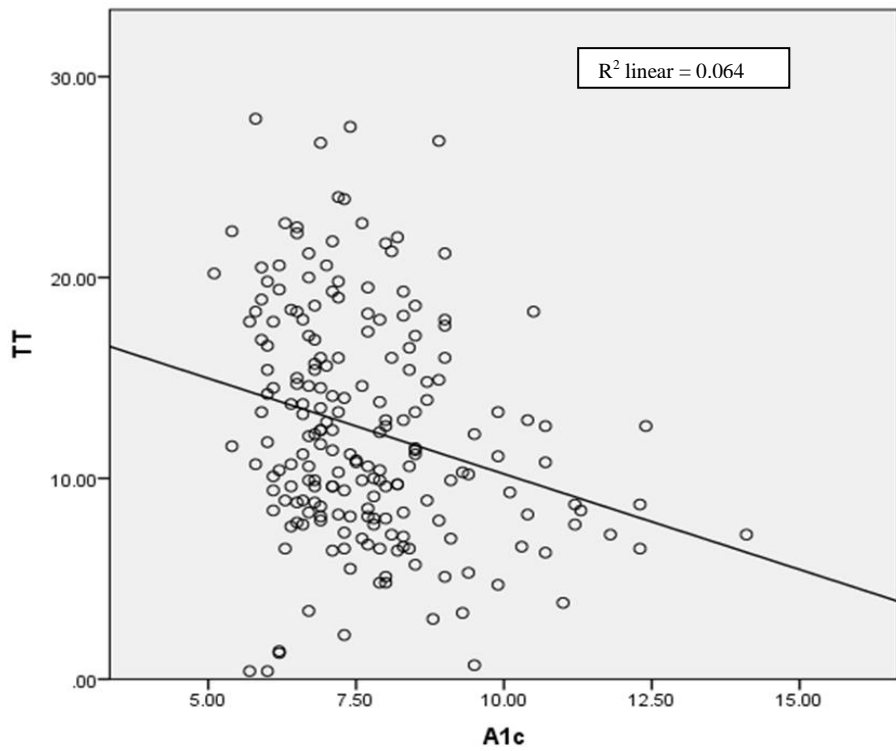
Variables	Group1 T $\leq$ 10.4 nmol/l n=77	Group2 T $>$ 10.4nmol/l n=81	Group3 Patients had TRT n=45	Whole cohort n=203	Significance p=
HbA1c*(%)	8 $\pm$ 1.7	7.2 $\pm$ 1.1	7.6 $\pm$ 1.4	7.6 $\pm$ 1.4	0.001
Weight*	98.2 $\pm$ 20.7	91.8 $\pm$ 16.8	103.9 $\pm$ 16.5	97 $\pm$ 18.8	0.006
HIP*(cm)	110.8 $\pm$ 10.2	107.7 $\pm$ 8.3	112 $\pm$ 10.33	109.9 $\pm$ 9.6	0.033
Waist*(cm)	114.1 $\pm$ 16	107.8 $\pm$ 12.1	119.14 $\pm$ 13.6	112.8 $\pm$ 14.6	0.002
Waist Hip* Ratio	1.03 $\pm$ 0.08	1 $\pm$ 0.07	1.1 $\pm$ 0.08	1.02 $\pm$ 0.08	0.019
BMI*	33.4 $\pm$ 5.9	30.1 $\pm$ 4.7	34.4 $\pm$ 5.6	32 $\pm$ 5.6	0.001
% Fat	32 $\pm$ 7.7	30 $\pm$ 6.8	33.5 $\pm$ 9.5	31.5 $\pm$ 7.9	0.073
SBP(mmHg)	135.4 $\pm$ 16.8	136.8 $\pm$ 17.1	140.6 $\pm$ 17.5	137.2 $\pm$ 17.1	0.697
DBP(mmHg)	77.2 $\pm$ 11.3	77.5 $\pm$ 10.1	75.2 $\pm$ 11.4	76.9 $\pm$ 10.8	0.517
TC(mmol/l)	3.7 $\pm$ 0.7	3.9 $\pm$ 0.6	3.8 $\pm$ 0.8	3.8 $\pm$ 0.7	0.63
HDL(mmol/l)	1.03 $\pm$ 0.2	1.2 $\pm$ 0.3	1 $\pm$ 0.2	1.1 $\pm$ 0.3	0.051
TG(mmol/l)	1.7 $\pm$ 0.9	1.7 $\pm$ 0.9	1.8 $\pm$ 0.8	1.7 $\pm$ 0.9	0.991
LDL(mmol/l)	1.9 $\pm$ 0.6	2 $\pm$ 0.5	2 $\pm$ 0.7	2 $\pm$ 0.6	0.921
AST(mmol/l)	24.6 $\pm$ 8.8	25.7 $\pm$ 8.2	28 $\pm$ 14	25.8 $\pm$ 10.1	0.238
ALT(mmol/l)	26.4 $\pm$ 14.2	29.3 $\pm$ 14.9	31.1 $\pm$ 18.3	28.6 $\pm$ 15.5	0.457

\*Significant at p= $<$ 0.05 after multivariate analysis adjusting for age, medications and smoking,  $\pm$ standard deviations, Hip- Hip circumference, Waist- Waist circumference, BMI- body mass index, % fat- Percentage body fat, SBP- Systolic Blood pressure, DBP- Diastolic Blood pressure, TC- Total cholesterol, HDL- High density lipoprotein Cholesterol, TG- Triglycerides, LDL- Low density lipoprotein cholesterol, AST-Aspartate amino transferase, ALT- Alanine amino transferase.

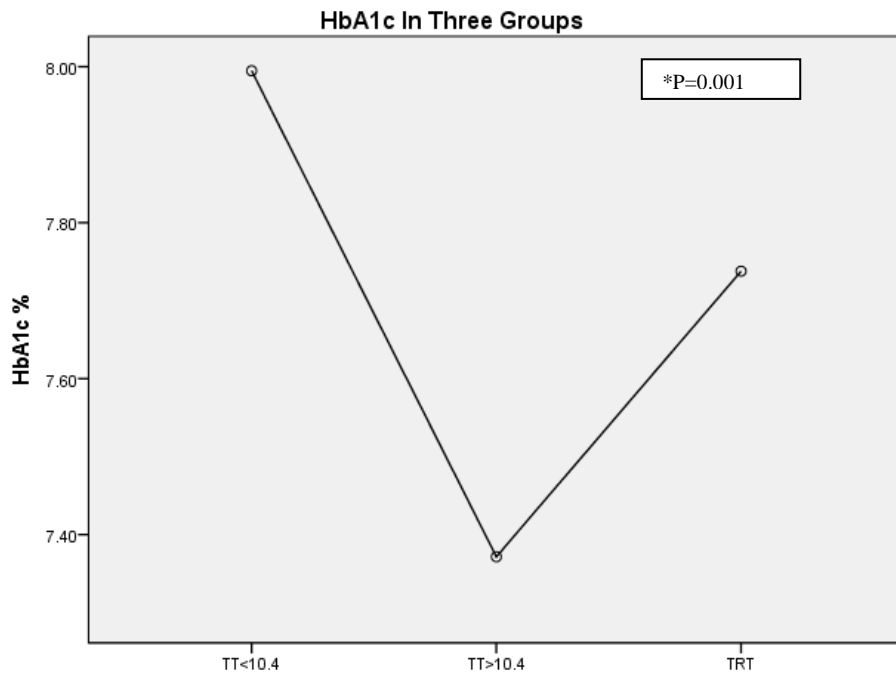


The HDL (Group1=1.03±0.2mmol/l; Group2=1.2±0.3mmol/l; Group3=1.±0.26mmol/l p=0.009) was significantly higher in Group2 when analysed adjusting for age and SHBG, but lost the significance in multivariate analysis (p=0.051). There were no significant difference in total cholesterol (Group1=3.7±0.7; Group2=3.9±0.6mmol/l; Group3=3.8±0.8mmol/l). LDL cholesterol (Group1=1.9±0.64mmol/l; Group2=2±0.5mmol/l; Group3=2±0.7mmol/l) or triglyceride (Group1=1.7±0.9mmol/l; Group2=1.6±0.9mmol/l; Group3 1.8±0.8mmol/l) levels between these groups.

**Figure 4.2 Correlation between HbA1c and total testosterone**



**Figure 4.3 HbA1c levels in the three groups of patients analysed**



HbA1c in three groups 1.low testosterone( $T<10.4 \text{ nmol/l}$ ), 2. Normal testosterone( $T>10.4\text{nmol/l}$ ) and 3.those with testosterone replacement (TRT). \*adjusted for age, medications, smoking and comorbidities

#### 4.8.2.2 Effect of SHBG on cardiovascular risk profile

Effect of SHBG on cardiovascular risk factors were analysed using quartiles (Q1- lowest Q4- Highest) of SHBG. After adjusting for age and testosterone, HbA1c, weight, waist circumference, BMI percentage fat, and triglyceride levels showed significant inverse correlation with SHBG and HDL showed a positive correlation with SHBG. This is summarised in table 4.3

HbA1c was highest in the lowest quartile and significantly ( $p=0.037$ ) lower in the highest quartile (Q1= $8.4\pm 1.5\%$ ; Q2= $7.4\pm 1.5$  Q3= $7.7\pm 1.5\%$ ; Q4= $7.2\pm 1.4\%$ ). Percentage body fat was highest in Q1 ( $35.8\pm 8.7\%$ ) and lowest in Q4 ( $29.7\pm 8.4\%$ ). Similarly weight (Q1= $108.4\pm 17.9\text{kg}$  and Q4= $92.7\pm 23.5\text{kg}$ ), BMI (Q1= $35.8\pm 6.2$  and Q4= $30.6\pm 6.4$ ), waist circumference (Q1= $119.5\pm 14.9\text{cm}$  and Q4= $109.7\pm 17.4\text{cm}$ ) and triglycerides (Q1= $3.7\pm 4\text{mmol/l}$  and Q4= $1.3\pm 0.7\text{mmol/l}$ ) showed inverse relation with SHBG levels. Conversely HDL was higher in the lowest quartile (Q1= $0.96\pm 0.2\text{mmol/l}$  and Q4= $1.2\pm 0.3$ ) There was no significant effect of SHBG on LDL, total cholesterol, ALT, AST, hip circumference, systolic and diastolic blood pressure.

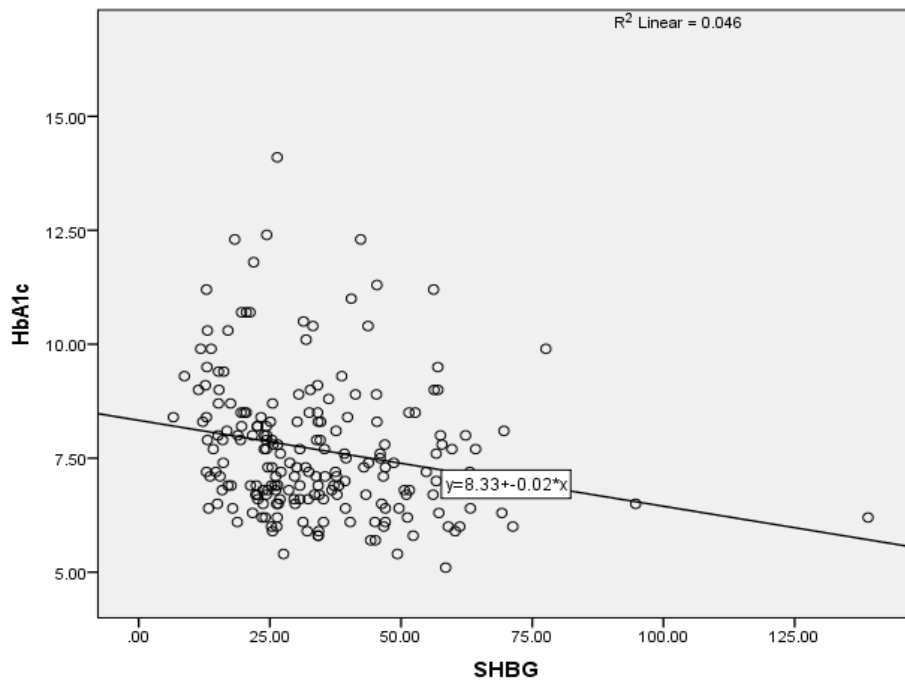
This correlation was further analysed after adjusting for concomitant medications. After adjusting for metformin, insulin, sulphonyl urea, statins, aspirin, ACE and ARB the significance persisted except for HbA1c.

**Table 4.3 Cardiovascular risk profile in SHBG quartiles adjusted for age and testosterone levels**

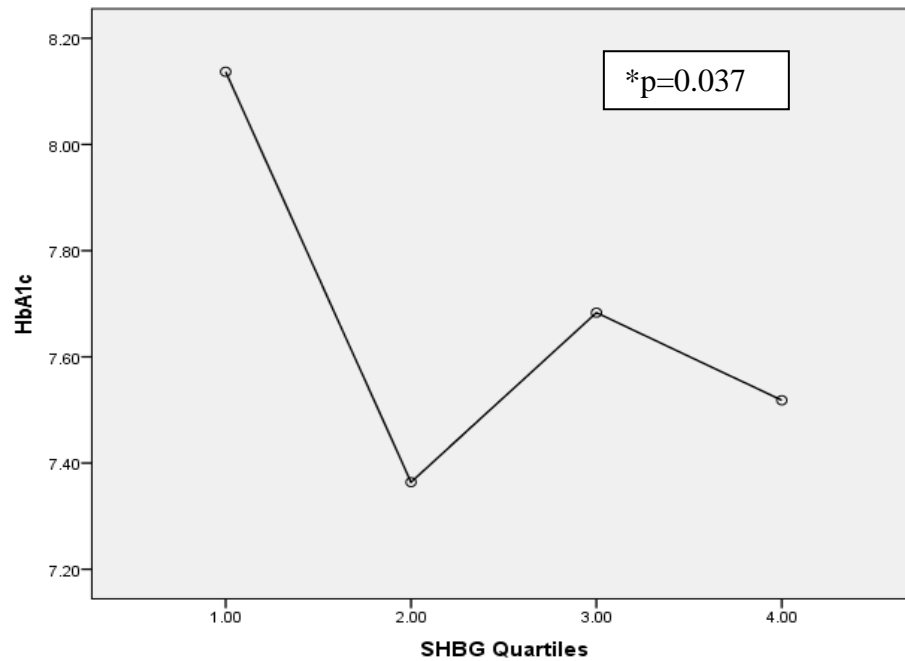
Variables	SHBG Q1	SHBG Q2	SHBG Q3	SHBG Q4	Whole cohort	p=
HbA1c*(%)	8.4±1.5*	7.4±1.5*	7.7±1.5	7.2±1.4	7.6±1.4	0.037
Weight*	108.4±17.9	98.5±17.3	91.7±12.5	92.7±23.5	97±18.8	0.002
HIP(cm)	113.7±10.6	110.5±8.1	107.8±6.6	109.6±12.9	109.9±9.6	0.11
Waist*(cm)	111.9±14.9	114.4±13.2	109.3±10.9	109.7±17.4	112.8±14.6	0.013
BMI*	35.8±6.2	32.4±5.4	30.7±3.8	30.6±6.4	32±5.6	0.001
% Fat*	35.8±8.7	31.1±7.3	30.4±7.1	29.7±8.4	31.5±7.9	0.009
HDL(mmol/l)*	0.96±0.2	1.04±0.3	1.1±0.24	1.2±0.3	1.1±0.3	0.000
TG*(mmol/l)	3.2±4	1.9±1.7	1.9±2	1.3±0.7	1.7±0.9	0.02
TC(mmol/l)	4.1±1.2	3.8±1	3.9±0.8	3.9±0.6	3.8±0.7	0.663
SBP(mmHg)	138.4±17.6	135. ±15.6	137.9±16.2	137.2±20.5	137.2±17.1	0.648
DBP(mmHg)	78.8±13.8	74.8±11.4	77.6±8.5	78.2±11.6	76.9±10.8	0.923
LDL(mmol/l)	1.9±0.7	1.9±0.5	2±0.6	2.1±0.6	2±0.6	0.977
AST(mmol/l)	27.1±12.5	26±10.9	26.3±9.9	24.6±7.4	25.8±10.1	0.939
ALT(mmol/l)	33±19.3	26.9±11.8	30±20.3	25.4±12.1	28.6±15.5	0.857

Quartile range Q1= up to 22.6, Q2 22.7 to 30.7, Q3= 30.8 to 44.9, Q4= 45 and above \*Significant at p<0.05 after adjusting for age and Testosterone levels, ±standard deviations, Hip- Hip circumference, Waist- Waist circumference, BMI- body mass index, % fat- Percentage body fat, SBP- Systolic Blood pressure, DBP- Diastolic Blood pressure, TC- Total cholesterol, HDL- High density lipoprotein Cholesterol, TG- Triglycerides, LDL- Low density lipoprotein cholesterol, AST-Aspartate amino transferase, ALT- Alanine amino transferase.

**Figure 4.4** Correlation between HbA1c and SHBG levels

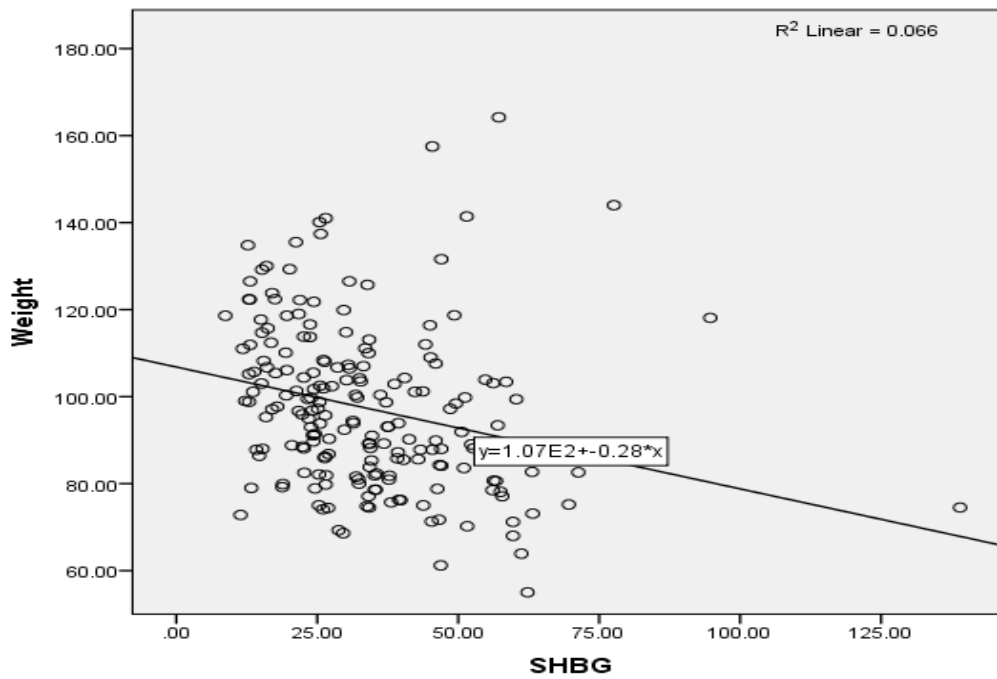


**Figure 4.5** HbA1c in quartiles of SHBG

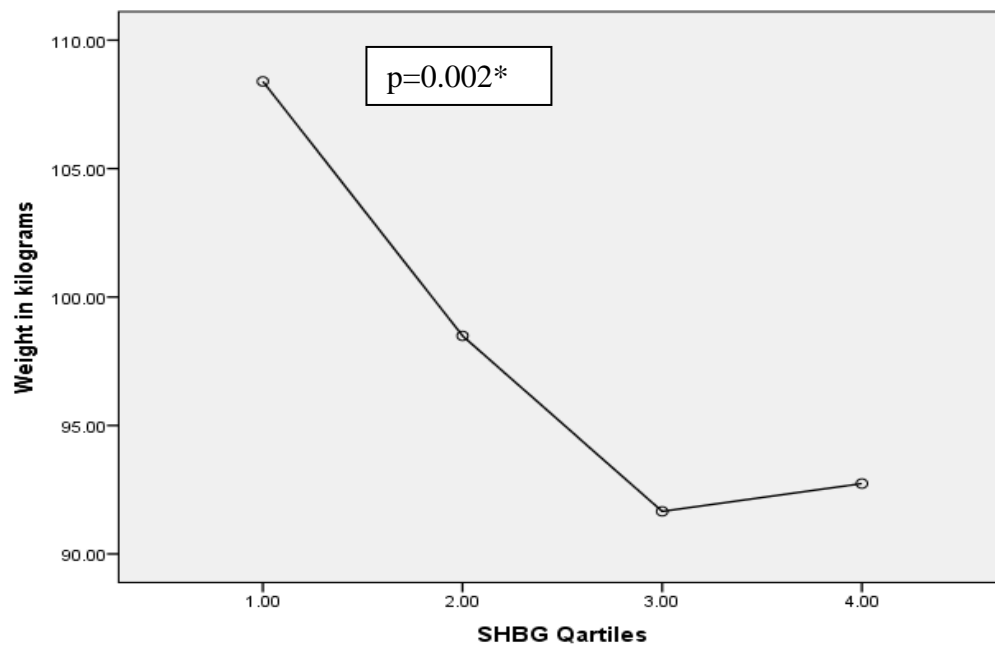


HbA1c in quartiles of SHBG. \*adjusted for age and testosterone levels

**Figure 4.6** Correlation between weight and SHBG levels

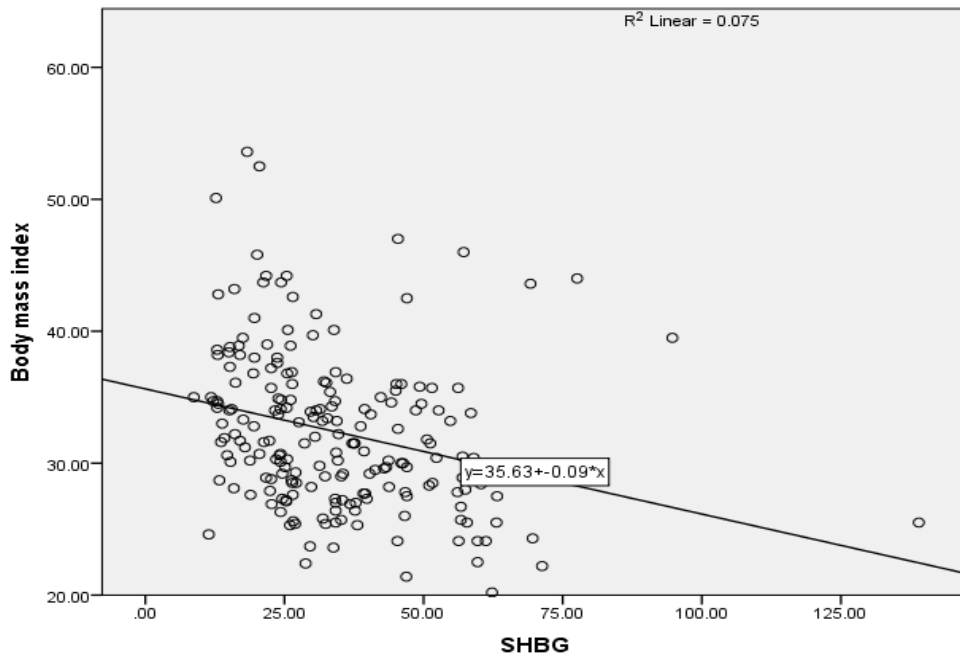


**Figure 4.7** Weight in quartiles of SHBG

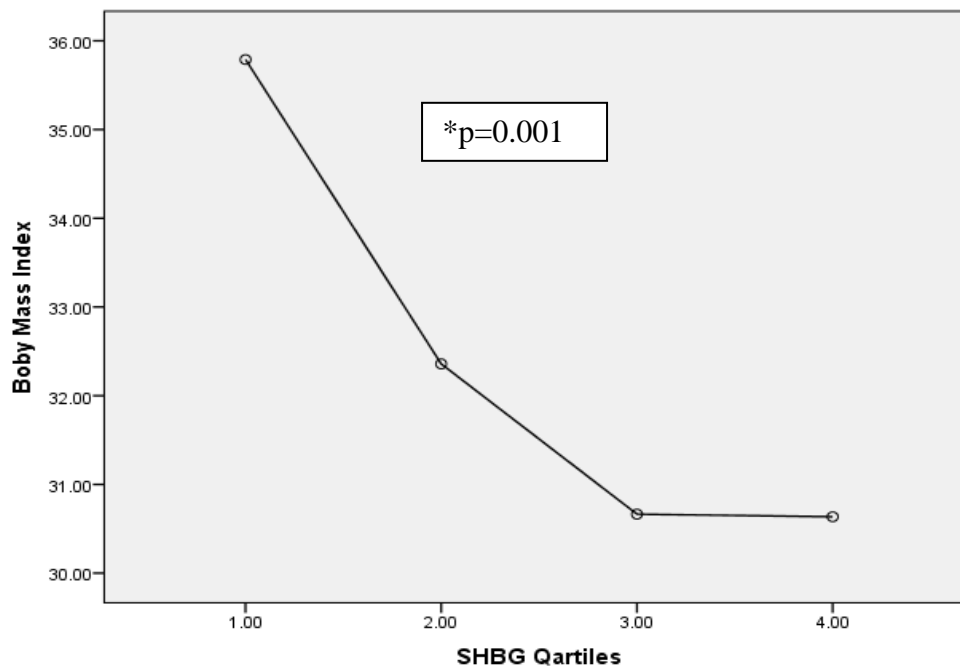


Body weight in quartiles of SHBG. \*adjusted for age and testosterone levels

**Figure 4.8** Correlation between body mass index and SHBG levels

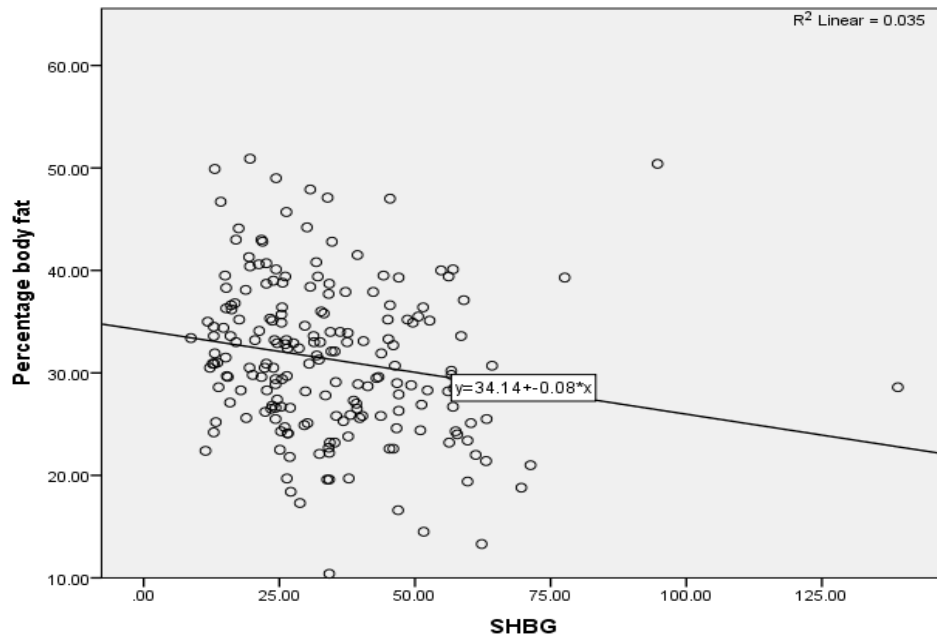


**Figure 4.9** Body mass index in quartiles of SHBG

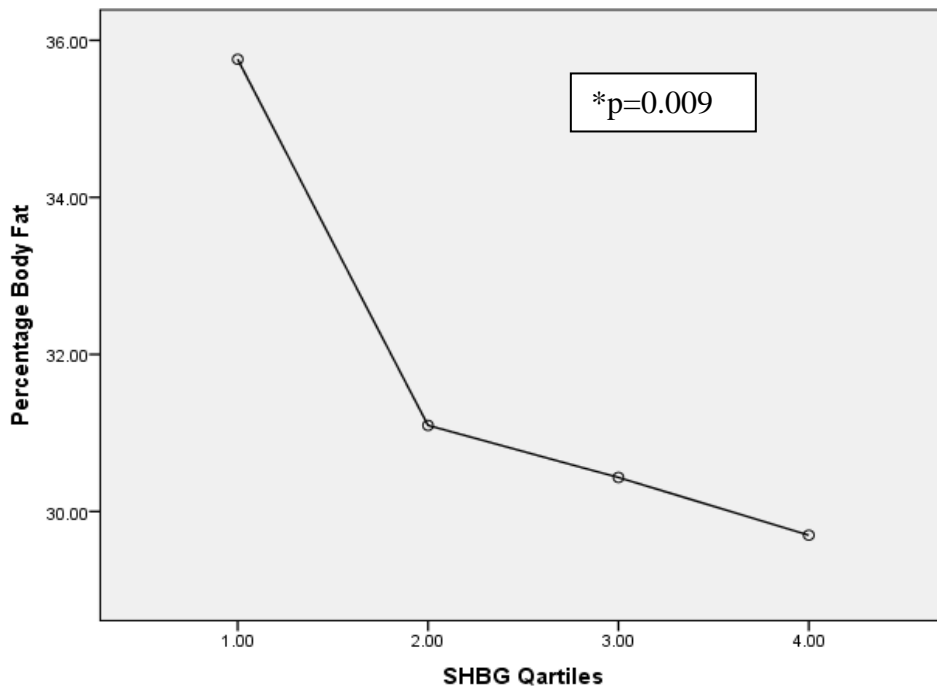


Body mass index in quartiles of SHBG. \*adjusted for age and testosterone levels

**Figure 4.10** Correlation between percentage body fat and SHBG levels



**Figure 4.11** Percentage Body fat in quartiles of SHBG



Percentage body fat in quartiles of SHBG. \*adjusted for age and testosterone levels



### 4.8.3 CIMT and Carotid Stiffness index $\beta$

Of the 203 patients, 148 had carotid Doppler examination performed. CIMT was not significantly different in the three groups (Group1 ( $T \leq 10.4 \text{ nmol/l}$ )  $0.85 \pm 0.15$ ; Group2 ( $> 10.4 \text{ nmol/l}$ )  $0.81 \pm 0.17$ ; Group (TRT)  $0.82 \pm 0.13$ ,  $p=0.640$ ). The relationship was further explored using a lower cut off for low testosterone at  $8 \text{ nmol/l}$ , calculated bioavailable testosterone (cut-off of  $2.6 \text{ nmol/l}$  for low testosterone) and calculated free testosterone (cut-off of  $225 \text{ pmol/l}$ ). There was no significant difference noted between different levels of testosterone in these analyses. Apart from age, CIMT did not show any significant correlation with other cardiovascular risk factors. Similarly the effect of SHBG was analysed using quartiles of SHBG comparing against CIMT. No significant changes in CIMT measurements were noted for different levels of SHBG (Q1= $0.8 \pm 0.12$ ; Q2= $0.8 \pm 0.15$ ; Q3= $0.86 \pm 0.2$ ; Q4= $0.82 \pm 0.13$ ,  $p=0.34$ ).

Carotid Stiffness index  $\beta$  showed a significant correlation with the three groups of testosterone in unadjusted analysis (Group1= $9.7 \pm 5.7$ ; Group2= $8.4 \pm 3.4$ ; Group3= $10.9 \pm 3.3$   $p=0.018$  with the highest being in the group). The significance was lost when adjusted for SHBG ( $p=0.109$ ).

There was a significant inverse correlation between SHBG quartiles and the  $\beta$  stiffness index when analysed adjusting for age and testosterone levels (Q1= $10.53 \pm 3.8$ ; Q2= $9.8 \pm 6$ ; Q3= $8.1 \pm 2.6$ ; Q4= $9 \pm 4$ ). However the significance was lost when adjusted for other co variates of age and testosterone levels ( $p=0.064$ ).

#### **4.5 Discussion and conclusion**

This cross sectional study showed that low testosterone state is associated with worsening the cardiovascular risk profile.

Glycaemic control was significantly worse in the low testosterone group and there is an inverse relation between the HbA1c and the testosterone levels. The mean HbA1c was 7.2% in the high testosterone group compared to a significantly high level of 8% in the low testosterone group. The HbA1c was 7.6% in the testosterone treated group. Although this does not reach significance compared the other two groups separately the results suggest that physiological testosterone replacement might have a positive impact on glycaemic control. This is compatible with the function testosterone exhibits on glucose, adipose tissue, liver and muscles.

Hip circumference, waist circumference, BMI and percentage body fat also were worse in the low testosterone group. However these does not seems to be affected significantly by testosterone replacement. This may be a reflection of the routine clinical practice experience of extreme difficulty in losing weight once gained. The lack of any significance with the testosterone replacement might also be explained by the duration of treatment with testosterone which widely varies in different patients on replacement therapy.

Lipid profile did not show any significant differences between the different testosterone groups. Total cholesterol, LDL cholesterol, HDL cholesterol and Triglycerides remained same in all three groups.

The CIMT which had shown to have an inverse relation with testosterone levels did not show any significant differences in this study. However the stiffness index  $\beta$ , another surrogate marker for atherosclerosis was low in the high testosterone group and the testosterone replacement group had the highest index in the unadjusted model. The significance was lost when SHBG and other co variates were added. The reason for this negative finding for CIMT could be due to the fact that these studies have a higher operator dependency.

There was no significant difference between groups in the incidence of ischaemic heart disease or CVA.

SHBG has shown an inverse relationship with HbA1c, weight, BMI, waist circumference, hip circumference, waist hip ratio, and percentage body fat. The HDL showed a positive correlation with SHBG. Except for HbA1c these relationships persisted after adjustment for testosterone levels and medications. SHBG has been linked to metabolic syndrome and type 2 diabetes. This suggests SHBG may be an independent predictor of cardiovascular risk profile in men. Whether SHBG has any causal role in cardiovascular disease needs to be established through further studies.

This study shows that low testosterone level is associated with worsening of cardiovascular risk profile in men and testosterone replacement might improve some of the cardiovascular risk profile. There was no increase in cardiovascular events with testosterone replacement therapy. The study also showed SHBG levels are independently associated with cardiovascular risk. Testosterone deficiency should be evaluated and treated, if appropriate, in men with metabolic syndrome and diabetes to improve the cardiovascular risk profile. The role of SHBG in cardiovascular risk profile needs to be evaluated further.

#### **4.6 Limitations**

This is a cross sectional study and any associations seen does not necessarily mean causation. However, this is a study from routine clinical practice with all the other co morbidities and likely to reflect day to day practice. The duration of TRT may not be long enough for all the cardio-metabolic effects to express. Long term randomised controlled trials are needed to confirm the findings



## **Chapter five**

### **Cross- sectional study: The relationship between the androgen receptor CAG polymorphism, ratio of testosterone to AR CAG and cardiovascular risk profile in men with type 2 diabetes**

*My contribution to this chapter includes design, majority of patient recruitment for reassessment from the previous cohort, all of clinical assessment, blood sampling, all of the carotid ultrasound, and measurement of carotid intima media thickness, calculation of stiffness index  $\beta$  and statistical analysis. The AR CAG repeat results for the same cohort from previous researchers were used with additional DNA extraction and AR CAG measurement for about 10% of the patients done by me. The baseline data for analysis used is from the previous researchers.*

#### **5.1 Introduction**

Testosterone plays an important role in metabolic syndrome, type 2 diabetes and adiposity (Kapoor, Malkin et al. 2005). As previously discussed, studies have shown that in men with type 2 diabetes, serum levels of testosterone and SHBG are lower (Andersson, Marin et al. 1994). Studies from our research group were the first to highlight the correlation between low testosterone and type 2 diabetes patients (Kapoor, Aldred et al. 2007). The study further suggested that levels of free testosterone and bioavailable testosterone are low and symptomatic hypogonadism was highly prevalent in diabetic men. These findings suggest availability of active form of testosterone to the target tissues is important.

There is evidence to suggest low testosterone levels are associated with increased risk of development of metabolic syndrome and type 2 diabetes (Haffner, Shaten et al. 1996, Tibblin, Adlerberth et al. 1996, Oh, Barrett-Connor et al. 2002). Results from Massachusetts Male aging study reported that low levels of testosterone and SHBG were associated with development of insulin resistance and subsequent type 2 diabetes (Stellato, Feldman et al. 2000). More recently a study of 702 Finnish middle aged men reported that low total testosterone and SHBG levels independently predict development of metabolic syndrome and diabetes (Laaksonen, Niskanen et al. 2004). These studies suggest low testosterone could be an early marker for derangement of the metabolic pathways of insulin and glucose metabolism which may progress to develop metabolic syndrome or frank diabetes.

Results from clinical trials show that testosterone replacement therapy in hypogonadal men reduces fat mass and central adiposity (Isidori, Giannetta et al. 2005, Kapoor, Goodwin et al. 2006). Our research team published a recent multicentre, prospective, randomised, double-blind, placebo-controlled study of 220 men with type 2 diabetes or metabolic syndrome which showed improvements in the overall study population in insulin resistance (HOMA-IR) and improvements in body fat percentage, waist circumference and serum lipids in subgroup analysis (Jones, Arver et al. 2011).

The classic androgen actions are mediated via the high affinity nuclear receptor the androgen receptor (AR). This receptor acts as a transcription factor after association with an appropriate ligand. The AR gene is located on the long arm of the X-chromosome. Exon 1 of the AR gene contains a polymorphic CAG repeat sequence which encodes a variable length polyglutamine stretch (AR CAG) (Klocker, Eder et al. 2004). Studies show that the length of AR CAG repeats correlates with the transcriptional capacity of the AR (Chamberlain, Driver

et al. 1994). The longer sequences are associated with impaired transcriptional activity. Conversely shorter AR CAG are associated with a high rate of prostate cancer and increased prostate growth during testosterone treatment (Hsing, Gao et al. 2000, Zitzmann, Depenbusch et al. 2003). In men with Klinefelter's Syndrome it has been reported that longer AR CAG repeat lengths are associated with gynaecomastia and smaller testes (Zitzmann, Depenbusch et al. 2004)(Zitzmann, Depenbusch et al. 2004).

There are many studies which looked into the AR CAG length and cardio-metabolic risk profile in men. One of the inherited neurodegenerative conditions, Kennedy Syndrome, is caused by abnormal AR CAG lengths (>15) (Mariotti, Castellotti et al. 2000). A link between carbohydrate metabolism and androgenicity is supported by the association of Kennedy syndrome with the development of type 2 diabetes (Mariotti, Castellotti et al. 2000). Studies have reported a relationship between insulin levels leptin levels and fat mass (Zitzmann, Gromoll et al. 2003). Previous study from our group reported a relationship between AR CAG repeat number with leptin and waist circumference as well as BMI in type 2 diabetic men. The greater the number of CAG repeats the higher the circulating level of testosterone which is required to maintain a normal state of androgen in an individual (Stanworth, Kapoor et al. 2008). From the above studies it is becoming clear that there is a link between AR CAG and androgen levels as well as androgenic actions. This leads to the hypothesis that a ratio of circulating testosterone levels to AR CAG length could be a more sensitive marker of the biological effects of androgen actions.

Testosterone however also exhibits actions independent of the androgen receptor and there is evidence that testosterone has direct action via a non-genomic mechanism as reported from the studies which showed a direct vasodilatory action of testosterone (Hall, Jones et al. 2006).



The present study is a cross sectional study looking at the relationship of AR CAG and cardiovascular risk profile in 195 men who were followed up from the original study of 355 men with type 2 diabetes (Kapoor, Aldred et al. 2007).

## **5.2 Hypothesis**

My primary hypothesis is that longer AR CAG, with less transcriptional activity, would predict worsening cardiovascular risk profile. My secondary hypothesis is that the ratio of testosterone to AR CAG, which could be a more sensitive marker of androgenic action, is positively associated with cardiovascular risk profile.

## **5.3 Research Design and Methods**

An initial cross-sectional study of 355 men over the age of 30 with type 2 diabetes was conducted at Barnsley Hospital NHS Foundation Trust, Barnsley, UK (Kapoor, Aldred et al. 2007). This is a cross sectional study of 203 patients from the cohort described in the initial study described above who attended for further review. The study was approved by the South Yorkshire Research Ethics Committee. Subjects for the original cohort were recruited from their attendance at the district wide Retinopathy Screening Service as well as hospital clinic appointments at the Centre for Diabetes and Endocrinology and gave informed consent. The study population contained patients with diabetes usually managed in primary as well as secondary care. One man was of Arabian ethnicity; all the other subjects were white Caucasian.

## **5.4 Clinical assessment**

### **5.4.1 Recording of demography, medical history and drug history**

Age, medical history and drug history were specifically recorded at the start of the study. This was confirmed with details in the medical notes where possible and in the event of any doubt the patient's GP was contacted, with their permission, to confirm details. For those patients who were added to the study the above data were collected from the hospital records.

### **5.4.2 Measurement of weight and body composition**

Height was measured after removal of shoes. Participants stood with the backs of their ankles, gluteal region and shoulders touching a wall which had a calibrated scale attached. A horizontal plane was applied to the crown of the head to be read against the scale.

Weight and body composition were assessed using Tanita BF-300 body fat analyser (Tanita Corporation, Japan). 'Standard Male' body type measurement was selected and the participants height and age were entered. After removing outer clothing, shoes and socks participants were asked to step onto the weighing platform so the plantar surface of heels and forefeet were in firm contact with the posterior and anterior electrodes respectively. The device measures weight directly and measures bioelectrical impedance by applying a 50 kHz, 500mA electrical current between the feet to derive body fat percentage on the principle that fat has lower electrical conductivity compared to other body tissues. This method has been validated against under water weighing for body composition analysis (Cable, Nieman et al.

2001). Body fat mass was calculated by multiplying weight by body fat percentage. Lean body mass was calculated by subtracting body fat mass from weight.

#### **5.4.3 Measurement of waist and hip circumference**

Waist circumference was measured at the point midway between the iliac crest and the costal margin. Hip circumference was measured at the widest point around the buttocks. Waist-hip ratio was calculated by dividing waist circumference by hip circumference.

#### **5.4.4 Measurement of blood pressure**

Blood pressure was measured using Colin Press-Mate BP8800C non-invasive blood pressure monitor (Colin Medical Instruments Corporation, Japan). Measurements were taken by placing the appropriately sized cuff around the upper arm such that the pressure sensor was applied to the skin close to the brachial arterial pulse to derive blood pressure from an automated oscillometric technique.

### **5.5 Biochemical measurements**

#### **5.5.1 Sampling technique**

In the cross-sectional study samples were taken between 0800 and 1000h at a fasting state (8hours minimum). Smokers were asked not to smoke on the morning of an assessment visit. Venous blood was drawn by venepuncture from the antecubital fossa. Samples were taken in appropriate bottles and mixed by inverting the bottles three times. After the sample had

clotted serum was obtained by centrifugation (10 minutes at 3500 revolutions per minute) and immediately frozen at  $-20^{\circ}\text{C}$  pending further analysis. Patients were asked to provide a first morning urine sample.

### **5.5.2 Measurement of samples in Barnsley hospital clinical chemistry laboratory**

Measurement of total testosterone was done using competitive chemiluminescence assay (Bayer Advia Centaur- Siemens Medical Solutions Diagnostics). The SHBG measurement was done by solid phase two-site chemiluminescent enzyme immunometric assay (Siemens Immulite, Siemens Medical Solutions Diagnostics).

Measurement of total cholesterol, HDL cholesterol and triglycerides was by Olympus AU640 Analyser (Olympus Diagnostics, Germany). LDL cholesterol was calculated using the Friedewald equation (Friedewald, Levy et al. 1972);  $\text{LDL cholesterol} = \text{Total cholesterol} - (\text{HDL cholesterol} + \text{triglycerides}/2.19) \text{ mmol/l}$ .

Glucose and urinary microalbumin were measured by Olympus analysers (Olympus Diagnostics, Germany).  $\text{HbA}_{1\text{C}}$  was measured by Menarini Analyser HA8160 (Menarini Diagnostics, Italy). Haemoglobin and haematocrit was measured by Cell-Dyn 4000 analyser (Abbott Laboratories, USA). PSA was measured by chemiluminescent micro particle immunoassay (Abbott Laboratories, USA).

### **5.5.3 Measurement of serum bioavailable testosterone- summary**

The principle of this technique is to remove testosterone from a test sample using activated charcoal.  $^3\text{H}$ -labelled testosterone is then added to the serum on the basis that it will equilibrate between binding with SHBG and other proteins or remain unbound in the same proportions as the test sample testosterone. The SHBG bound portion is then precipitated out of the sample and the remaining concentration of  $^3\text{H}$ -testosterone is assessed using a beta-counter. The result is compared to a control sample where the SHBG bound  $^3\text{H}$ -testosterone is not precipitated and this proportion is used to calculate the bioavailable testosterone from the total testosterone which is assessed separately. The technique is adapted from that described by Tremblay and Dube (Tremblay and Dube 1974).

### **5.5.4 Measurement of serum bioavailable testosterone- method**

Samples were allowed to come to room temperature. 400  $\mu\text{l}$  of sample was dispensed into each of three Eppendorf tubes comprising duplicate test samples and a single control. 400  $\mu\text{l}$  of activated charcoal was added to each Eppendorf tube and samples incubated at room temperature for 30 minutes. At the completion of incubation samples were spun in a centrifuge at 3500 rpm for 20 minutes at 4°C. 200  $\mu\text{l}$  of the resulting supernatant was dispensed into new Eppendorf tubes and 50  $\mu\text{l}$   $^3\text{H}$ -testosterone solutions added. The samples were applied to a vortex to ensure good mixing, incubated for 120 minutes at 37°C and then chilled on ice. 200  $\mu\text{l}$  cold, saturated ammonium sulphate solution was added to the test samples with 200  $\mu\text{l}$  saline added to the control. The samples were mixed with a vortex and then spun in a centrifuge at 3500 rpm for 20 minutes at 4°C. 200  $\mu\text{l}$  of the resulting supernatant was dispensed into counting vials and 3 ml of OptiPhase Hisafe 2 scintillation

fluid (Fisher Scientific, UK) added. Activity of the samples was measured by Beta counter and was proportional to the <sup>3</sup>H-testosterone content. The percentage of bioavailable testosterone was calculated according to the formula.

% Bioavailable testosterone = Average of duplicate sample (dpm) x 100/ control (dpm).

Total testosterone was measured by ELISA (see section 2.3.2.1) and the concentration of bioavailable testosterone was then calculated as follows;

Bioavailable testosterone = % Bioavailable testosterone x Total Testosterone/100

### 5.5.5 Calculation of free testosterone and bio available testosterone

Due to the considerable technical challenge concerning accurate measurement of free testosterone and oestradiol formulae have been developed which aim to estimate free sex hormone levels based on total serum levels of testosterone, oestradiol and SHBG. In our studies we used the formula of Vermeulen to calculate free testosterone (Vermeulen, Stoica et al. 1971) and free oestradiol was calculated from total oestradiol, SHBG and TT by the formula of Sodergard et al (Sodergard, Backstrom et al. 1982).

Formula for free testosterone (Vermeulen, Stoica et al. 1971);

$$\text{Free testosterone} = (-b + \sqrt{b^2 + 4a [\text{Total Testosterone}]}) / 2a$$

$$A = K_{at} + K_{st} + (K_{at} \times K_{st})([\text{SHBG}] + [\text{Albumin}] - [\text{Total Testosterone}])$$

$$B = 1 + K_{st}[\text{SHBG}] + K_{at}[\text{Albumin}] - (K_{at} + K_{st})[\text{Total Testosterone}]$$

$K_{at}$  = Affinity constant for testosterone with albumin =  $3.6 \times 10^4$  in this equation  $K_{st}$  = Affinity constant for testosterone with SHBG =  $1 \times 10^9$  in this equation.

The equation used to calculate bioavailable testosterone was from Morris et al (Morris, Malkin et al. 2004)

$$\text{Ln Bioavailable Testosterone} = -0.266 + (0.995 \times \text{Ln Total Testosterone}) - (0.228 \times \text{LnSHBG})$$

Ln=log Normal.

Measurements using different formulae for calculation of the total and free testosterone revealed similar results, although the absolute values were different as noted in studies (De Ronde, Van der Schouw et al. 2006).

## **5.6 Measurement of AR CAG - summary**

DNA was extracted from peripheral lymphocytes in whole blood and success was confirmed with spectrophotometry. DNA amplified by polymerase chain reactions (PCR) to amplify the region of the AR gene containing AR CAG and success was confirmed by gel electrophoresis. Samples required magnetic separation of PCR products for optimisation and were analysed by automated sequencer which produced electropherograms from which the DNA sequence could be derived.

### **5.6.1 Extraction of DNA from human lymphocytes**

Samples were allowed to come to room temperature. 1 ml blood was dispensed into 15 ml polypropylene conical bottomed tubes and 8 ml solution A added (red cell lysis buffer; Appendix 2 for constituents). Samples were left for 5 minutes at room temperature with occasional mixing and were then spun at 3000 rpm for 10 minutes in a centrifuge at room

temperature. The supernatant was discarded and the pellet and residue re-suspended in 4 ml solution A and mixed for 4 minutes at room temperature. The samples were then spun at 1700 rpm in a centrifuge for 4 minutes at room temperature. The supernatant was poured off and discarded. Pellet and residue were re-suspended in 400 µl solution B (leucocyte lysis buffer; Appendix 2 for constituents) using a vortex. 100 µl 5M Sodium perchlorate was added and the tube inverted 8 times. 400 µl pre-cooled chloroform was added and tube inverted 10 times. Samples were then spun at 2200 rpm for 3 minutes in a centrifuge at room temperature. At this stage the contents had separated into three layers. The top aqueous layer was carefully pipetted into 1 ml cold ethanol and gently inverted 15 times leading to precipitation of DNA which was now visible. DNA was removed from the ethanol with a sealed Pasteur pipette, allowed to dry for five minutes and then re-suspended in 100 µl distilled water within in a 1.5 ml Eppendorf tube. Samples were left on rotating wheel for 24-72 hours to allow full resuspension and then stored at -20°C.

### **5.6.2 Polymerase chain reaction (PCR) to amplify section of androgen receptor gene containing AR CAG (adapted from ABGene the Master mix PCR protocol)**

DNA was amplified in 25 µl reactions containing 22.5 µl PCR Master mix (ABGene, Epsom, UK- 1.25U DNA polymerase, 75mM Tris-HCl (pH 8.8 at 25°C), 20mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% Tween 20c, 200µM of each dATP, dCTP, dGTP, dTTP, 1.5mM MgCl<sub>2</sub>), 0.5 µl 10pmol/µl each primer, 0.5 µl distilled water and 1 µl DNA containing sample. The primers used for AR CAG amplification were 5'-GCT GTG AAG GTT GCT GTT CCT CAT-3' and 5'-TCC AGA ATC TGT TCC AGA GCG TGC-3'. The amplifications for AR CAG were performed using



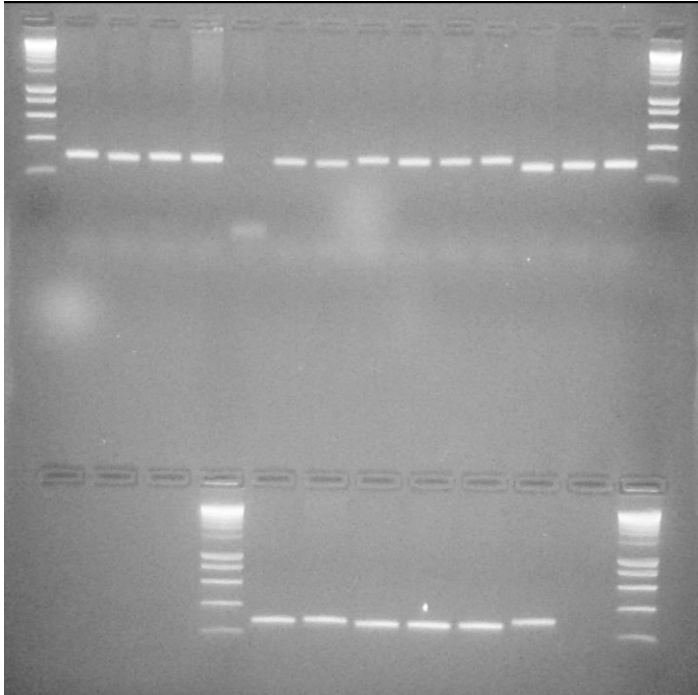
an automated thermal cycler applying the following PCR conditions: initialization at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute and primer extension at 72°C for 1 minute. This was followed by a 7 min final extension at 72°C.

### **5.6.3 Agarose gel electrophoresis to confirm PCR product**

A 2% agarose gel was made by heating 3g agarose and 150 ml 1x Tris-Borate EDTA (see Appendix for constituents) in a microwave for about 1 minute until fully dissolved. After allowing to cool slightly, 2 µl of 10mg/ml ethidium bromide was added and mixed. The gel was poured into a template and allowed to set.

When set, the templates were removed and the gel was submerged in 1x Tris-Borate EDTA. 10 µl sample plus 2 µl loading buffer was added to each well within the gel. Samples of DNA ladder were entered in the end well. The gel tank was closed and the gel was run at 90 Volts for 45 minutes. The gel was inspected in UV light to confirm samples that had DNA product from the PCR (see Figure 5.1).

**Figure 5.1** Agarose gel electrophoresis showing PCR results from a group of AR CAG samples



#### **5.6.4** Magnetic separation and use of automated sequencer (Core Facility)

After magnetic separation to remove non-specific products and excess primers PCR products were analysed by the capillary based AB3730 automated sequencer (Applied Biosystems, Warrington, UK). The primer for sequencing were AR CAG; 5'-GCT GTG AAG GTT GCT GTT CCT CAT-3'. Electropherograms were produced which were viewed with Finch TV software (Geospiza, USA) allowing length of AR CAG repeat sequence to be counted. The majority of samples returned appropriate quantities of DNA and the gel allowed identification of those samples needing repeat PCR.

## **5.7 Assessment of arterial wall parameters-carotid artery ultrasound**

Local arterial stiffness and intima-media thickness of the common carotid arteries was assessed in the clinical studies for the longitudinal and the cross sectional studies.

Participants were examined in the supine position. With the neck slightly extended views were obtained of the common carotid artery immediately before the artery dilates to form the carotid bulb using the Sonosite Titan High Resolution Portable Ultrasound System (Sonosite Inc., USA) and L38 Vascular/Small Parts Transducer (Sonosite Inc., USA).

Recordings of this section of artery were taken and images selected with the artery at minimal (end-diastolic) and maximal (systolic) diameter. The minimal and maximal diameters of the artery were measured and recorded. The diastolic image was uploaded into a specialised IMT measurement programme (SonoCalc IMT Version 3.0, Sonosite Inc., USA) and a part of artery not showing evidence of overt atherosclerotic plaque was analysed for IMT. Three sets of vessel diameter and IMT measurements were taken from each common carotid artery.

The ultrasound probe was removed from the patient between each set of images. Blood pressure was checked at the brachial artery on three consecutive occasions in the dominant arm when the ultrasound measurements were complete and the average of the three measurements was calculated.

### **5.7.1 Carotid ultrasound Measurement for carotid Intima media thickness**

The patients were examined in the supine position with the neck slightly extended to allow optimal ultrasound views. Images were taken from the common carotid artery immediately before the artery dilates to form the carotid bulb, using the Sonosite Titan High Resolution Portable Ultrasound System (Sonosite Inc., USA) and L38 Vascular/Small Parts Transducer (Sonosite Inc., USA).

Recordings of this section of artery were taken and images selected with the artery at minimal (end-diastolic) and maximal (systolic) diameter. The minimal and maximal diameters of the artery were measured and recorded. The diastolic image was uploaded into a specialised IMT measurement programme (SonoCalc IMT Version 3.0, Sonosite Inc., USA) and a part of artery not showing evidence of overt atherosclerotic plaque was analysed for IMT. Three sets of vessel diameter and IMT measurements were taken from each carotid artery and the ultrasound probe was removed from the patient between each set of images.

### **5.7.2 Calculation of Carotid Stiffness index $\beta$**

Blood pressure was checked on three consecutive occasions when the ultrasound measurements were complete and average measurements was calculated. Stiffness index  $\beta$  was then calculated from the diastolic carotid artery diameter (Dd), systolic carotid artery diameter (Ds), diastolic blood pressure (BPd) and systolic blood pressure (BPs) using the formula;

Stiffness index  $\beta = (\ln(Ps/Pd)) \times Dd / (Ds - Dd)$ .

## 5.8 Statistical analysis

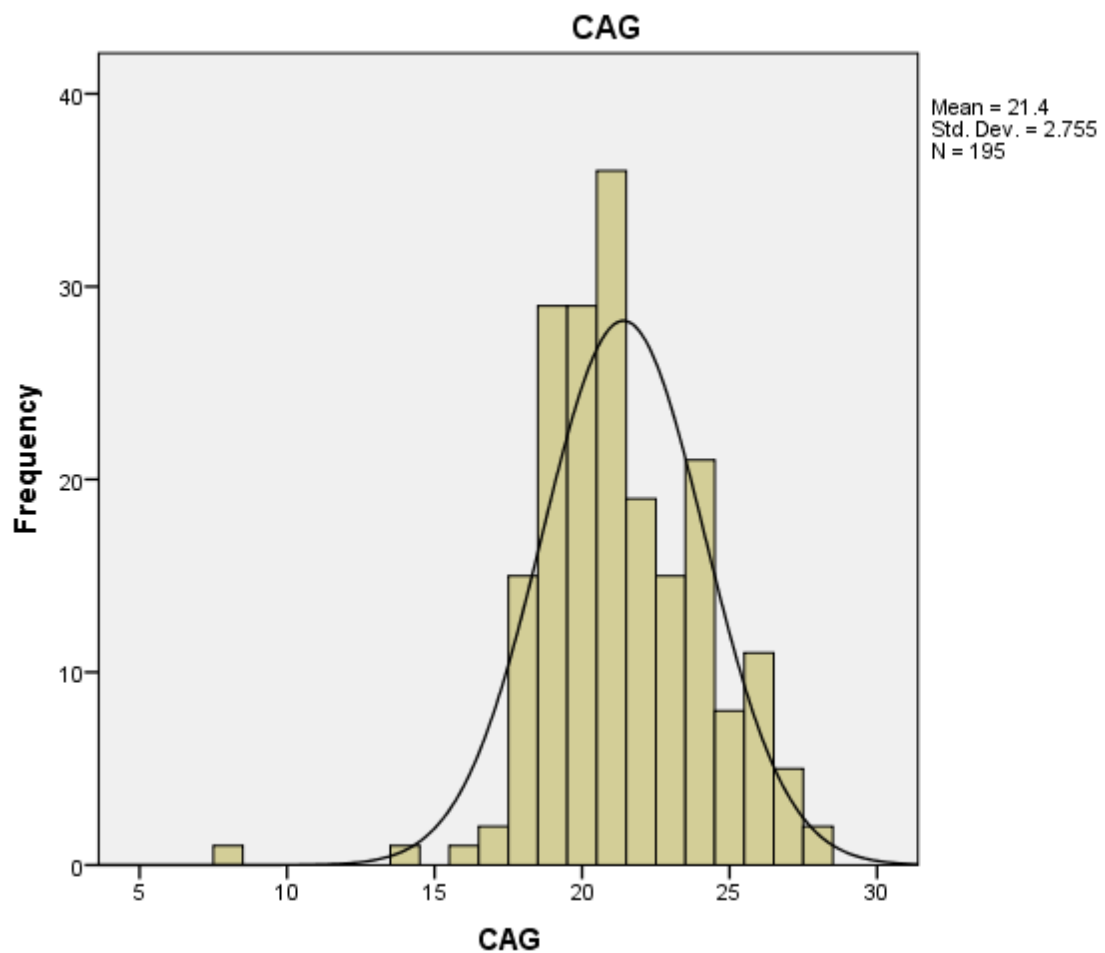
Statistical analysis was carried out using the SPSS 19.0 statistical package (Chicago, USA). All descriptive data are expressed as mean +/- standard deviation. Correlations were assessed and expressed as a Pearson correlation coefficient (r) with associated p value. Results were adjusted for age which is known to affect serum testosterone and SHBG levels. Univariate and multivariate analysis was performed using general linear model to see significant differences between AR CAG and various cardiovascular risk factors in quartiles of AR CAG lengths. Further the ratio of total testosterone (T/AR CAG) or bio available testosterone (BioT/AR CAG) were used to compare the risk profile. The correlation was tested first by looking at the correlation coefficients and then multivariate analysis in quartiles of the ratio (T/AR CAG or BioT/AR CAG) similar to that done for AR CAG. Results were considered statistically significant at  $p < 0.05$ .

## 5.9 Results

### 5.9.1 AR CAG and cardiovascular risk profile

The mean AR CAG length was  $21.4 \pm 2.8$ , range 8-28. The median was 21 and inter quartile range of 19,21 and 23. The distribution is shown in Figure1

**Figure 5.2 AR CAG repeat distribution among the cohort**



The relationship between CAG and the CV risk profile were analysed using bi variate correlation analysis for each of the CV risk measures. The data was further analysed by dividing the patents based on quartiles of AR CAG length and multivariate general linear model to see any significant relation to any of the risk factors measured. There was no significant relationship noted between the cardiovascular risk profile and AR CAG repeats. The results are summarised in Table 1. The analysis was repeated after adjusting for those who had testosterone replacement for more than 3 months during the follow up period. Again no significant relationship was noted.

**Table 5.1 Cardiovascular risk factors in different quartiles of AR CAG repeat**

Variables	CAG Q1	CAG Q2	CAG Q3	CAG Q4	Significance p=
HbA1c(%)	7.5±1.6	7.6±1.3	7.3±1.3	7.6±1.4	0.86
Weight(kg)	94±17.8	98.2±19.7	97±24.9	99.1±14.7	0.594
HIP(cm)	108.7±9.8	110.7±10	111.4±6.4	110±6.4	0.585
Waist(cm)	111.5±14.2	113.8±15.5	112.1±18.3	114±11.8	0.885
Waist Hip ratio	1.03±0.08	1.02±0.09	1±0.08	1.04±0.08	0.487
BMI	31.4±5.2	32.4±6.1	31.9±7.4	32.3±4.2	0.887
% Fat	31.1±7.6	32.3±9.1	29.9±7.4	32.3±5.9	0.613
HDL(mmol/l)	1.07±0.3	1.09±0.31	1.12±0.3	1.08±0.3	0.915
TG(mmol/l)	1.6±0.8	1.7±0.9	1.7±1	1.9±0.9	0.45
TC(mmol/l)	3.7±0.7	4±0.9	3.7±0.7	3.9±0.6	0.361
LDL(mmol/l)	2±0.6	2.1±0.6	1.8±0.6	2±0.5	0.342
SBP(mmHg)	136.7±14.9	134.9±17.9	137±21.2	140.3±18.5	0.575
DBP(mmHg)	76.9±10.2	77.1±11.5	77.1±12.4	76.5±11.2	0.988
AST(mmol/l)	25.4±8.4	27.6±12.1	24±8	25.5±9.9	0.497
ALT(mmol/l)	27.4±11.9	31.8±18.2	22.9±8.7	30.4±17.4	0.116

Quartile range 8-19; 20-21; 22-23; 24-28, ±standard deviations, Hip- Hip circumference, Waist- Waist circumference, BMI- body mass index, % fat- Percentage body fat, SBP- Systolic Blood pressure, DBP- Diastolic Blood pressure, TC- Total cholesterol, HDL- High density lipoprotein Cholesterol, TG- Triglycerides, LDL- Low density lipoprotein cholesterol, AST-Aspartate amino transferase, ALT- Alanine amino transferase.



### **5.9.2 Total testosterone AR CAG ratio and cardiovascular risk profile**

The testosterone AR CAG ratio (T/AR CAG ratio) was calculated by dividing total testosterone by the AR CAG repeat length. The individual risk profiles were analysed for correlation using bivariate correlation analysis.

There were highly significant negative correlation between T/AR CAG ratio and HbA1c (correlation coefficient  $-0.255$   $p < 0.000$ ); T/AR CAG ratio and the hip circumference (correlation coefficient  $-0.232$   $p = 0.001$ ). There was also significant negative correlation between the ratio and weight (correlation coefficient  $-0.176$   $p = 0.014$ ). The HDL cholesterol correlated positively with the T/ARCAG ratio (correlation coefficient  $+0.189$   $p = 0.009$ ). Body mass index showed a trend towards negative correlation (correlation coefficient  $-0.14$   $p = 0.052$ ). There was no significant correlation for other risk profiles analysed with the T/AR CAG ratio. The results are summarised in table 5.2

**Table 5.2 Correlation Coefficients comparing calculated T/AR CAG ratio and CV risk factors**

Variable	Correlation Coefficients	Significance p=
Age(years)	-0.035	0.626
HbA1c (%)	-0.255	<0.000*
Hip Circumference (cm)	-0.232	0.001*
Waist Circumference(cm)	-0.113	0.119
Waist Hip ratio	-0.034	0.639
Weight(kg)	-0.176	0.014*
Body Mass index	-0.14	0.052
Percentage Body Fat	-0.104	0.151
Total Cholesterol	-0.087	0.229
HDL Cholesterol	0.189	0.009*
LDL cholesterol	0.018	0.813
Triglycerides	-0.122	0.09
Systolic Blood pressure	0.49	0.501
Diastolic Blood pressure	0.018	0.807
Carotid Intima Media thickness	-0.012	0.887
Carotid Stiffness index $\beta$	-0.019	0.827
All cardiovascular events	0.078	0.281
Myocardial infarction	-0.072	0.319
Other ischaemic heart disease	0.053	0.462
Cerebrovascular disease	0.032	0.658
Peripheral vascular disease	0.072	0.321

(\* significant at p<0.05)

The correlation was further analysed by comparing the risk profile in quartiles of T/CAG ratio the means using the general linear model adjusted for age. The HbA1c was significantly lower in the upper quartiles compared to lower quartile (Q1=8.2±1.6, Q2=7.7±1.4, Q3=7.7±1.4, Q4=7.1±1.2 p=<0.000). Similarly weight (Q1=103.7±23.3, Q2=96.7±18.3 , Q3=98.3±19, Q4= 93.3±16.1 p=0.037) and Hip circumference (Q1=114.4±12.2, Q2=109.6±8.3, Q3=110.3±8.3, Q4=107.6±9.8 p=0.006) were also significantly lower in the upper quartiles. The HDL was significantly higher in the higher quartiles of T/AR CAG ratio (Q1=1±0.23, Q2=1±0.23, Q3=1.13±0.32, Q4=1.18±0.32 p=0.008). There were no significant differences in the other cardiovascular risk profiles measured. The results are summarised in table 5.3.

**Table 5.3 Cardiovascular risk profile for different quartiles of T/AR CAG ratio adjusted for age**

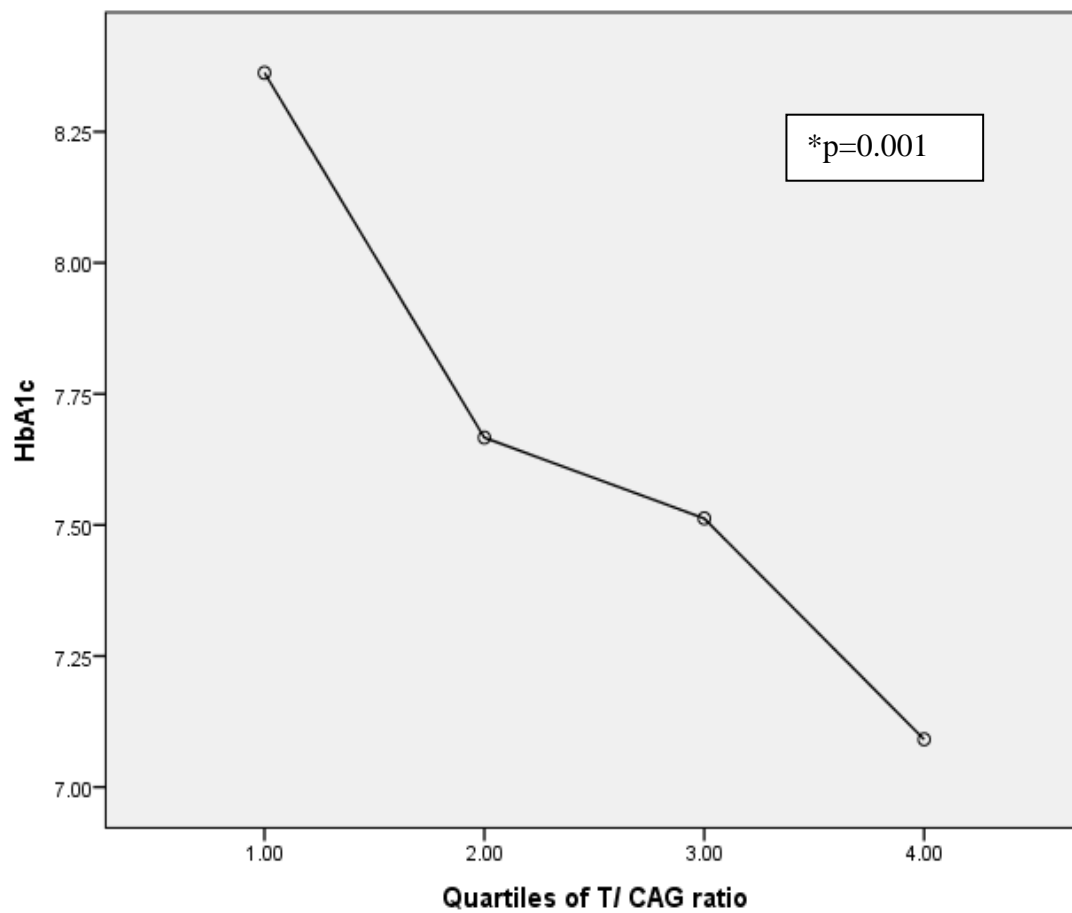
Variables	T/CAG Q1	T/CAG Q2	T/CAG Q3	T/CAG Q4	Significance p=
HbA1c(%)	8.2±1.6	7.7±1.4	7.7±1.4	7.1±1.2	<0.000*
Weight	103.7±23.3	96.7±18.3	98.3±19	93.3±16.1	0.037*
HIP(cm)	114.4±12.2	109.6±8.3	110.3±8.3	107.6±9.8	0.006*
Waist(cm)	116.9±17.5	113.1±13.2	112.9±14.61	10.8±13.8	0.19
Waist Hip ratio	1.02±0.09	1.03±0.07	1.02±0.08	1.03±0.08	0.855
BMI	34.4±7.2	32.2±5.3	31.9±5.7	31±5	0.01*
% Fat	34±8.5	31.8±8.9	31.4±7.2	29.9±7.4	0.066
CIMT	0.84±0.15	0.82±0.14	0.82±0.18	0.81±0.1	0.381
Stiffness index β	10.1±5.9	9.4±3.7	8.9±3.6	9.5±3.9	0.802
HDL(mmol/l)	1±0.23	1±0.23	1.13±0.32	1.18±0.32	0.008*
TG(mmol/l)	2.6±4	2.2±2.2	1.94±1.4	1.7±1.1	0.204
TC(mmol/l)	4±1.1	4±1	4±0.9	3.9±0.7	0.903
LDL(mmol/l)	2±0.6	2.1±0.7	2±0.57	2±0.58	0.709
SBP(mmHg)	133.9±16.9	137.7±15.9	137.1±18.1	138.1±18.9	0.633
DBP(mmHg)	76.7±13.5	77.2±10.1	80.2±9.2	74.2±12.9	0.199
AST(mmol/l)	26.1±12.8	25.8±10.6	27.4±10.3	25.9±8.3	0.991
ALT(mmol/l)	28.7±19.1	30.7±19.3	31.1±17.3	27±10.6	0.721

Quartile range Q1 up to 0.4, Q2 0.41 to 0.56, Q3 0.56 to 0.75, Q4 0.76 and above, \* Significant at p<0.05) ±standard deviations, Hip- Hip circumference, Waist- Waist circumference, BMI- body mass index, % fat- Percentage body fat, SBP- Systolic Blood pressure, DBP- Diastolic Blood pressure, TC- Total cholesterol, HDL- High density lipoprotein Cholesterol, TG- Triglycerides, LDL- Low density lipoprotein cholesterol, AST-Aspartate amino transferase, ALT- Alanine amino transferase

In the multivariate adjusted model, adjusting for age, BMI, percentage body fat, hip circumference, waist circumference ,SHBG and concomitant medications (insulin, sulphonylureas, Metformin, ACE, ARB, Statins ,CCB and testosterone replacement) the

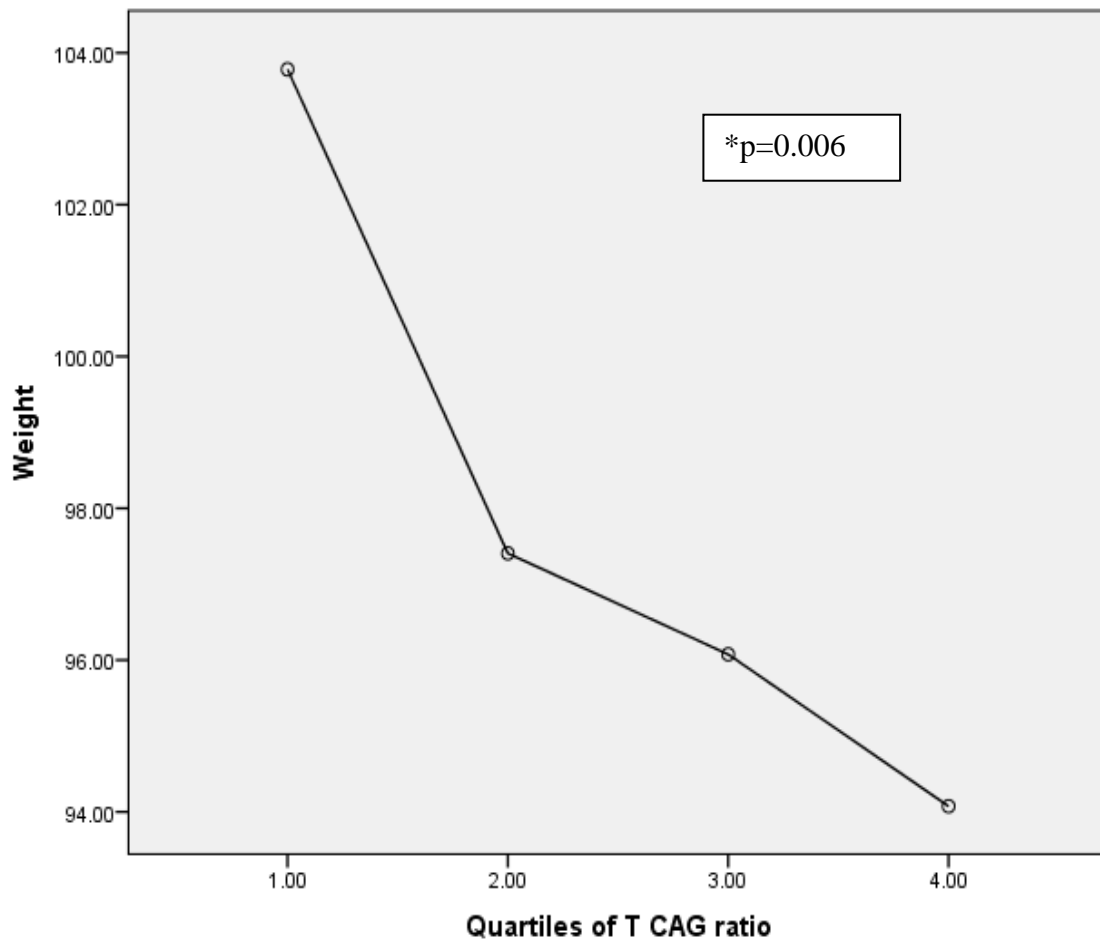
HbA1c was still significantly lower in the higher quartile compared to the lower quartiles ( $p < 0.001$ , figure 5.4)

**Figure 5.3 HbA1c change in quartiles of T/ AR CAG ratio**



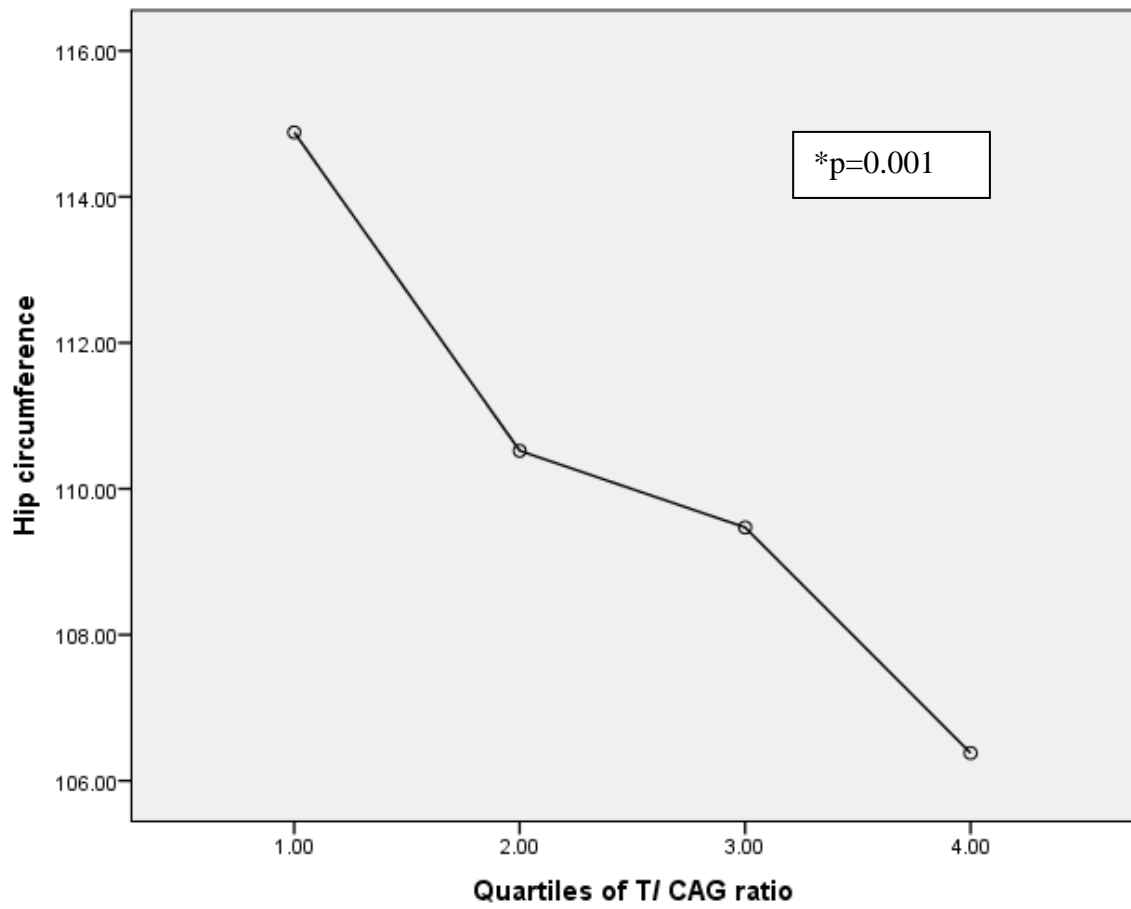
\*Adjusted for Age, SHBG, BMI, percentage body fat and concomitant medications including testosterone replacement

**Figure 5.4** Weight change in quartiles of T/ AR CAG ratio



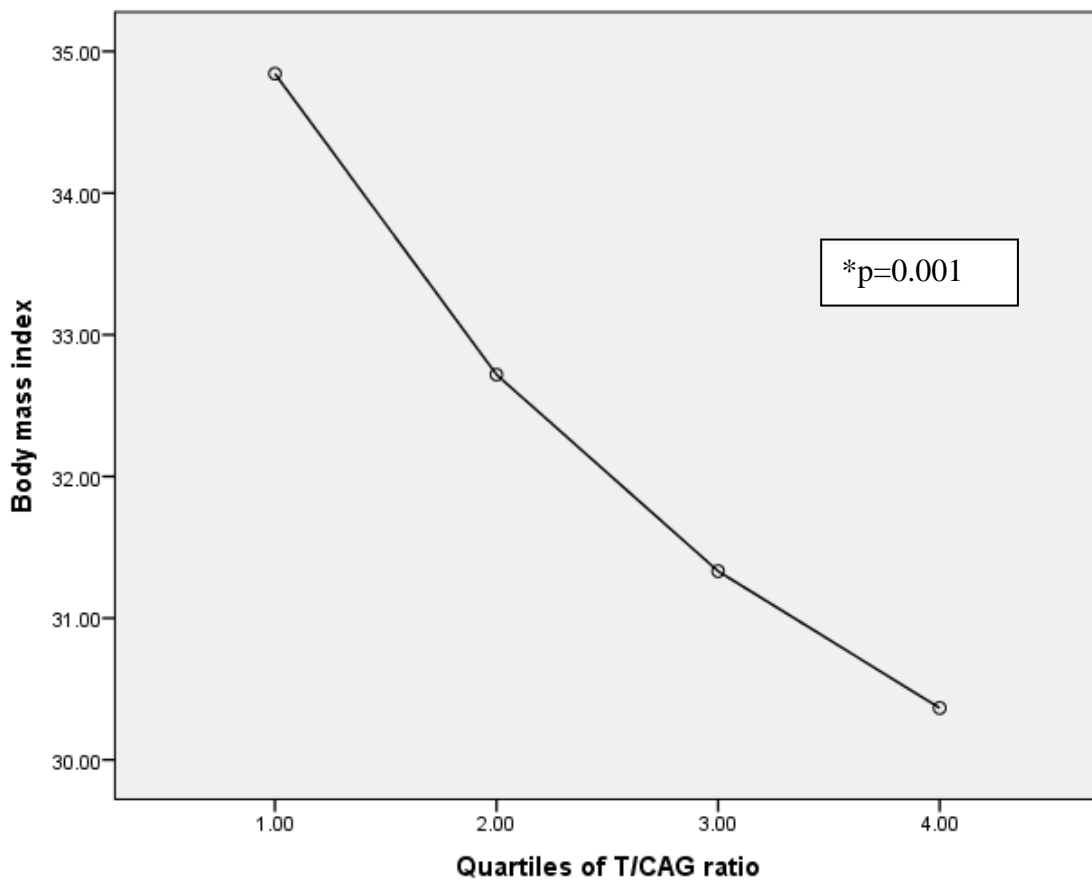
\*Adjusted for age, SHBG, and concomitant medications including testosterone replacement

**Figure 5.5 Hip circumference changes in quartiles of T/ AR CAG ratio**



\*Adjusted for age, SHBG, and concomitant medications including testosterone replacement

**Figure 5.6** Body mass index change in quartiles of T/ AR CAG ratio



\*Adjusted for age, SHBG, and concomitant medications including testosterone replacement

HDL change between the quartiles remain significant with multivariate analysis except when BMI was added as covariates ( $p=0.043$ ). When BMI added to co variates the significance was lost ( $p=0.085$ ). Similarly percentage body fat change was significant in the multivariate analysis (0.001), except weight, BMI or waist circumferences were added as covariates. When any of the latter were added, the significance was lost ( $=0.15$ )



### **5.9.3 Bioavailable Testosterone AR CAG ratio (cBioT/AR CAG) and Cardiovascular risk profile**

The cBioT/AR CAG ratio was calculated by dividing calculated bio available testosterone by AR CAG. Similar to the previous analysis the data was analysed for correlation using bivariate correlation analysis for each of the CV risk profile measures. The correlation coefficients and the significance are summarised in Table 5.4.

There was a significant ( $P < 0.05$ ) negative correlation between the cBioT/AR CAG ratio and HbA1c, hip circumference and percentage body fat. The HDL cholesterol had a positive correlation with the cBioT/AR CAG ratio. BMI showed a trend towards significance ( $p = 0.065$ ). The significance of HbA1c, Hip Circumference and HDL cholesterol were high at  $\leq 0.01$ .

**Table 5.4 Correlation Coefficients comparing calculated cBioT/AR CAG ratio and CV risk factors**

Variable	Correlation Coefficients	Significance p=
Age(years)	-0.111	0.123
HbA1c (%)	-0.203	0.004*
Hip Circumference (cm)	-0.192	0.008*
Waist Circumference(cm)	-0.09	0.215
Waist Hip ratio	0.035	0.633
Weight(kg)	-0.103	0.153
Body Mass index	-0.135	0.065
Percentage Body Fat	-0.169	0.019*
Total Cholesterol	-0.35	0.628
HDL Cholesterol	0.186	0.01*
LDL cholesterol	0.029	0.702
Triglycerides	-0.35	0.628
Systolic Blood pressure	0.046	0.527
Diastolic Blood pressure	0.02	0.769
Carotid Intima Media thickness	-0.031	0.71
Carotid Stiffness index $\beta$	0.015	0.857
All cardiovascular events	0.103	0.364
Myocardial infarction	-0.069	0.241
Other ischaemic heart disease	0.034	0.634
Cerebrovascular disease	0.033	0.649
Peripheral vascular disease	0.076	0.294

(\* significant at p<0.05)

When the means were analysed with quartiles of the cBioT/AR CAG ratio adjusting for age, most of the above significance were confirmed. Moreover there was a significant change in weight, BMI and LDL cholesterol. The results are summarised in Table5.5.

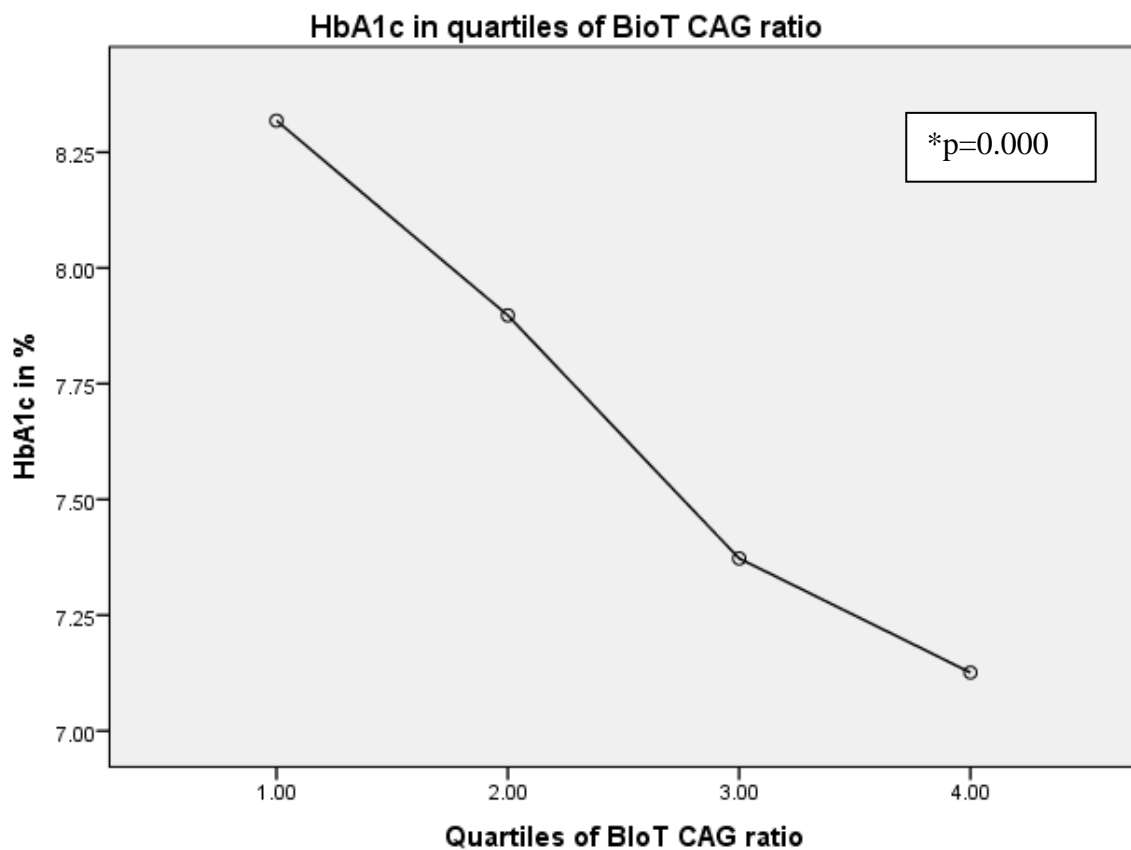
**Table 5.5 Cardiovascular risk profile for different quartiles of cBioT/AR CAG ratio adjusted for age**

Variables	cBT/CAG Q1	cBT/CAG Q2	cBT/CAG Q3	cBT/CAG Q4	Significance p=
HbA1c(%)	8.1±1.5	7.7±1.5	7.4±1.4	7.1±1	0.001*
Weight	101.5±22.9	94.5±17.3	96.8±20.2	95.9±16	0.025*
HIP(cm)	113.4±12	109.6±8.8	109.9±8.8	107.6±9.4	0.002*
Waist(cm)	116.7±17.2	110.9±11.4	112±16.3	112.4±13.4	0.17
Waist Hip ratio	1.02±0.09	1.02±0.07	1.01±0.08	1.05±0.08	0.087
BMI	33.6±6.7	31.3±4.6	31.5±6.4	31.7±4.9	0.006*
% Fat	33.3±7.2	31±7.7	31.6±8.9	30.6±7.2	0.131
CIMT	0.85±0.15	0.81±0.17	0.84±0.17	0.8±0.11	0.332
Stiffness index $\beta$	10.2±5.5	8.9±4.2	9.4±3.6	9.4±3.8	0.666
HDL(mmol/l)	1.02±0.2	1.03±0.25	1.2±0.3	1.12±0.3	0.001*
TG(mmol/l)	1.8±0.2	1±0.3	1.2±0.3	1.1±0.3	0.117
TC(mmol/l)	3.7±0.75	3.9±0.74	3.8±0.7	4±0.7	0.225
SBP(mmHg)	134.9±16.8	137.5±18.1	137.7±16.9	138.8±19	0.719
DBP(mmHg)	75.7±11	79±10.7	77.9±9.4	75.3±13.1	0.344
LDL(mmol/l)	1.9±0.6	2±0.65	1.9±0.5	2.1±0.6	0.046*
AST(mmol/l)	25.4±12.1	25.4±9.6	25.8±8	27.3±10.5	0.897
ALT(mmol/l)	28.9±20.4	25.4±12.6	27.9±15.1	29.7±10.5	0.6

Quartile range 0.01-0.13; 0.14-0.18; 0.19-0.24; 0.25-0.93, \* Significant at p<0.05) ±standard deviations, Hip- Hip circumference, Waist- Waist circumference, BMI- body mass index, % fat- Percentage body fat, SBP- Systolic Blood pressure, DBP- Diastolic Blood pressure, TC- Total cholesterol, HDL- High density lipoprotein Cholesterol, TG- Triglycerides, LDL- Low density lipoprotein cholesterol, AST- Aspartate amino transferase, ALT- Alanine amino transferase.

In the age adjusted model, glycaemic control was worse in the lower quartile as indicated by HbA1c which was significantly high in the lower quartile ( $Q1=8.1\pm1.5$   $p=0.001$ ) compared to the upper quartile ( $7.1\pm1$ ). This significance persisted after adjusting for medications and those who had testosterone replacement during follow up ( $p=0.000$ ; Figure 5.8).

**Figure 5.7 HbA1c in quartiles of cBioT/CAG ratio**

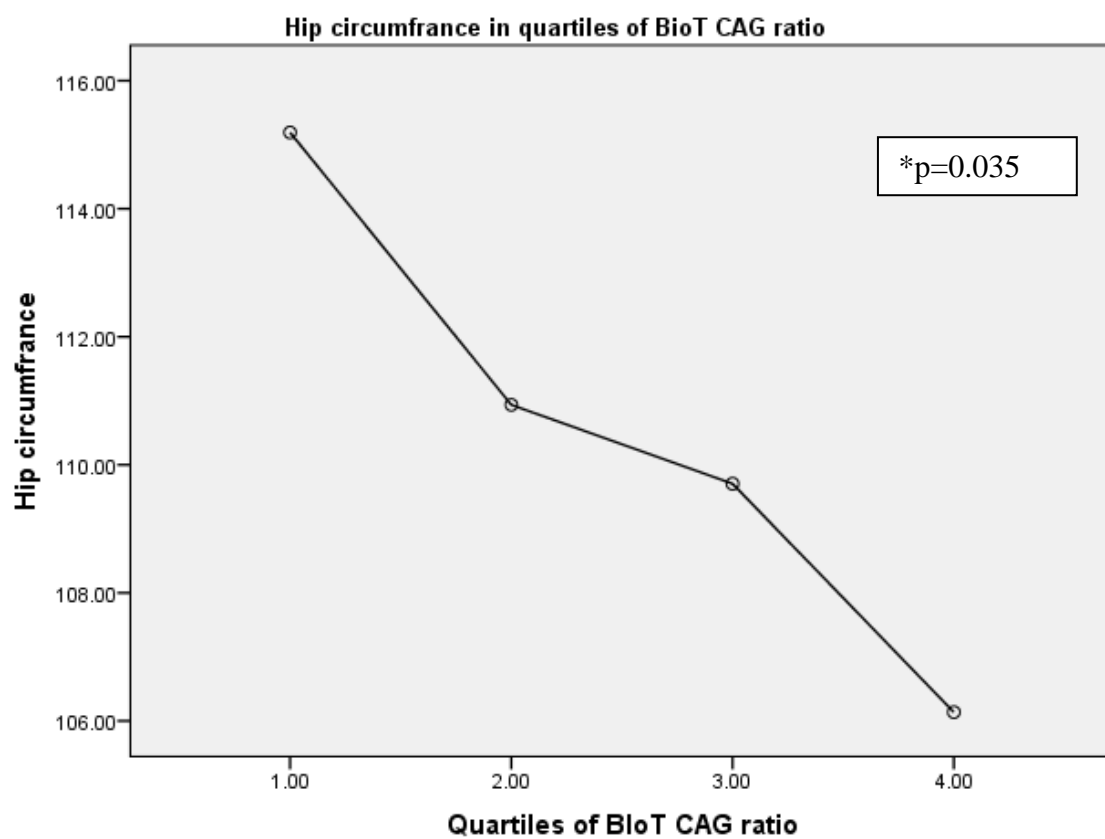


\*Adjusted for age, body fat percentage, hip circumference, waist circumference, BMI and concomitant medications including testosterone

Hip circumference ( $Q1=113.4\pm12$  and  $Q4=107.6\pm9.4$ ,  $p=0.002$ , weight ( $Q1=101.5\pm22.9$  and  $Q4=95.9\pm16$ ,  $p=0.025$ ) and BMI ( $Q1=33.6\pm6.7$  and  $Q4=31.7\pm4.9$ ,  $p=0.006$ ) were lower in the upper quartile in the age adjusted model. These changes also persisted after adjusting for medications and those who had TRT during follow up (Figure 5.9  $p=0.035$  for hip circumference, Figure 5.10  $p=0.003$  for weight and Figure 5.11  $p=0.001$  for BMI). However

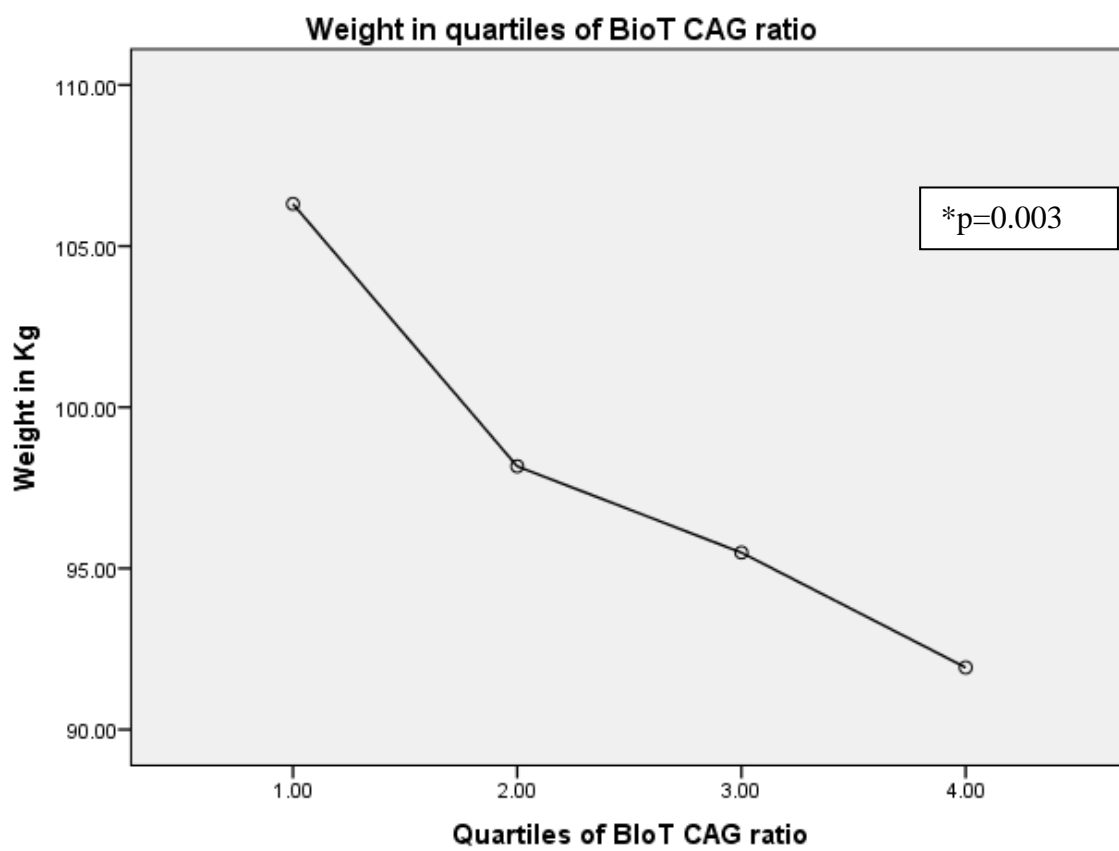
the significance for change in hip circumference was lost when weight, waist circumference, BMI or body fat percentage added as co variates in the analysis. Similarly BMI and weight changes also lost significance when body fat percentage, hip circumference or waist circumference were added as co variates.

**Figure 5.8 Hip circumferences in quartiles of cBioT/CAG ratio**



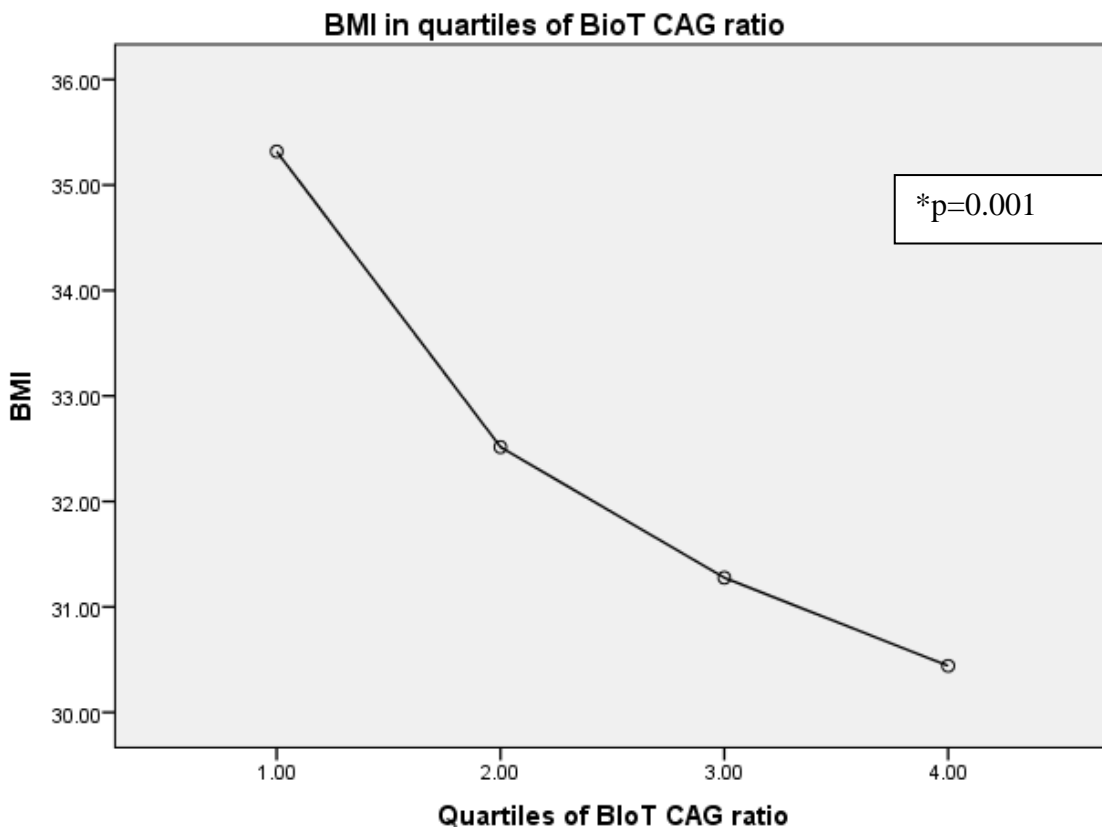
\* adjusted for age, medications including testosterone

**Figure 5.9 Weight in quartiles of cBioT/CAG ratio**



\*Adjusted for age, medications including testosterone

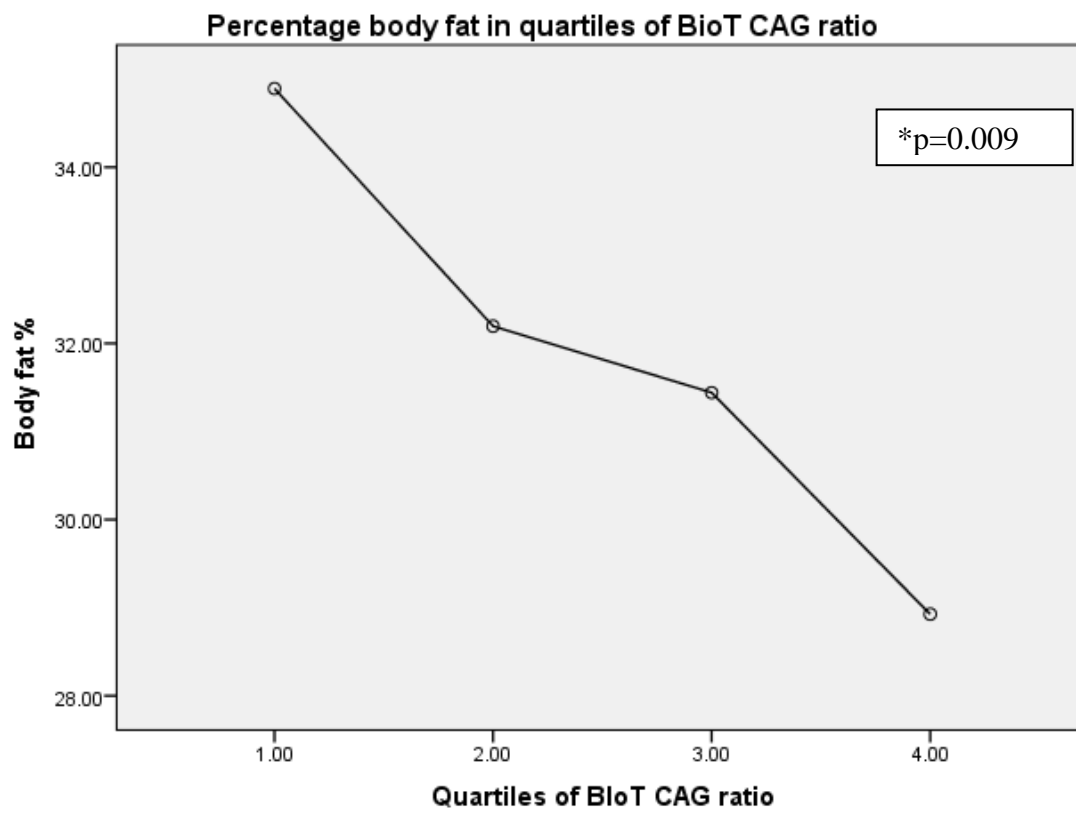
**Figure 5.10 Body mass index quartiles of cBioT/CAG ratio**



\*Adjusted for age, medications including testosterone

The change in percentage body fat was not significant in the age adjusted model between the quartiles (Q1=33.3±7.2 and Q4=30.6, p=0.131). However this became highly significant when adjusted for those who had TRT (p=0.009 Figure 5.12). Similarly waist circumference was not significant when adjusted only for age (Q1=116.7±17.2 and Q4=112.4±13.4 p=0.21) but became highly significant when adjusted for those who had TRT (p=0.002 Figure 5.13). These significances persisted after adjusting for medications.

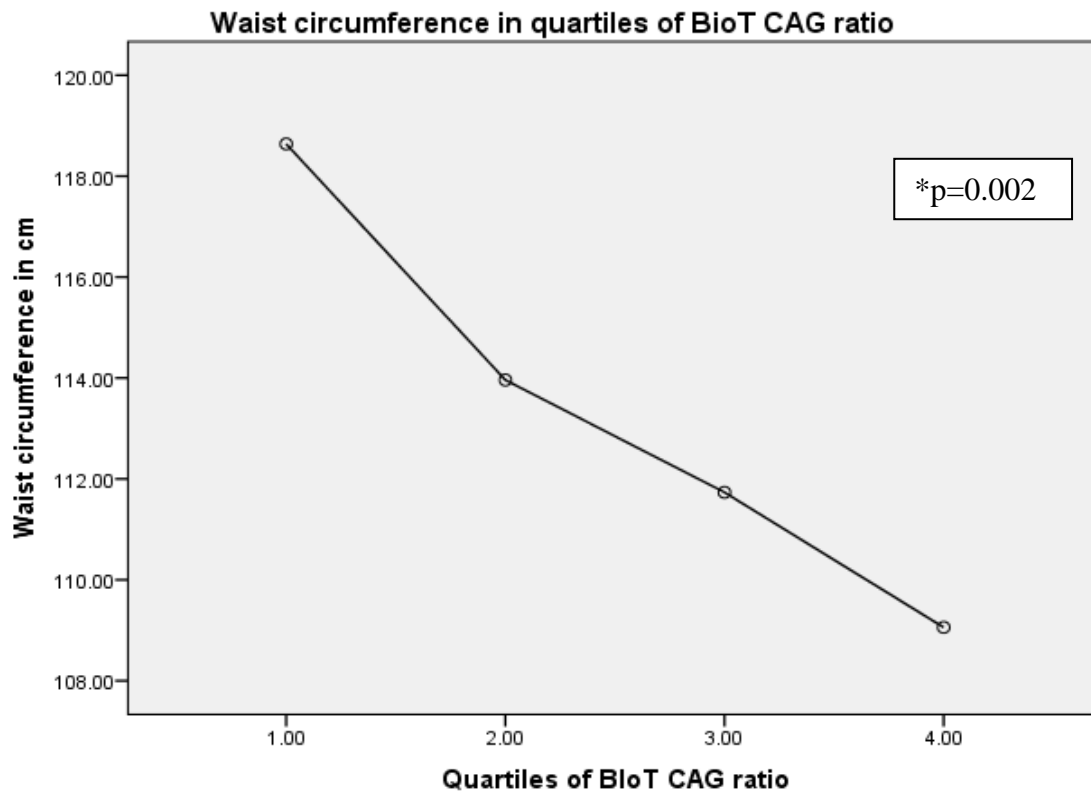
**Figure 5.11** Percentage body fat in quartiles of BioT/CAG ratio



\*Adjusted for age, medications including testosterone



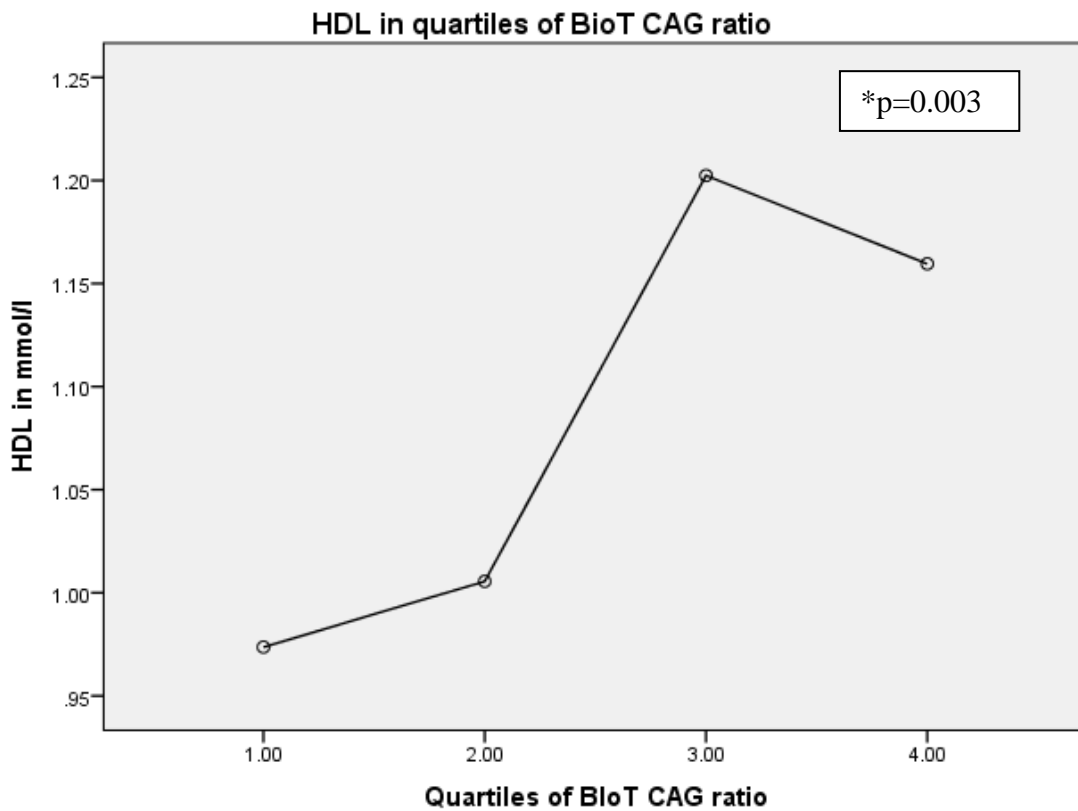
**Figure 5.12** Waist circumference in quartiles of BioT/CAG ratio



\*Adjusted for age, medications including testosterone

The HDL was significantly higher in the upper quartile ( $Q1=1.02\pm0.2$  and  $Q4=1.12\pm0.3$ ,  $p=0.001$ ). The significance persisted when adjusted for medications and those who had TRT ( $p=0.003$  Figure 5.14). In the age adjusted model there was a small but significant increase in LDL in the upper quartile ( $Q1=1.9\pm0.6$  and  $Q4=2.1\pm0.6$ ,  $p=0.046$ ). However this change became non-significant when adjusted for medications and those who had TRT ( $p=0.941$ ).

**Figure 5.13 HDL in quartiles of cBioT/AR CAG ratio**



\*Adjusted for age, medications including testosterone

There were no significant changes in total cholesterol (Q1=3.7±0.75 and Q4=4±0.7, p=0.225) or triglycerides (Q1=1.8±0.2 and Q4=1.1±0.3, p= 0.225) in either age adjusted or multivariate adjusted models.

Similarly there were no difference in systolic blood pressure (Q1=134.9±16.8mmHg, and Q4= 138.8±19 mmHg p=0.719), diastolic blood pressure (Q1=75.7±11mmHg and (Q4=75.3±13.1mmHg p=0.344), AST (Q1=25.4±12.1mmol/l and Q4=27.3±10.5mmol/l p=0.897), ALT (Q1=28.9±20.4mmol/l and Q4=29.7±10.5mmol/l p=0.6).

## **5.10 Haematocrit, Haemoglobin and cardiovascular risk profile in relation to testosterone**

Haematocrit (correlation coefficient 0.369,  $p < 0.000$ ) and haemoglobin (correlation coefficient 0.352,  $p < 0.000$ ) showed a direct correlation with testosterone levels. The data were further analysed using age adjusted model for the ratio of total testosterone and calculated bioavailable testosterone with both haematocrit and haemoglobin. In multivariate analysis there was no significant correlation between the ratio and individual cardiovascular risk profile or cardiovascular events.

## **5.11 Discussion and conclusions**

Previous research from our group have published data showing a negative correlation between testosterone and AR CAG and a positive correlation with obesity and leptin independent of testosterone, oestradiol, gonadotropins and age (Stanworth, Kapoor et al. 2008). It was also shown that AR CAG was correlated negatively with total, bioavailable and free testosterone, waist circumference, body mass index, leptin and systolic blood pressure. Furthermore there was an association between shorter AR CAG with low HDL-C and testosterone levels in men (Stanworth, Kapoor et al. 2011). Paradoxically this study found low testosterone levels were associated with a low HDL-C suggesting a divergent action of testosterone via AR dependant and AR no dependant pathways. More recently a sub study from the TIMES2 study reported AR CAG was independently positively associated with change in fasting insulin, triglycerides and diastolic blood pressure during testosterone replacement therapy with a trend to association with HOMA-IR but found no association of AR CAG with change in other glycaemic variables, other lipid variables or obesity (Stanworth, Akhtar et al. 2014).

In present study we did not find any significant changes in the cardiovascular risk profile in this cohort when analysed for different AR CAG lengths. The results were analysed after adjusting for those who had testosterone replacement during the follow up. There were still no significant effects on cardiovascular risk profile.

However there were significant changes in glycaemic control, lipid profile, weight, hip and waist circumference and body fat percentage when analysed using the ratio of testosterone to AR CAG length (T/CAG and BioT CAG ratio). This was suggested by the correlation analysis and confirmed using multivariate ANOVA for change in the mean values for each measure. The people in the highest quartile had the best cardiovascular risk profile compared to those in the lower quartiles. In the case of waist circumference and body fat percentage the changes became significant only when the effect of testosterone replacement therapy were added to the analysis. For BMI and weight the significance became more pronounced after the effect of testosterone replacement was added. HbA1c was highly significant at  $p < 0.01$  on all the analysis.

These results suggest that CAG in itself may be less important in the modulation of CV risk profile in the long term, a possibly more sensitive measure of androgenicity which takes into consideration of the biologically active testosterone levels may be more important marker for cardiovascular risk profile. The T/ CAG or BioT CAG ratio is seen to be linked closely to the cardio metabolic risk profile than CAG alone. Lower ratios were associated with higher HbA1c, weight, Body mass index, waist and hip circumference and body fat percentage. It also has the lower HDL compared to the higher ratio groups.

Further the results showed that when the effect of testosterone therapy was considered, the risk profile (BMI, weight, body fat percentage, waist circumference) showed more significant changes with more favourable profile in those with higher ratio. This suggests that testosterone therapy has a significantly beneficial effect on the cardiovascular risk profile and the cardio metabolic actions of testosterone are in part mediated through the androgen receptor. At the same time the changes in hip circumference, HbA1c, and HDL did not markedly change after adjusting for those with testosterone therapy. This may be due to the fact that they were highly significant to start with and no further statistically significant improvement were detectable. However it might also suggest that the action of testosterone on these parameters may not be mediated through the AR but mediated through other mechanisms.

In conclusion the ratio of testosterone to AR CAG (T/AR CAG or BioT/ AR CAG) is a better marker for cardiovascular risk profile than AR CAG alone and a higher ratio, reflecting better androgen action has a significantly positive outcome in relation to cardiovascular risk profile. This is novel concept and has the potential for wider clinical application for predicting the individual response to the testosterone action. This also suggests the cardio metabolic actions of testosterone are at least in part mediated through the androgen receptor.

## **5.12 Limitations**

This study shows a cross sectional correlation between testosterone/AR CAG ratio and cardiovascular risk profile. This is a new concept and need to be verified through prospective longitudinal studies.



## **Chapter six**

### **Animal Study: The effect of testosterone on the glucose and lipid metabolism in liver, muscle and adipose tissue of Tfm mice**

*My contributions to this chapter include initial design for the target molecule analysis, all of the tissue collections from Tfm mice ( liver, muscle, visceral adipose tissue and subcutaneous adipose tissue) and initial RNA extraction and QPCR analysis of the target molecules ( about a third of the total analysis). I also analysed and presented the initial data and contributed to its publications. The data presented includes further repeat analysis done by other members of the research team. The Animal Husbandry work were done by Dr Daniel Kelly from our research team.*

#### **6.1 Introduction**

As discussed in the previous chapters there is evidence to suggest that testosterone deficiency in men is an independent cardiovascular risk factor and is associated with obesity, metabolic syndrome and type-2 diabetes (Jones 2010, Kelly and Jones 2013). Insulin resistance, common to the three disease states correlates negatively with serum testosterone (Marin, Holmang et al. 1992, Pitteloud, Mootha et al. 2005). Studies have clearly demonstrated that testosterone is a multi-system hormone serving a wide range of hitherto unsuspected biological functions (Saad 2015). Studies have also shown that patients who receive androgen deprivation therapy are at a higher risk of diabetes, cardiovascular disease and increased mortality (Jones 2011). Some studies also reported testosterone replacement

therapy improves cardiovascular risk profile including central adiposity, percentage body fat, hepatic steatosis, lipid profile and insulin resistance (Kelly and Jones 2013).

Although there are many studies looking at testosterone action at molecular level both in vivo and in vitro, tissue specific action remain poorly understood (Kelly and Jones 2013). Our research team has previously published studies in testicular feminised mouse (Tfm) mouse (which has non-functional androgen receptor and low testosterone levels) showing low testosterone levels are associated with increased fatty streaks in aortic root and increased lipid deposition in liver when mice were fed lipid rich diet (Nettleship, Jones et al. 2007, Kelly, Sellers et al. 2013, Kelly, Nettleship et al. 2014). It has also shown that testosterone replacement reverses the metabolic abnormalities induced by the high fat diet (Kelly, Nettleship et al. 2014).

There is evidence of heterogeneity of glucose and lipid metabolism in different tissues (Rask-Madsen and Kahn 2012). However the mechanism by which testosterone affects metabolic syndrome, type 2 diabetes and cardiovascular risk are unknown.

The target enzymes in the glucose metabolism investigated are major rate limiting enzymes in the glycolysis pathway (Hexokinase 2, Glucokinase, and Phosphofructokinase), the major enzyme of cellular glucose transport - GLUT4, rate limiting enzyme in the pentose phosphate pathway (glucose 6 phosphate dehydrogenase and the major rate limiting enzyme of glycogen synthesis) glycogen synthase.



In the lipid metabolism the target enzymes are the key enzymes in the fatty acid synthesis (Fatty acid synthase, Acetyl CoA carboxylase and Stearoyl-CoA desaturase 1), fatty acid hydrolysis (Lipoprotein lipase and Hormone Sensitive lipase), ATP-binding cassette transporter A1 which promotes cellular phospholipid and cholesterol efflux and two important transcription factors linked to cholesterol and fatty acid synthesis, Sterol regulatory element-binding protein 1 and Sterol regulatory element-binding protein 2

We also studied the master regulatory receptors of carbohydrate and lipid metabolism, liver X receptor, Peroxisome proliferator-activated receptor alpha and Peroxisome proliferator-activated receptor gamma. The target genes studied are summarised in table 6.1.

The animal model experiments investigated here examines three physiological situations Tfm mouse which has non-functional androgen receptor and a low testosterone, littermate control which has normal functioning androgen receptor and the testosterone treated Tfm mouse which has non-functioning androgen receptor with physiological testosterone levels

**Table 6.1 Target genes involved in carbohydrate and lipid metabolism studied**

Target	Gene	Function
<b>Targets enzymes of Carbohydrate metabolism</b>		
Hexokinase 2	Hk2	Phosphorylates glucose to glucose 6-phosphate in the glycolytic pathway
Hexokinase 4 (Glucokinase)	Gck	Phosphorylates glucose to glucose 6-phosphate in the glycolytic pathway
Phosphofructokinase	Pfk	Converts fructose-6-phosphate to fructose-1,6-bisphosphate, one of the most important step in glycolytic pathway
Glucose transporter 4	Glut4	Cellular glucose transport
Glucose-6-phosphate dehydrogenase	G6pdx	Enzyme in the pentose phosphate pathway, often for tissues actively
Glycogen synthase	Gys	Converts glucose to glycogen for storage, regulating glycogen/glucose levels
<b>Targets enzymes of Lipid metabolism</b>		
Fatty Acid Synthase	Fasn	Catalyses the formation of long-chain fatty acids in fatty acid synthesis
Acetyl CoA carboxylase	Acaca	Essential role in regulating fatty acid synthesis
Stearoyl-CoA desaturase 1	Scd1	Catalyses a rate-limiting step in the synthesis of unsaturated fatty acids. Key enzyme in fatty acid metabolism.
Lipoprotein lipase	Lpl	Hydrolysis of triglycerides into free fatty acids
Hormone sensitive lipase	Lipe	Hydrolyses stored triglycerides to free fatty acids
Sterol regulatory element-binding protein 1	Srebf1	Cholesterol biosynthesis and uptake, and fatty acid biosynthesis
Sterol regulatory element-binding protein 2	Srebf2	Cholesterol biosynthesis and uptake, and fatty acid biosynthesis
Apolipoprotein E	Apoe	Lipoprotein metabolism and transport.
ATP-binding cassette transporter A1	ABCA1	Major regulator of cellular cholesterol efflux and phospholipid homeostasis
<b>Regulatory receptors</b>		
Liver X receptor alpha	Nr1h3	Nuclear receptor transcription factor regulating cholesterol, fatty acid, and glucose homeostasis
Peroxisome proliferator-activated receptor alpha	Ppara	Transcription factor and major regulator of lipid metabolism
Peroxisome proliferator-activated receptor gamma	Pparg	Regulates fatty acid storage and glucose metabolism

## **6.2 Hypothesis**

I hypothesised that testosterone differentially regulates the enzymes modulating carbohydrate, lipid metabolism and the master regulators of metabolism in liver, muscle and adipose tissues of Tfm mouse with low testosterone adversely affecting the metabolic profile. I further hypothesised that testosterone replacement reverses the adverse metabolic profile induced by testosterone deficient state through the actions independent of classic androgen receptor.

## **6.3 Materials and methods**

### **6.3.1 The Testicular Feminised Mouse**

This mouse is discussed in detail in chapter 1. The Tfm mouse originates from the C57BL/6 strain which has developed a mutation in the gene encoding the classical androgen receptor and a deficiency of the enzyme 17 $\alpha$  hydroxylase (Charest, Zhou et al. 1991, Murphy and Oshaughnessy 1991, Legoascogne, Sananes et al. 1993). Tfm mice express a non-functional AR and low levels of circulating testosterone and consequently have severe testosterone deficiency. Therefore Tfm mouse is considered a suitable model for investigating the effects of AR in testosterone function.

### 6.3.2 Animal Husbandry

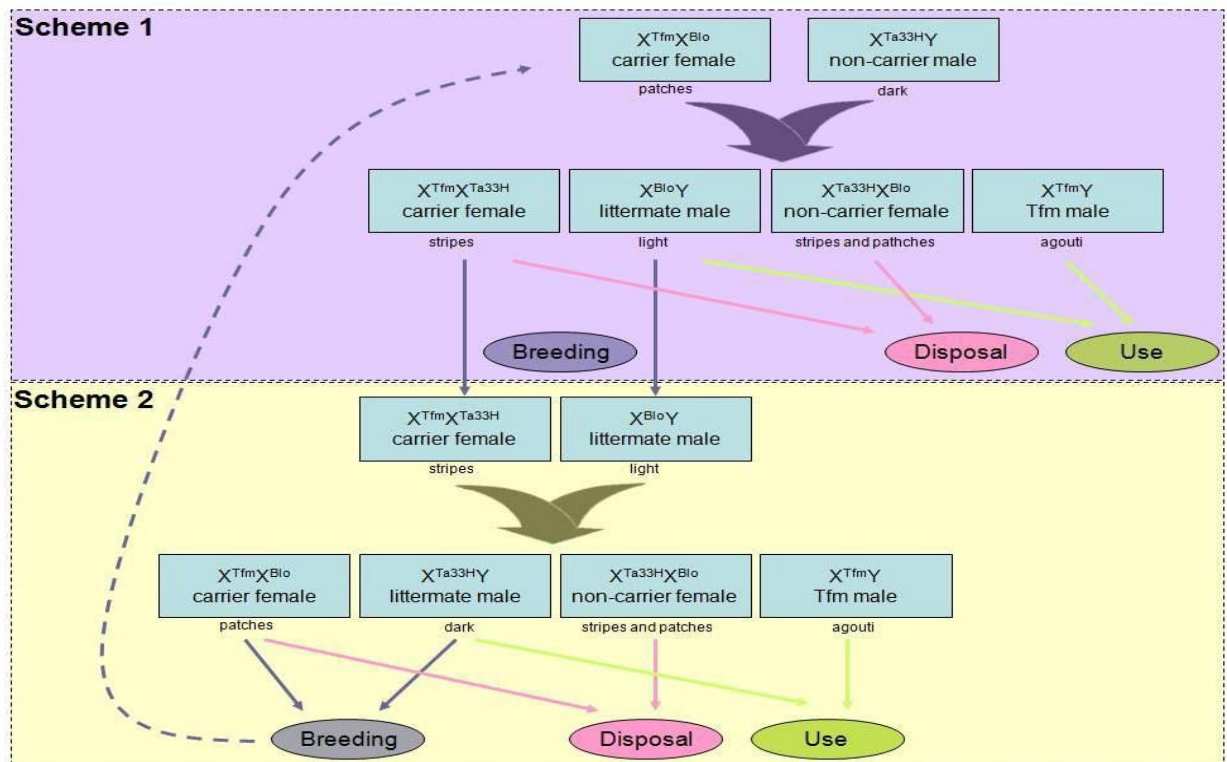
**(The section 2.7.2 to 2.7.4 work were done by Dr Daniel Kelly from our research team)**

The inherited mutation in the AR of Tfm mice is X-linked resulting in only one quarter of the progeny from a breeding pair being affected. Two breeding schemes were employed in the present study using mice of specified genotype. The mice were identified by inherent coat colour markers and gender phenotype (Figure 3.1). Tfm mice (XTfmY males) exhibit a dark agouti coat colour which is female fur phenotype. Unaffected littermate males (XBloY or XTa33HY) have a male phenotype and have either light coloured coats (XBloY) or dark agouti coats, with bald patches behind the ears and dark patches around the eyes (XTa33HY). Carrier females (XTfmX) have light fur and exhibit prominent transverse stripes or have dark fur with irregular patches whereas non-carrier females (XTa33HX or XBloX) have both stripes and light coloured patches (figure 6.1).

The breeding colony of Tfm mice (strain C57BL/6J-A) used in this study was derived from frozen embryos obtained from the Medical Research Council genome project (MRC Harwell, UK). Mice were bred in sterile barrier conditions at The University of Sheffield Field Laboratories and XTfmY and male littermate controls (XBloY or XTa33HY) were transferred to the holding room upon weaning (6 weeks old) where they were maintained for the duration of the experiment. Non-carrier females and carrier females not required for breeding were sacrificed via a UK Home Office-approved schedule 1 method once weaned. All animals used for the experimental procedures were maintained in enclosures containing up to 3 animals on a twelve-hour light/dark cycle in a temperature (between 19 and 23°C) and

humidity controlled ( $55 \pm 10\%$ ) environment. All procedures were carried out under the jurisdiction of UK Home Office personal and project licences (project licence number 40/3165, personal licence number 60/11754), governed by the Animals Scientific Procedures Act 1986.

**Figure 6.1 Schematic representation of the two breeding schemes used to generate Tfm mice**



Carrier females ( $X^{Tfm}X^{Blo}$  and  $X^{Tfm}X^{Ta33H}$ ) and non-carrier males ( $X^{Ta33HY}$  and  $X^{Blo}Y$ ) are bred producing a 1:4 ratio of affected offspring ( $X^{Tfm}Y$ ), identified by coat-colour and markings. Adapted from Jones et al. 2003(Jones, Pugh et al. 2003).

### 6.3.3 Experimental treatments

Animals were randomly assigned to specific diet and treatment groups after weaning until desired group numbers were reached. The experiment groups are summarised in the table 6.2.

**Table 6.2 Mouse experiment treatment groups**

Strain	Diet	Treatment	Abbreviation
<b>XY Littermate</b>	Normal chow diet	No treatment	XY ND
	High-cholesterol diet	No treatment	XY D
		10µl intra-muscular saline injection	XY S
<b>Testicular feminised mouse</b>	Normal chow diet	No treatment	Tfm ND
	High-cholesterol diet	No treatment	Tfm D
		10µl intra-muscular	Tfm S
		10µl intra-muscular sustanon100® injection	Tfm T

### **6.3.3.1 Promotion of Metabolic Syndrome State**

At age 8 weeks Tfm and littermate controls were placed on a diet containing 42% butterfat and 1.25% cholesterol, along with 0.5% cholate, which is required for cholesterol absorption in the mouse (Special Diet Services, UK) for a period of 28 weeks, ad libitum. Prior to receiving this high-cholesterol diet animals received a normal chow diet. Control mice received normal chow diet for the duration of the study.

### **6.3.3.2 Testosterone treatment**

Mice that underwent testosterone or saline treatment received this via intramuscular injections. The hind leg of the mouse was held to immobilise the quadriceps and the injection site shaved with a hair trimmer previously cleaned with hibitane (SSL International Plc, UK). The shaven area was then gently wiped clean using a 1:200 dilution of hibitane. A sterile 0.3mL 30G needle (BD, UK) was introduced at right angle to the skin surface into the centre of the muscle mass and 10 $\mu$ L of either Sustanon® 100 (20mg/mL testosterone propionate, 40mg/mL testosterone phenylpropionate, 40mg/mL testosterone isocaproate; equivalent to 74mg per mL testosterone) or physiological saline was injected. Animals were then returned to cages. Mice were injected once fortnightly from 7 weeks of age alternating the leg injected to minimise discomfort and irritation.

Animals were carefully monitored for the duration of the study and were weighed on a weekly basis.

#### **6.3.4 Sry gender determination of animals**

Sry (sex determining region Y) gene is a locus located on the Y chromosome. The Sry protein is a testis-specific transcription factor that promotes several genes leading to the formation of the testis and subsequent sexual differentiation and development in males (Sekido 2010). This phenotypic differentiation and male development is driven by the production and secretion of testosterone. Tfm mice are phenotypically female as they possess a non-functional AR and express only low levels of testosterone both elements necessary for male differentiation (Murphy et al. 1991), but they remain genetically male. Female mice lack the Sry gene whereas males express the Sry gene regardless of their phenotype. Therefore testing for the presence or absence of the Sry gene is very useful in the molecular assessment of mice with abnormal sexual differentiation.

The following methods were carried out in conjunction with BioServ UK Ltd, part of the University of Sheffield.

#### **6.3.5 Collection of animal tissues**

At the end of the treatment period (36 weeks old) mice were killed by cervical dislocation, a Home Office approved Schedule 1 technique.

All samples collected from the mice after this point were labelled numerically, corresponding to the individual animals. Details of the experimental procedures that each animal underwent were not revealed until all sample analysis was complete so that the investigator was blind to the test groups throughout sample processing.



### **6.3.5.1 Serum Collection**

Following cervical dislocation a mid-line sternotomy was performed, the diaphragm was slit open and the thoracic aorta severed. Whole blood for serum measurements was collected from the chest cavity using a 2ml syringe (BD, UK) collected into 1.5ml Eppendorf tubes and allowed to clot for a minimum of 30 minutes at room temperature. Whole blood was then centrifuged at  $0.8 \times g$  for 10 minutes at room temperature and the serum removed and frozen in 60 $\mu$ l aliquots at  $-80^{\circ}\text{C}$  until analysis. All analyses were carried out on non-pooled serum and samples underwent only one freeze-thaw cycle to maintain sample integrity.

### **6.3.5.2 Tissue collection**

The heart and the aorta were used for further experiments by colleagues at the Sheffield Hallam University. These tissues were removed following mid-line sternotomy and removal of whole blood from the chest cavity. The rib cage was opened and the lungs dissected clear. The heart with the thoracic aorta attached was carefully dissected free from the adventitia.

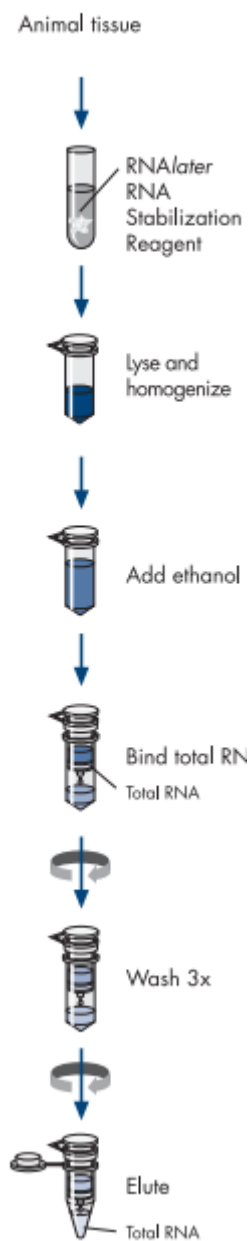
The subcutaneous fat was dissected from under skin of thoracic and abdominal wall and the visceral fat was removed from the omentum. The liver tissues were removed and muscle samples were collected from the thigh muscles. This was then transferred into saline and RNAlater® solution. All these tissues were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further analysis. The RNAlater® solution is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. RNAlater® solution minimizes the need to immediately process tissue samples. Tissue pieces can be harvested and submerged in RNAlater® solution for storage without jeopardizing the quality or quantity of RNA obtained after subsequent RNA isolation.

### **6.3.6 RNA extraction procedure from the liver, subcutaneous tissues and muscle (Adapted from QIAzol Lysis Reagent Handbook; Figure 6.2)**

Small amount of the tissue, approximately 100mg, was excised from the stored sample. This was placed in in 2ml of Phenol/guanidine-based QIAzol Lysis Reagent. QIAzol Lysis Reagent is used to remove the genomic DNA from the tissue. The combination of organic extraction and chaotropic disruption contributes to efficient lysis and higher yields of total RNA. The organic extraction step removes both proteins and DNA improving the efficiency of further purification steps. The tissue was homogenised using Tissue Lyser Probe. After placing the tube at room temperature for 5 minutes Chloroform was added and shook vigorously for 15 seconds. The addition of chloroform causes phase separation where protein is extracted to the organic phase, DNA resolves at the interface and RNA remains in the aqueous phase. The tube was then placed at room temperature for 15minuts and then centrifuged at 12000Xg for 15 minutes at  $-4^{\circ}\text{c}$ .The aqueous phase which contain the RNA was then transferred to a new tube and 1 volume of 70% ethanol and vortex was added. The sample was then transferred to RNAeasy column in 2 ml tube. This was centrifuged for 15seconds at 13000Xg at room temperature. The follow through is discarded. Repeated the above using the residue of the solution. 700 microliters of Buffer RW was added. Buffer RW1 contains a guanidine salt as well as ethanol and is used as a stringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins, fatty acids etc., that are non-specifically bound to the silica membrane. The tube is then centrifuged for 15 seconds at 13000xg. 500 Microliters of Buffer RPE to RNeasy spin column. Buffer RPE is a mild washing buffer and the main function is to remove traces of salts Centrifuged for 15sec at 13000xg to wash the membrane. The follow through is discarded. 500microlitre of Buffer RPE was added to RNeasy column, centrifuged for 15 seconds at 13000xg, follow through is discarded. 500microlitres of Buffer RPE was added to RNAeasy Spin column centrifuged for

2 minutes at 13000xg. The RNeasy Spin column was placed in a new 1.5ml collection tube 30 microlitres of RNase free water added to the spin column membrane. To elute the RNA, centrifuge for 1 minute at 13000xg. The RNA yield was checked using agarose gel and Nano Graph.

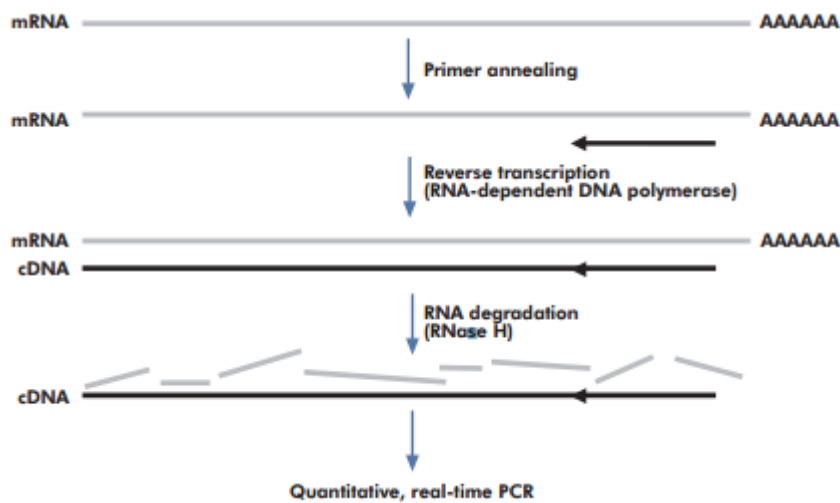
**Figure 6.2 RNA extraction procedure**



### 6.3.7 cDNA preparation using QuantiTect Reverse Transcription Procedure (Figure 6.3)

Reverse transcriptase enzymes are generally derived from RNA-containing retroviruses such as avian myeloblastosis virus (AMV), Moloney murine leukaemia virus (MMLV), or human immunodeficiency virus (HIV). Quantiscript Reverse Transcriptase is from a new source.

**Figure 6.3** Enzymatic activities of reverse transcriptase

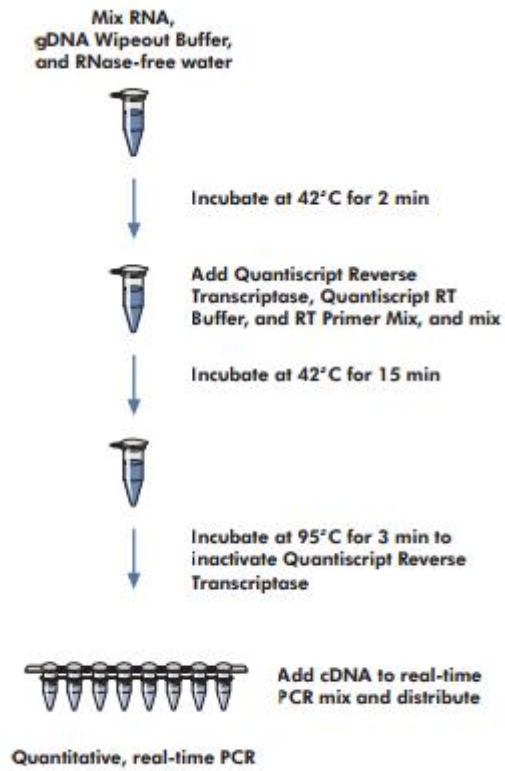


The reverse transcriptase enzymes have 3 different enzymatic activities. First action is an RNA-dependent DNA polymerase, the second is hybrid-dependent exoribonuclease (RNase H) and the third a DNA-dependent DNA polymerase. In the retroviral genome all the three actions allow the RNA virus to produce double stranded DNA. For in vitro studies we use the first two activities of the enzyme to produce a cDNA which is single stranded.

### **6.3.7.1 Reverse transcription protocol (Adapted from QuantiTect® SYBR® Green PCR Handbook; Figure 6.4)**

1. Thaw template RNA on ice. Thaw gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water at room temperature (15–25°C).  
Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes and then store on ice.
2. Prepare the genomic DNA elimination reaction on ice according to Table 6.3. Mix and then store on ice.
3. Incubate for 2 min at 42°C. Then place immediately on ice. Note: Do not incubate at 42°C for longer than 10 min.
4. Prepare the reverse-transcription master mix on ice according to Table 6.4 Mix and then store on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA.
5. Add template RNA from step 3 (14 µl) to each tube containing reverse-transcription master mix. Mix and store on ice.
6. Incubate for 15 min at 42°C.
7. Incubate for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase.
8. Add an aliquot of each finished reverse-transcription reaction to real-time PCR mix

**Figure 6.4** QuantiTect reverse transcription procedure



**Table 6.3 Genomic DNA elimination reaction components**

Component	Volume/reaction	Final concentration
gDNA Wipeout Buffer, 7x	2 $\mu$ l	1x
Template RNA	Variable (up to 1 $\mu$ g*)	
RNase-free water	Variable	
Total volume	14 $\mu$ l	–

\* This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present, and regardless of the primers used or cDNA analysed.

**Table 6.4 Reverse-transcription reaction components**

Component	Volume/reaction	Final concentration
<b>Reverse-transcription master mix</b>		
Quantiscript Reverse Transcriptase*	1 $\mu$ l	
Quantiscript RT Buffer,	5x†‡ 4 $\mu$ l	1x
RT Primer Mix‡	1 $\mu$ l	
<b>Template RNA</b>		
Entire genomic DNA elimination reaction (step 3)	14 $\mu$ l (add at step 5)	
Total volume	20 $\mu$ l	-

\* Also contains RNase inhibitor. † Includes Mg<sup>2+</sup> and dNTPs. ‡ For convenience, premix RT Primer Mix and 5x Quantiscript RT Buffer in a 1:4 ratio if RT Primer Mix will be used routinely for reverse transcription. This premix is stable when stored at –20°C. Use 5  $\mu$ l of the premix per 20  $\mu$ l reaction.

**6.3.7.2 Protocol for Quantitative, Real-Time PCR using SYBR Green I  
(Adapted from QuantiTect® SYBR® Green PCR Handbook)**

1. Thaw 2x QuantiTect SYBR Green PCR Master Mix (if stored at  $-20^{\circ}\text{C}$ ), template DNA or cDNA, primers, and RNase-free water. Mix the individual solutions.
2. Prepare a reaction mix according to Table 6.5.
3. Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
4. Add template DNA or cDNA (500 ng/reaction) to the individual PCR tubes or wells containing the reaction mix.
5. Program real-time cycler according to the program outlined in Table 6.6. Data acquisition should be performed during the extension step.
6. Place the PCR tubes or plates in the real-time cycler and start the cycling program.



**Table 6.5 Reaction setup Real-Time PCR using SYBR Green I**

<b>Component</b>	<b>Volume/reaction</b>	<b>Final concentration</b>
2x QuantiTect SYBR Green PCR Master Mix*	25 $\mu$ l <sup>†</sup>	1x
Primer A	Variable	0.3 $\mu$ M <sup>‡</sup>
Primer B	Variable	0.3 $\mu$ M <sup>‡</sup>
Template DNA or cDNA (added at step 4)	Variable	500 ng/reaction
RNase-free water	Variable	
Total reaction volume	50 $\mu$ l	

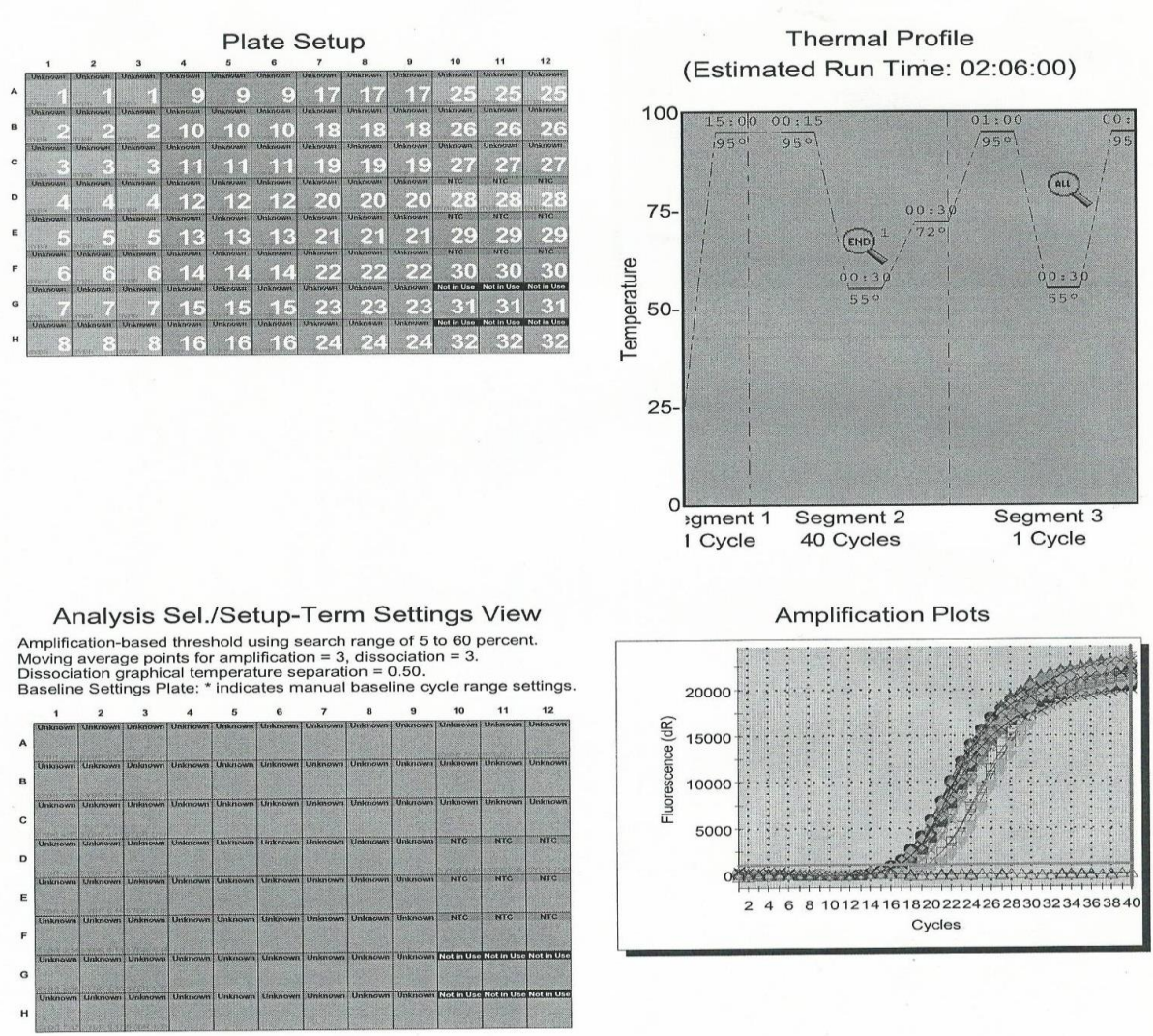
\* Provides a final concentration of 2.5 mM MgCl<sub>2</sub>. † If using a total reaction volume other than 50  $\mu$ l, calculate the volume of 2x master mix required using this formula: Volume of 2x master mix ( $\mu$ l) = 0.5 x [Total reaction volume ( $\mu$ l)] ‡ A final primer concentration of 0.3  $\mu$ M is usually optimal. However, for individual determination of optimal primer concentration, a primer titration from 0.2  $\mu$ M to 1  $\mu$ M can be performed. SmartCycler users should use a final primer concentration of 0.5  $\mu$ M for each primer; if necessary, a primer titration from 0.5  $\mu$ M to 1  $\mu$ M can be performed to determine the optimal primer concentration.

**Table 6.6 Real-time cyclers conditions**

Step	Time	Temperature	Additional comments
PCR initial activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step
3 (4)-step cycling:			
Denaturation*	15 s	94°C	
Annealing	30 s	50–60° C	Approximately 5–8°C below $T_m$ of primers
Extension Perform fluorescence data collection, unless an additional data acquisition step has been integrated	30 s	72°C	
Number of cycles	35–45		The number of cycles depends on the amount of template DNA

\* SmartCycler users can reduce denaturation time to 1 s to take advantage of cycling capacities.

**Fig 6.5 Plate setup, Thermal profile and amplification plots of typical PCR experiment for RT PCR**



Each tissue sample is done in triplicate and a total of 9 tissues samples analysed in one experiment

The plate setup, thermal profile and amplification plots of typical PCR experiment for RT PCR are shown in figure 6.5. Results of QPCR were expressed as fold changes in the relative copy number  $2^{-\Delta\Delta CT}$ .

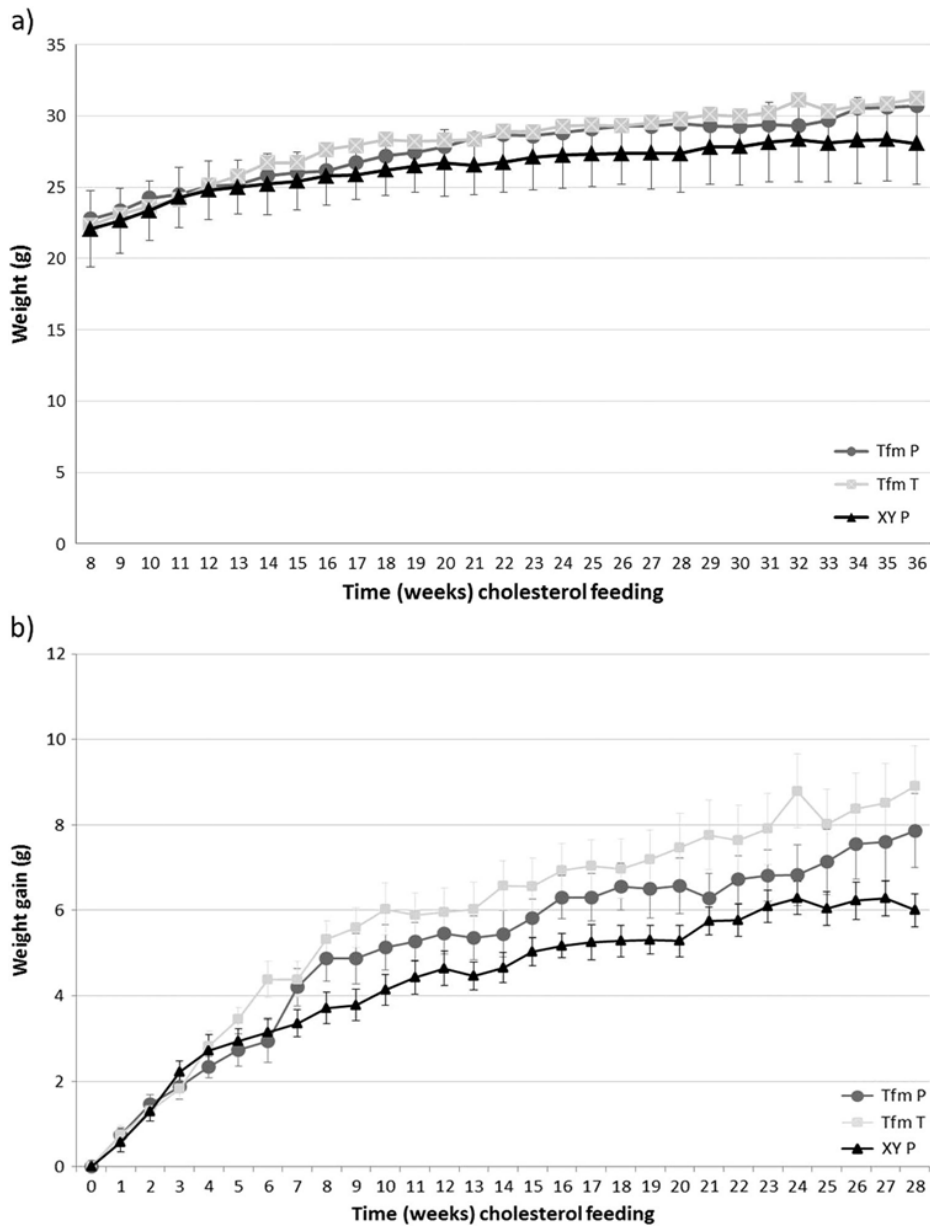
## 6.4 Statistical analysis

Results are presented as mean  $\pm$  standard error of mean. Differences between groups with normally distributed data were compared using unpaired tests without assuming consistent standard deviations of groups. Mann–Whitney U tests were used where data did not follow a normal distribution. Significance was accepted at  $p \leq 0.05$ .

## 6.5 Results

As expected the serum testosterone levels were greatly reduced in Tfm mice ( $2.2 \pm 1.2$  nM,  $p = 0.03$ ) compared to wild-type equivalents ( $16.5 \pm 4.3$  nM). There was no significant difference between the testosterone levels of treated and wild type animals ( $14.7 \pm 5.2$  nM,  $p = 0.98$ ). There were no significant changes in weight between groups over the duration of the 28 week feeding period. However, there was a trend towards Tfm mice gaining more weight ( $p = 0.066$ ,  $n = 14$ ) when compared to other groups toward the end of the study Figure 6.6(Kelly, Akhtar et al. 2016).

**Figure 6.6 Weight change and weight gain during the experiment in the mice**



Animal weights and weight gain. Tfm mice receiving either placebo (Tfm P) or testosterone (Tfm T) and wild-type XY littermates receiving placebo (XY P) had total body weight (a) measured at weekly intervals from the commencement of high- cholesterol diet feeding at week 8 through to the end of the study at week 36. Weight gain (b) was calculated from starting weights of individual animals. No significant differences were noted between groups (Kelly, Akhtar et al. 2016)

## **6.5.1 Target enzymes of carbohydrate metabolism**

The results of expression of the targets involved in the carbohydrate metabolism in high fat fed Tfm mice (placebo and testosterone treated), compared with wild type and XY littermates are summarised in table 7.2.

### **6.5.1.1 Effect of high fat diet on Tfm mice as compared to XY littermates**

Tfm mouse, when compared to XY littermates had significantly low gene expression for the regulatory enzymes in the glycolytic pathway, Hexokinase 2 (HK2), in the muscle ( $1.18 \pm 0.19$  and  $0.5 \pm 0.16$   $p=0.012$ ) and subcutaneous tissues ( $1.32 \pm 0.36$  and  $0.24 \pm 0.05$   $p=0.009$ ) and glucokinase (GCK or Hexokinase 4) in the liver ( $1.07 \pm 0.1$  and  $0.47 \pm 0.14$   $p=0.002$ ).

The phosphofructokinase (PFK) were significantly lower in the (muscle  $1.28 \pm 0.23$  and  $0.64 \pm 0.16$   $p = 0.032$ ), liver ( $0.62 \pm 0.1$  and  $1.19 \pm 0.11$   $p = 0.04$ ) and subcutaneous adipose tissue ( $1.76 \pm 0.68$  vs.  $0.16 \pm 0.05$   $p = 0.03$ ) of Tfm mouse compared to the XY littermates. Visceral adipose tissue, which mainly expresses the hexokinase2, did not show any significant difference of expression of this enzyme between the groups. PFK measurement in visceral fat could not be completed because of technical errors with the experiment and a repeat was not possible as there were no further visceral fat tissue available.

Glucose transporter4 (GLUT4) was similarly decreased in muscle ( $1.2 \pm 0.19$  and  $0.59 \pm 0.14$   $p=0.015$ ) and subcutaneous adipose tissue ( $1.31 \pm 0.32$  and  $0.37 \pm 0.12$   $p = 0.014$ ) of Tfm mice compared to their wild type counterparts.

G6PDx, was elevated in the liver of Tfm mice compared to XY mice  $1.03 \pm 0.07$  and  $1.99 \pm 0.2$  ( $p < 0.001$ ). Glycogen synthase (Gsy) which is expressed mainly in muscle and liver did not differ in the two groups of tissues in the two animal groups. Glycogen synthase was not expressed in adipose tissue and no experiment was performed in these tissues for the enzyme

#### **6.5.1.2 Effect of testosterone administration Tfm mice as compared to Tfm littermates without testosterone**

Testosterone administration increased glucokinase GCK expression ( $0.97 \pm 0.13$  and  $0.47 \pm 0.14$  ( $p = 0.015$ )) in the liver of Tfm. There was a trend towards significance in the reduction of the elevated G6PD expression of Tfm mouse in the testosterone treated mouse ( $1.99 \pm 0.2$  in Tfm mouse on placebo vs  $1.45 \pm 0.18$  the testosterone treated Tfm mouse  $p = 0.058$ ).

There were no significant changes in the treated group for the other enzymes. The major targets in carbohydrate metabolism in the three groups are summarised in table 6.7 and shown in figure 6.7.1 to figure 6.7.8.

**Table 6.7 Effect of testosterone on regulatory enzymes in carbohydrate metabolism**

Gene	Muscle			Liver			Subcutaneous fat			Visceral fat		
	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100
HK2	1.18 ±0.19	0.5 ±0.16*	0.54 ±0.1				1.32 ±0.36	0.24 ±0.05**	0.61 ±0.19	0.97 ±0.23	1 ±0.21	1.57 ±0.54
GCK				1.07 ±0.1	0.47 ±0.14**	0.97 ±0.13†						
PFK	1.28 ±0.23	0.64 ±0.16**	0.62 ±0.1	1.19 ±0.11	0.79 ±0.15*	0.77 ±0.06	1.76 ±0.68	0.16 ±0.05*	0.54 ±0.31			
GLUT4	1.2±0.19	0.59 ±0.14*	0.71 ±0.13				1.31 ±0.32	0.37±0.1 *	0.73 ±0.29	1.03 ±0.1	1.24 ±0.26	0.83 ±0.16
G6PDx	1.13 ±0.19	1.72 ±0.38	0.92 ±0.12	1.03 ±0.07	1.99 ±0.2***	1.45 ±0.18 <sup>α</sup>	1.06 ±0.23	2.67 ±1.36	1.09 ±0.16	1.57 ±0.59	2.53 0.87	1.69 ±0.4
Gys1	1.14 ±0.15	1.01 ±0.31	1.82 ±0.72	1.18 ±0.18	2.13 ±0.65	1.56 ±0.22						

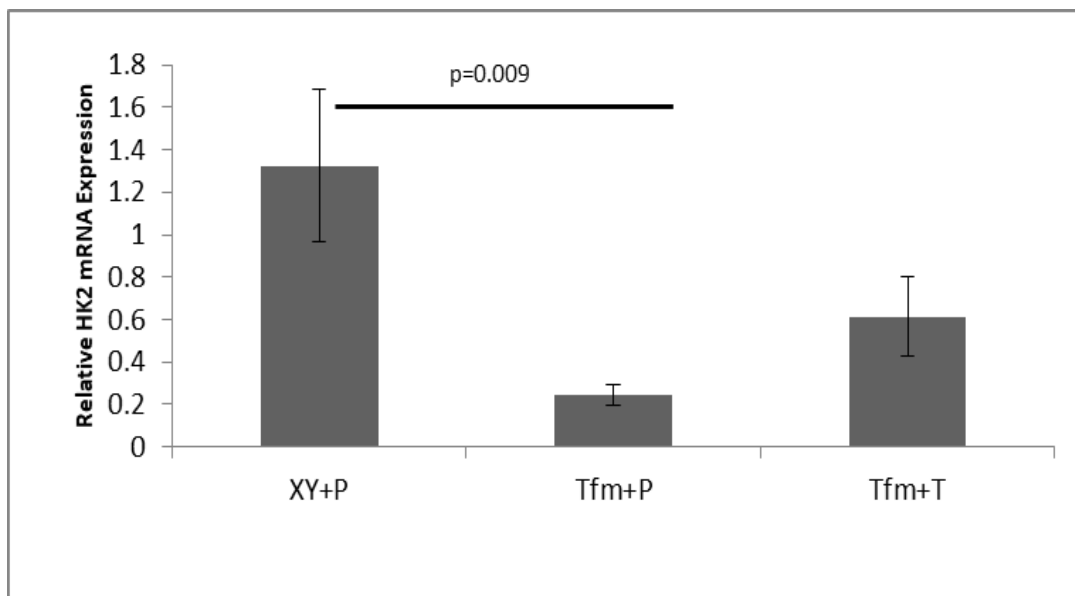
Relative tissue-specific qPCR end-point analysis of selected genes of carbohydrate metabolism between three groups of mouse N=11. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 versus XY placebo, †p < 0.05, ††p < 0.01 versus Tfm placebo, α- p = 0.058. ±standard error on mean



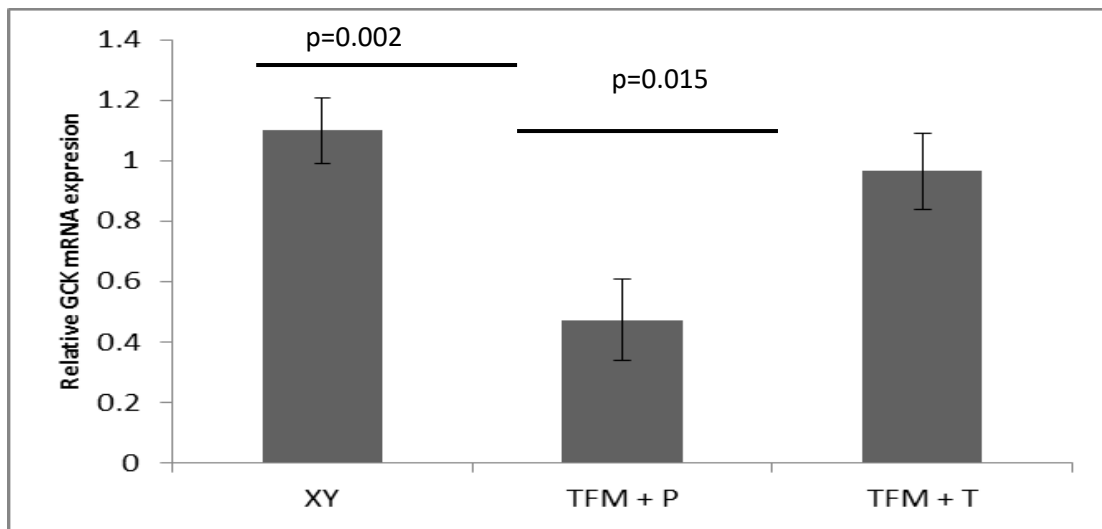
**Figure 6.7** Relative mRNA expressions of enzymes of carbohydrate metabolism in different tissue of Tfm mice comparing to XY littermates

The mean relative mRNA expression of the significant enzyme changes are depicted with error bars representing standard error of mean. p values are represented over the corresponding groups

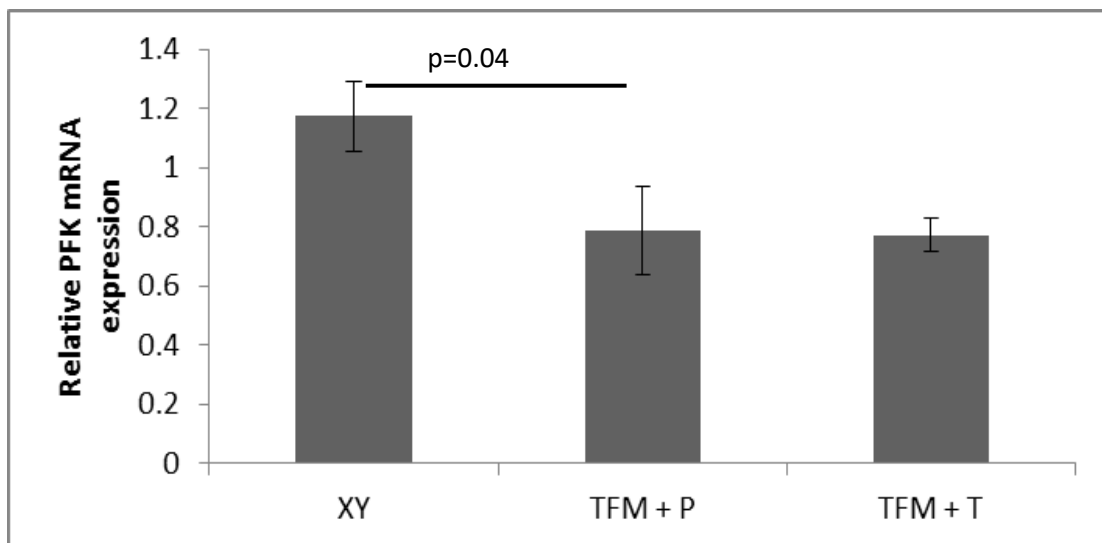
**Figure 6.7.1** Relative mRNA expression of Hexokinase 2 in subcutaneous fat of Tfm Mice



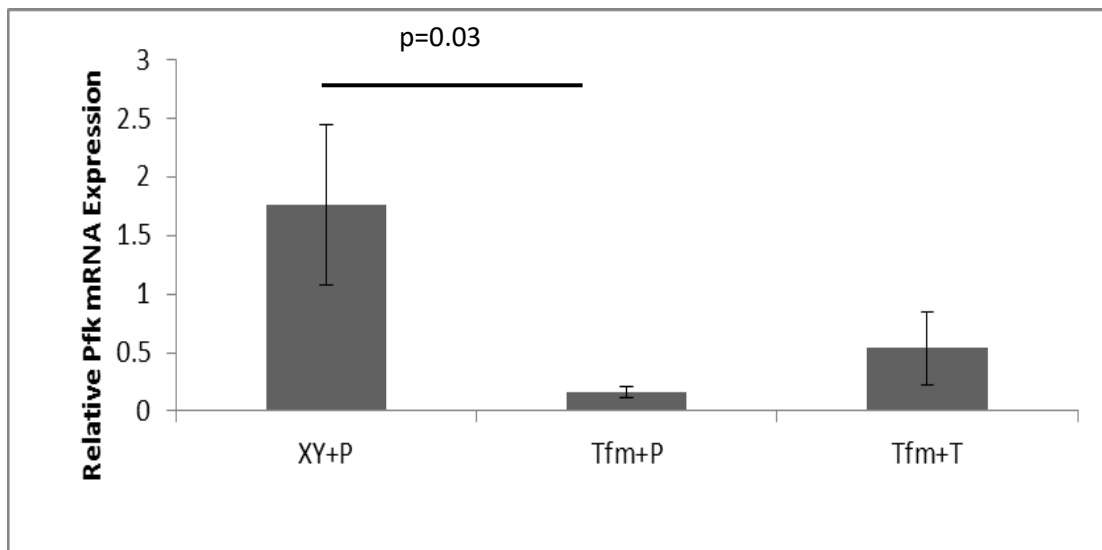
**Figure 6.7.2** Relative mRNA expression of Glucokinase in Liver of Tfm mice



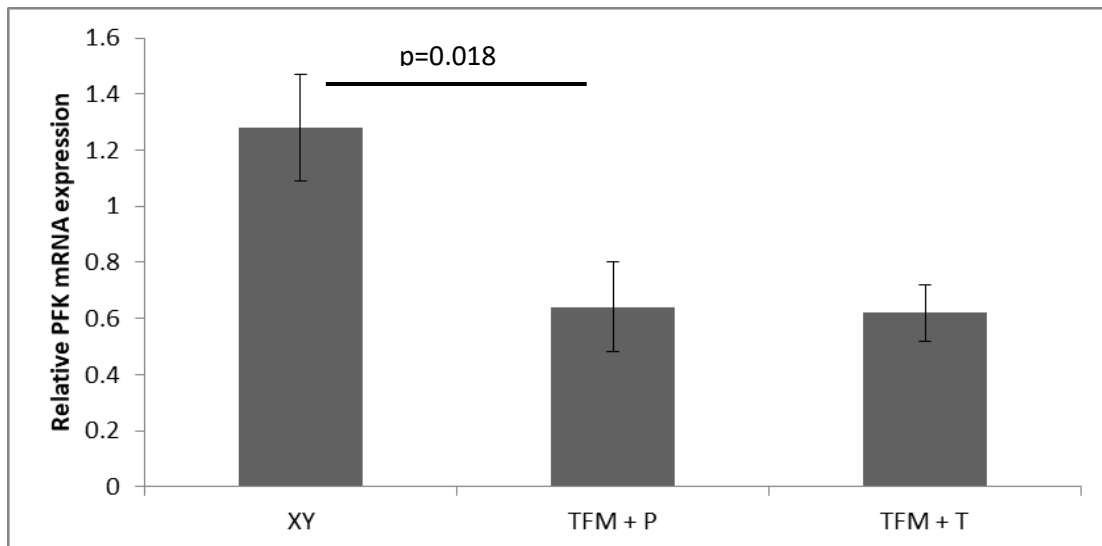
**Figure 6.7.3** Relative mRNA expression of Phosphofructokinase in liver of Tfm Mice



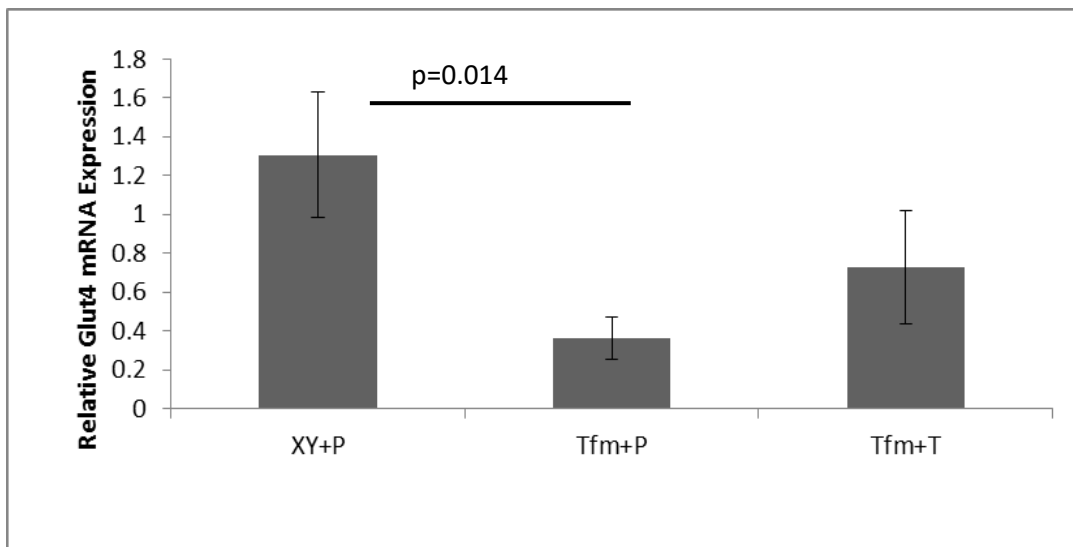
**Figure 6.7.4** Relative mRNA expression of Phosphofructokinase in subcutaneous adipose tissues of the Tfm mouse



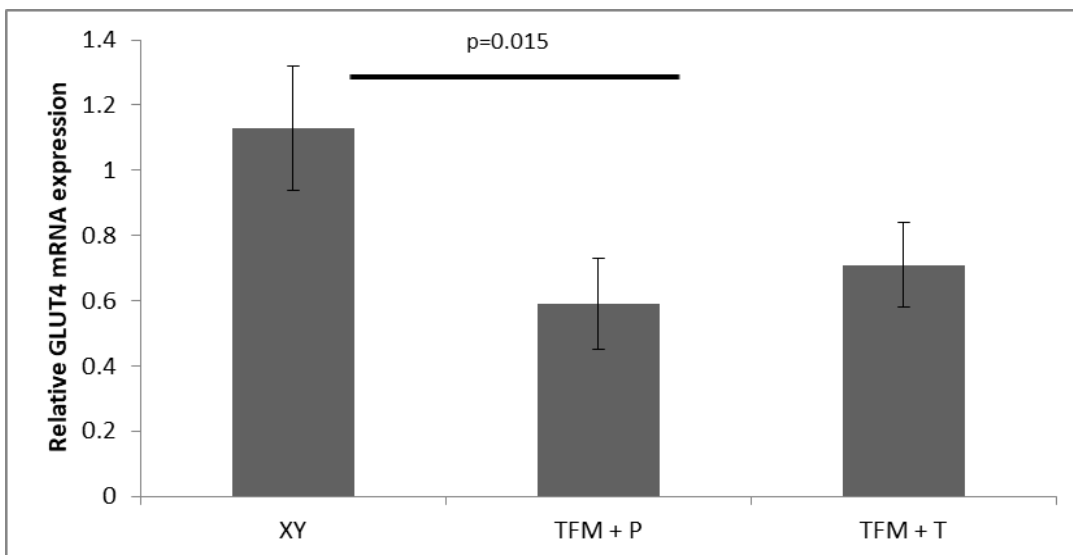
**Figure 6.7.5** Relative mRNA expression of Phosphofructokinase in muscle tissues of the Tfm mice



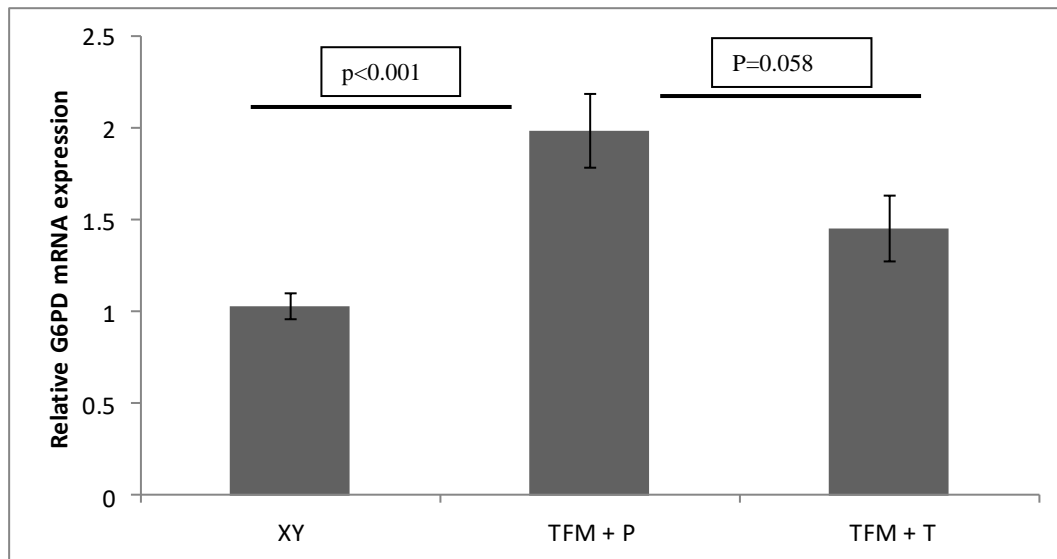
**Figure 6.7.6** Relative mRNA expression of GLUT4 enzyme in subcutaneous adipose tissue of Tfm mouse



**Figure 6.7.7** Relative mRNA expression of GLUT4 enzyme in muscle tissue of Tfm mouse



**Figure 6.7.8** Relative mRNA expression of G6PD enzyme in muscle tissue of Tfm mouse



## 6.5.2 Target enzymes of lipid metabolism

### 6.5.2.1 Target enzymes of fatty acid metabolism

The results of expression of the targets involved in the fatty acid metabolism in high fat fed Tfm mouse, compared with wild type and XY littermates are summarised in table 6.8 and figure 6.8.1 to figure 6.8.5.

#### **6.5.2.1.1 Effect of high fat diet on Tfm mice compared to wild type XY littermates**

Hepatic fatty acid synthase (FASN-TfmP=1.09 ± 0.13 and XYP=2.49 ± 0.64 p=0.049) and acetyl coenzyme A carboxylase (ACACA- TfmP=1.15 ± 0.17 and XYP=11.42 ± 4.93 p=0.042) were significantly increased in the Tfm mouse as compared to wild type.

Lipoprotein lipase (LPL) expression was decreased in the subcutaneous adipose tissue Tfm mouse as compared to the XY wild type (Tfm=1.03 ± 0.08 and 0.70 ± XYP=0.016).

The expression of Steroyl-CoA-desaturase-1 (SCD-1) was also significantly increased in the visceral adipose tissue of Tfm mice as compared to the XY wild type (Tfm=1.10 ± 0.23 and XYP=4.99 ± 1.64 p=0.034).

There was no significant difference in the expression of Hormone sensitive lipase (Lipe) in the different tissues of three groups of animals.

#### **6.5.2.1.2 Effect of testosterone administration Tfm mouse as compared to Tfm littermates without testosterone**

In the testosterone treated Tfm mouse the liver expression of FASN (TfmP =11.42 ± 4.93 and TfmT=2.99 ± 0.88 p=0.12) and ACACA (TfmP=2.49 ± 0.64 and TfmT=1.30 ± 0.29p=0.11) were decreased but they were not statistically significant.

There was a significant reduction in SCD1 expression in the visceral fat of the testosterone treated Tfm mice compared to the placebo treated Tfm mice (TfmT=0.94 ± 0.15 and TfmP=4.99 ± 1.64 p=0.027).

Lipoprotein lipase was significantly high in the visceral fat of testosterone treated Tfm mouse as compared to the Tfm littermates on placebo (TfmP=0.99±0.2 and TfmT=2.18±0.36 p=0.015).

**Table 6.8 Effect of testosterone on regulatory enzymes in fatty acid metabolism**

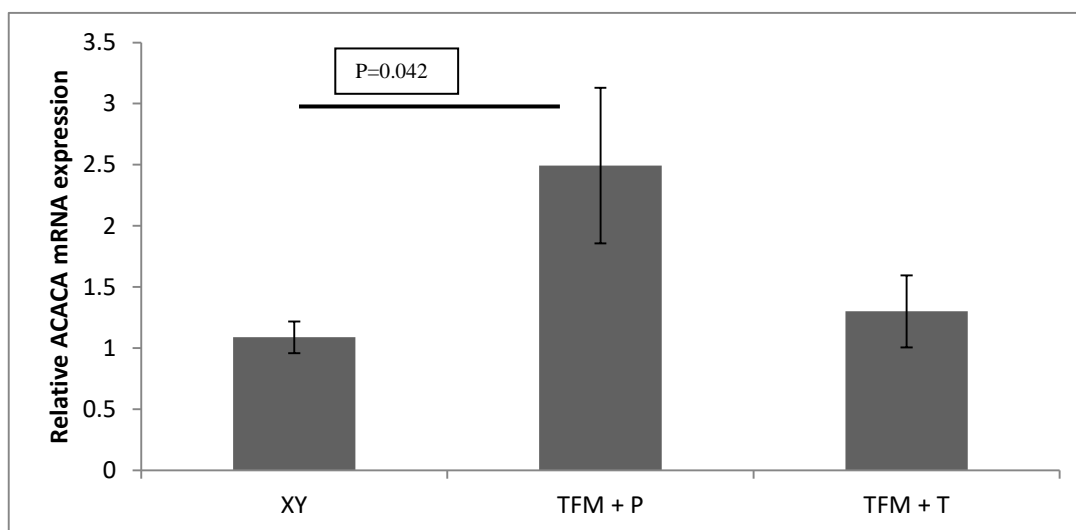
Gene	XY-P	Muscle			Liver		Subcutaneous fat			Visceral Fat		
		Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100
ACACA	1.28 ± 0.27	1.65 ± 0.46	0.77 ± 0.14	1.09 ± 0.13	2.49 ± 0.64*	1.30 ± 0.29	1.22 ± 0.28	1.15 ± 0.36	1.89 ± 0.62	1.07 ± 0.16	1.02 ± 0.27	0.95 ± 0.35
FASN	1.52 ± 0.43	1.48 ± 0.56	0.56 ± 0.14	1.15 ± 0.17	11.42 ± 4.93*	2.99 ± 0.88	1.20 ± 0.28	2.89 ± 1.49	4.76 ± 1.90	1.12 ± 0.23	1.35 ± 0.36	0.87 ± 0.30
SCD1	1.06 ± 0.11	1.30 ± 0.24	1.37 ± 0.29	1.04 ± 0.14	2.45 ± 1.15	1.38 ± 0.15	1.2 ± 0.29	3.05 ± 1.37	0.70 ± 0.15	1.10 ± 0.23	4.99 ± 1.64 <sup>‡</sup>	0.94 ± 0.15 <sup>‡</sup>
LPL	1.24 ± 0.31	0.90 ± 0.30	0.83 ± 0.20	1.07 ± 0.13	1.26 ± 0.45	0.90 ± 0.26	1.03 ± 0.08	0.70 ± 0.09*	0.81 ± 0.22	1.08 ± 0.13	0.99 ± 0.20	2.18 ± 0.36 <sup>‡</sup>
LiPe	1.20 ± 0.46	1.25 ± 0.24	1.18 ± 0.49	1.03 ± 0.08	1.24 ± 0.16	0.85 ± 0.10	1.17 ± 0.17	1.49 ± 0.29	1.14 ± 0.20	1.05 ± 0.14	1.10 ± 0.27	0.82 ± 0.21

Relative tissue-specific qPCR end-point analysis of selected genes of regulatory enzymes in fatty acid metabolism between Tfm placebo- treated versus XY littermates placebo-treated, and Tfm placebo-treated versus Tfm testosterone-treated. N=11. \*p < 0.05 Tfm-P versus XY-P, †p < 0.05 Tfm testosterone versus Tfm placebo, ±standard error on mean

**Figure 6.8** Relative mRNA expressions of enzymes and regulatory proteins of fatty acid metabolism in different tissue of Tfm mice comparing to XY littermates

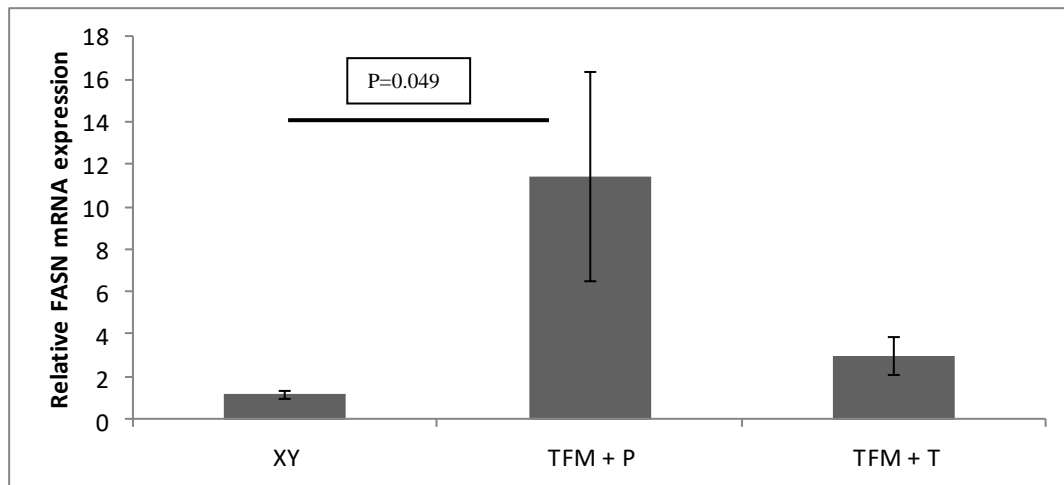
The mean relative mRNA expression of the significant enzyme and regulatory protein changes are depicted with error bars representing standard error of mean. p values are represented over the corresponding groups.

**Figure 6.8.1** Relative mRNA expression of Acetyl Coenzyme A Carboxylase enzyme in liver tissue of Tfm mice

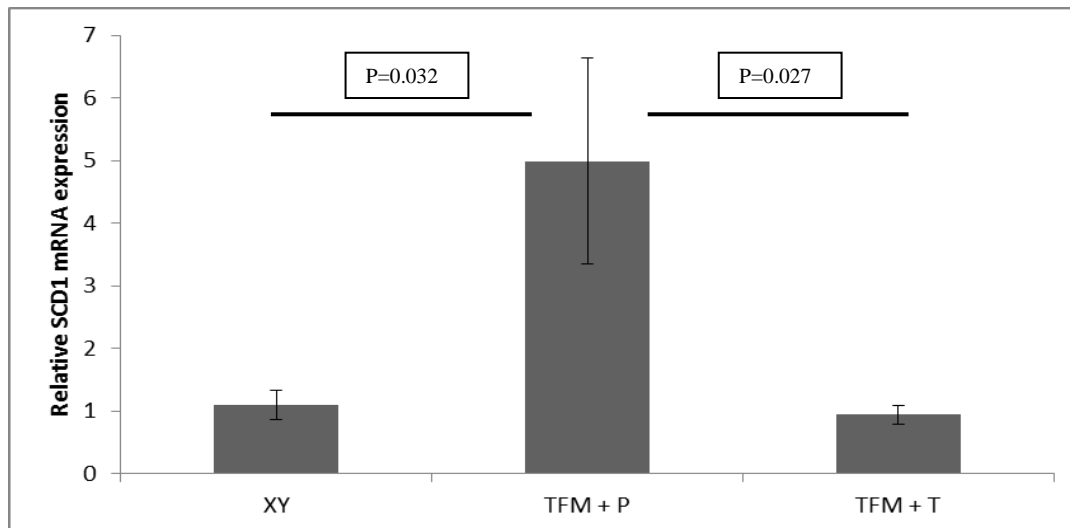




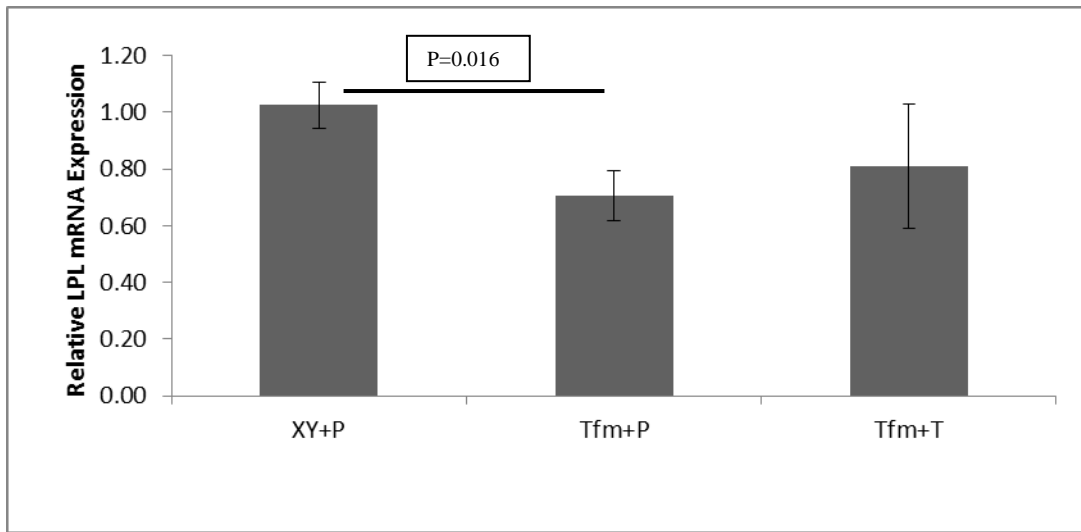
**Figure 6.8.2** Relative mRNA expression of Fatty Acid Synthase enzyme in liver tissue of Tfm mice



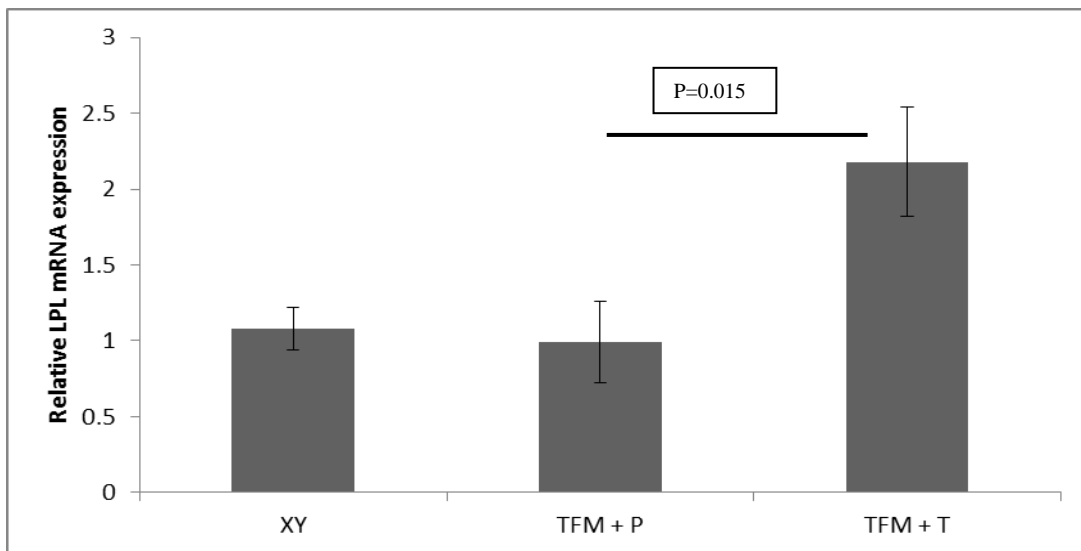
**Figure 6.8.3** Relative mRNA expression of Steroyl-CoA Carboxylase enzyme in visceral adipose tissue of Tfm mice



**Figure 6.8.4** Relative mRNA expression of Lipoprotein Lipase enzyme in subcutaneous adipose tissue of Tfm mice



**Figure 6.8.5** Relative mRNA expression of Lipoprotein Lipase enzyme in visceral adipose tissue of Tfm mice



### **6.5.2.2 Target enzymes of cholesterol metabolism**

The results of expression of the targets involved in cholesterol metabolism studied in high fat fed Tfm mouse, compared with wild type and XY littermates are summarised in table 6.9 and shown in figures 6.9.1 to 6.9.5.

#### **6.5.2.2.1 Effect of high fat diet on Tfm mice compared to wild type XY littermates**

In the liver of Tfm mice compared to the XY, there was a significant reduction in the expression of cholesterol acceptor transporter ApoE ( $1.07 \pm 0.05$  and  $0.77 \pm 0.09$   $p=0.009$ ) and cholesterol efflux transporter ABCA1 ( $1.05 \pm 0.04$  and  $0.71 \pm 0.09$   $p=0.002$ ).

In the subcutaneous adipose tissue ApoE was again showed a significant decrease in the Tfm mice compared to the XY mice ( $1.02 \pm 0.08$  and  $0.59 \pm 0.13$   $p=0.01$ ).

In the subcutaneous tissue the expression of SREBf1 ( $1.07 \pm 0.17$  and  $0.36 \pm 0.09$   $p=0.002$ ) and SREBf2 ( $1.07 \pm 0.17$  and  $0.42 \pm 0.08$   $p=0.003$ ) expression were significantly lower in the subcutaneous fat of Tfm mice as compared to XY littermates.

The visceral fat did not show any significant difference between the wild type and the Tfm mouse groups in the expression of ApoE, ABCA1, SREBPf1 and SREBPf2 enzymes.

#### **6.5.2.2.2 Effect of testosterone administration Tfm mice as compared to Tfm littermates without testosterone**

In the liver treatment with testosterone significantly increased this expression of ApoE ( $0.77 \pm 0.09$  and  $1.14 \pm 0.11$   $p = 0.027$ ) and ABCA ( $0.71 \pm 0.09$  and  $1.1 \pm 0.13$   $p = 0.02$ ), restoring the expression to that of the wild-type

Similarly in the subcutaneous adipose tissues testosterone treatment increased the level of ApoE expression (Tfm- $0.59 \pm 0.13$  and Tfm treated- $1.14 \pm 0.16$   $p = 0.015$ ) to a similar level seen in the wild type.

The expression levels of SREBPf1 showed a significant improvement in the subcutaneous tissue (TfmP- $0.36 \pm 0.09$  and TfmT- $0.86 \pm 0.16$   $p = 0.015$ ) similar to those demonstrated in wild-type mice. In the case of SREBPf2, again there was an improvement in subcutaneous tissue, which showed a trend towards significance (TfmP- $0.42 \pm 0.08$  and TfmT- $1.12 \pm 0.33$   $p = 0.053$ ).

**Table 6.9 Effect of testosterone on regulatory enzymes in cholesterol metabolism**

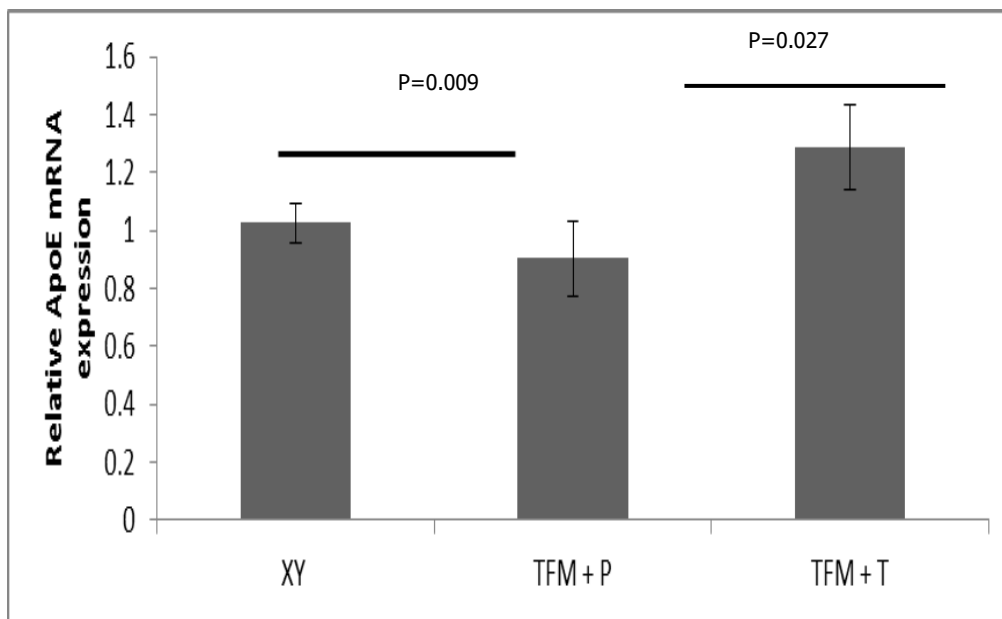
Gene	Muscle			Liver			Subcutaneous fat			Visceral fat		
	XY-P	Tfm-P	Tfm-T	XY-P	Tfm-P	Tfm-T	XY-P	Tfm-P	Tfm-T	XY-P	Tfm-P	Tfm-T
ApoE				1.07 ± 0.05	0.77 ± 0.09*	1.14 ± 0.11 <sup>†</sup>	1.02 ± 0.08	0.59 ± 0.13*	1.14 ± 0.16 <sup>†</sup>	1.03 ± 0.1	0.75 ± 0.16	0.86 ± 0.06
ABCA1				1.05 ± 0.04	0.71 ± 0.09*	1.1 ± 0.13 <sup>†</sup>	1.07 ± 0.14	0.77 ± 0.12	0.63 ± 0.11	1.14 ± 0.24	0.98 ± 0.2	1.91 ± 0.23
SREBPf1	1.29 ± 0.26	0.74 ± 0.21	1.47 ± 0.39	1.13 ± 0.16	1.01 ± 0.19	1.32 ± 0.29	1.07 ± 0.17	0.36 ± 0.09*	0.86 ± 0.16 <sup>†</sup>	0.81 ± 0.13	0.59 ± 0.25	0.78 ± 0.13
SREBPf2	1.17 ± 0.21	0.8 ± 0.28	1.99 ± 0.62	1.08 ± 0.13	1.44 ± 0.22	1.17 ± 0.16	1.07 ± 0.17	0.42 ± 0.08*	1.12 ± 0.33 <sup>α</sup>	0.8 ± 0.14	0.56 ± 0.21	0.79 ± 0.13

Relative tissue-specific qPCR end-point analysis of selected genes of regulatory enzymes in cholesterol metabolism between Tfm placebo-treated versus XY littermates placebo-treated, and Tfm placebo-treated versus Tfm testosterone-treated. N=11. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 Tfm placebo versus XY placebo, †p < 0.05, ††p < 0.01 Tfm testosterone versus Tfm placebo, α = 0.056. ± standard error on mean.

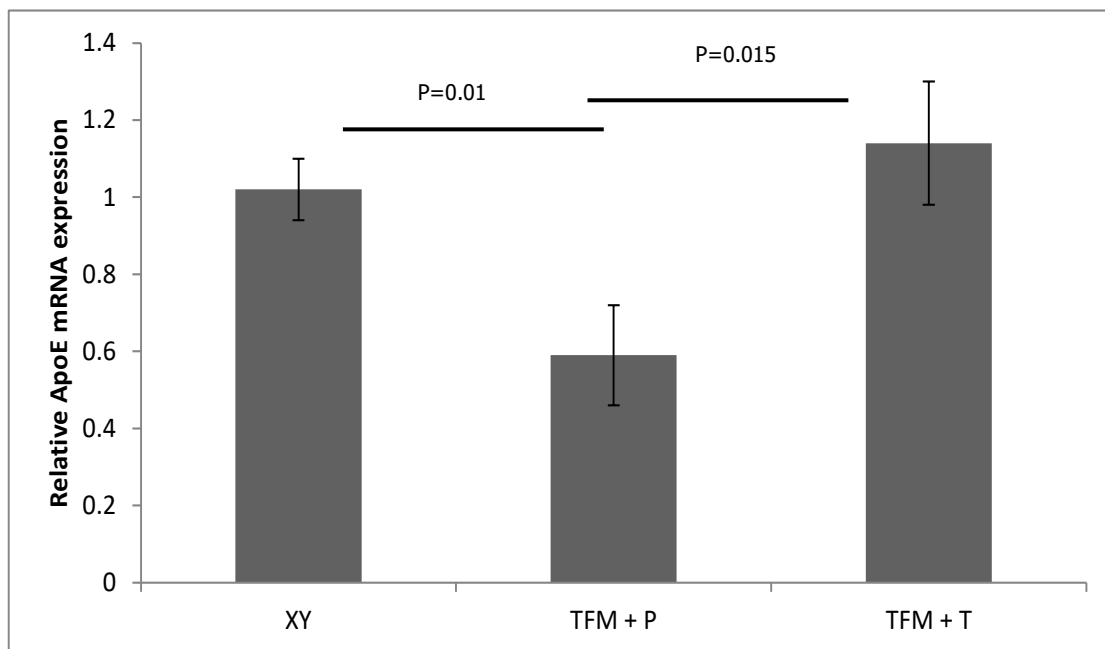
**Figure 6.9** Relative mRNA expressions of enzymes and regulatory proteins of cholesterol metabolism in different tissue of Tfm mice comparing to XY littermates

The mean relative mRNA expression of the significant enzyme and regulatory protein changes are depicted with error bars representing standard error of mean. p values are represented over the corresponding groups

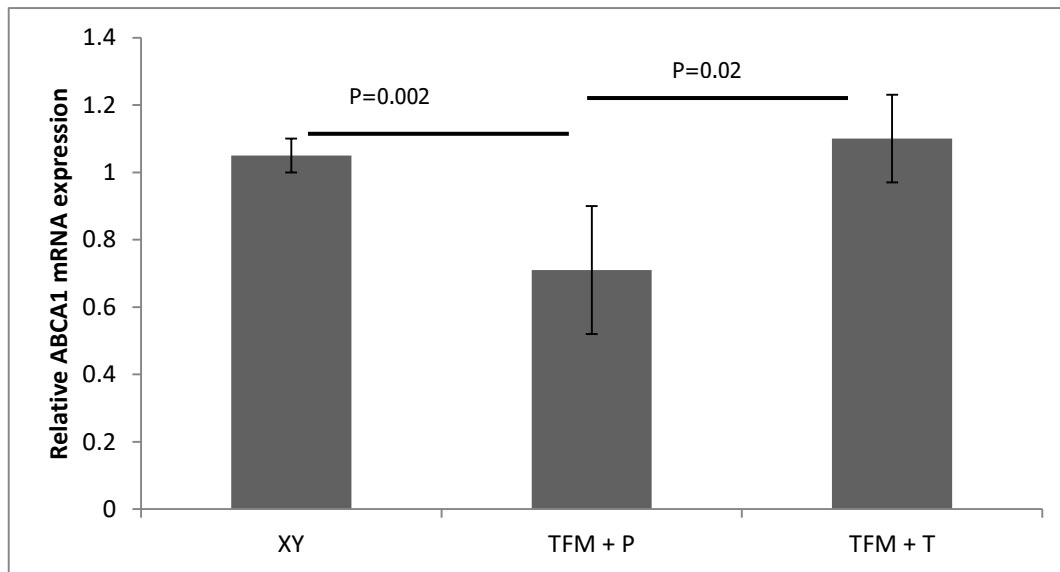
**Figure 6.9.1** Relative mRNA expression of ApoE in the liver tissue of Tfm mice



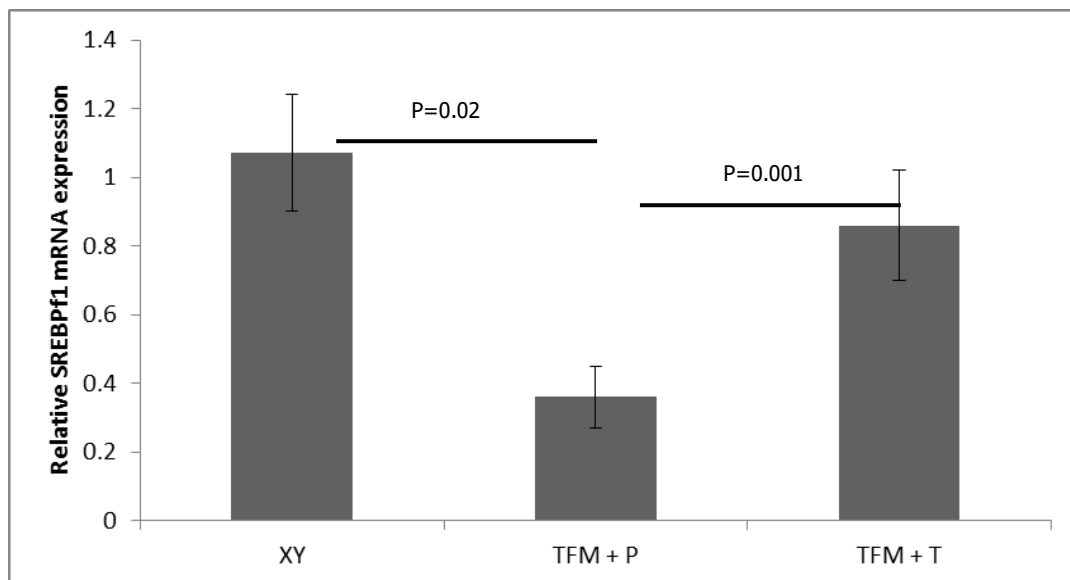
**Figure 6.9.2** Relative mRNA expression of ApoE in the subcutaneous tissue of Tfm mice



**Figure 6.9.3** Relative mRNA expression of ATP-binding cassette transporter A1 in the liver tissue of Tfm mice

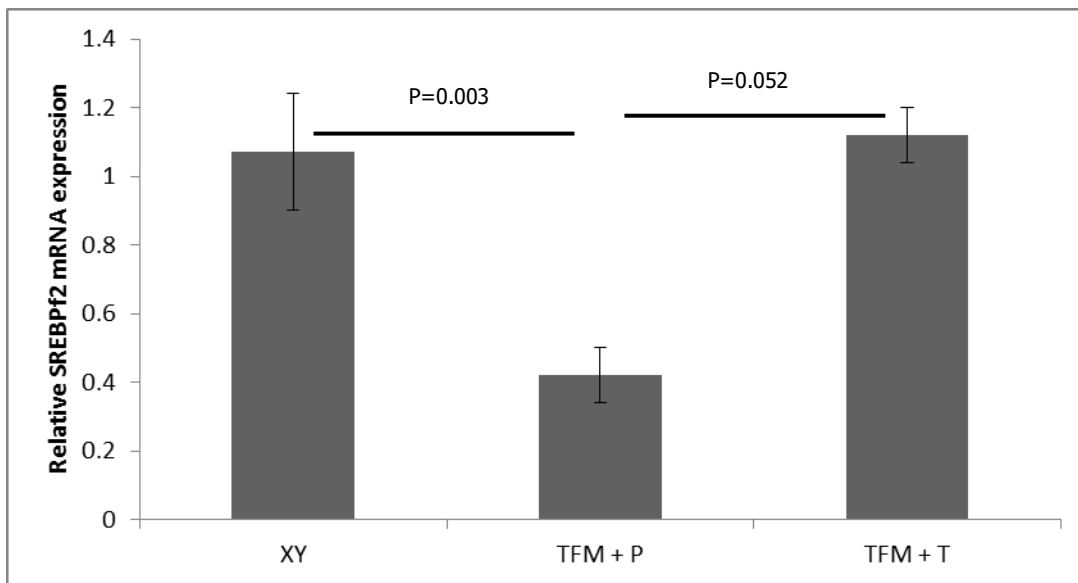


**Figure 6.9.4** Relative mRNA expression of Sterol regulatory element-binding protein1 in the subcutaneous tissue of Tfm mice





**Figure 6.9.5** Relative mRNA expression of Sterol regulatory element-binding protein2 in the subcutaneous tissue of Tfm mice



### 6.5.3 Nuclear receptors regulating metabolic pathways

The results of expression of the nuclear receptors involved in metabolic pathways in high fat fed Tfm mouse, compared with wild type and XY littermates are summarised in table 6.8 and figure 6.10.1 to figure 6.10.5.

#### 6.5.3.1 Effect of high fat diet on Tfm mice compared to wild type XY littermates

There was significant reduction in the expression of Liver X receptor alpha (LXR) in liver (TfmP=1.05±0.09 and XYP=0.66±0.08 p=<0.001), muscle (TfmP=1.35±0.29 and XYP=0.62±0.12, p=0.032) and subcutaneous fat (TfmP=1.07±0.14 and XYP=0.42±0.13p=0.003).

Expression of the nuclear receptors PPAR $\alpha$  (Tfm=1.13 $\pm$ 0.23 and XYP=0.41 $\pm$ 0.11 p=0.01) and PPAR $\gamma$  (TfmP=1.06 $\pm$ 0.14 and XYP=0.67 $\pm$ 0.06 p=0.02) were reduced in the subcutaneous adipose tissue of Tfm mice as compared to the XY littermates.

In the visceral adipose tissue, PPAR $\gamma$  showed a significant decrease compared to the XY littermate (TfmP=1.05 $\pm$ 0.11 and XYP= X0.49 $\pm$ 0.08 p=0.001).

#### **6.5.3.2 Effect of testosterone administration Tfm mice as compared to Tfm littermates without testosterone**

In the testosterone treated group, the expression of Liver X receptor increased significantly to reach that of the XY wild type mice levels in the liver (TfmP=0.66 $\pm$ 0.08 and TfmT=1.28 $\pm$ 0.24 p=0.024), muscle (0TfmP=0.62 $\pm$ 0.12 and TfmT=1.5 $\pm$ 0.27 p=0.008) and subcutaneous tissue (TfmP=0.42 $\pm$ 0.13 and TfmT=1.15 $\pm$ 0.28 p=0.03).

There were no significant changes in the expression of PPAR $\alpha$  and PPAR $\gamma$  in the testosterone treated Tfm mice compared to placebo treated Tfm mice.

**Table 6.10 Effect of testosterone on regulatory receptors of metabolic pathways**

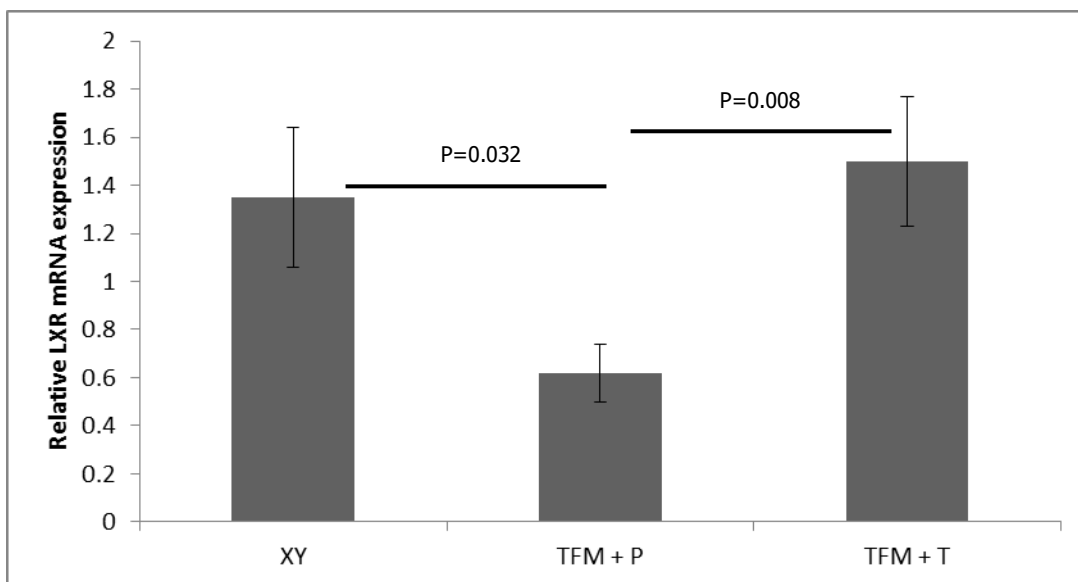
Gene	Muscle			Liver			Subcutaneous fat			Visceral fat		
	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100	XY-P	Tfm-P	Tfm-S100
LXR	1.35 ±0.29	0.62 ±0.12*	1.5 ±0.27 <sup>††</sup>	1.05 ±0.09	0.66 ±0.08**	1.28 ±0.24 <sup>†</sup>	1.07 ±0.14	0.42 ±0.13**	1.15 ±0.28 <sup>†</sup>	1.27 ±0.35	1±0.3	1.44 ±0.48
PPAR $\alpha$	1.16 ±0.2	1.65 ±0.78	0.94 ±0.21	1.02 ±0.07	1.84 ±0.55	0.93 ±0.14	1.13 ±0.23	0.41 ±0.11*	0.69 ±0.22	1.09 ±0.2	0.92 ±0.32	0.98 ±0.22
PPAR $\gamma$	1.29 ±0.31	1.29 ±0.62	0.63 ±0.17	1.09 ±0.14	0.99 ±0.19	1.29 ±0.23	1.06 ±0.14	0.67 ±0.06*	0.82 ±0.12	1.05 ±0.11	0.49 ±0.08**	0.66 ±0.1

Relative tissue-specific qPCR end-point analysis of selected genes of carbohydrate metabolism between Tfm placebo- treated versus XY littermates placebo-treated, and Tfm placebo-treated versus Tfm testosterone-treated. N=11. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 Tfm Placebo versus XY placebo, †p < 0.05, ††p < 0.01 Tfm testosterone treated versus Tfm placebo, ±standard error on mean

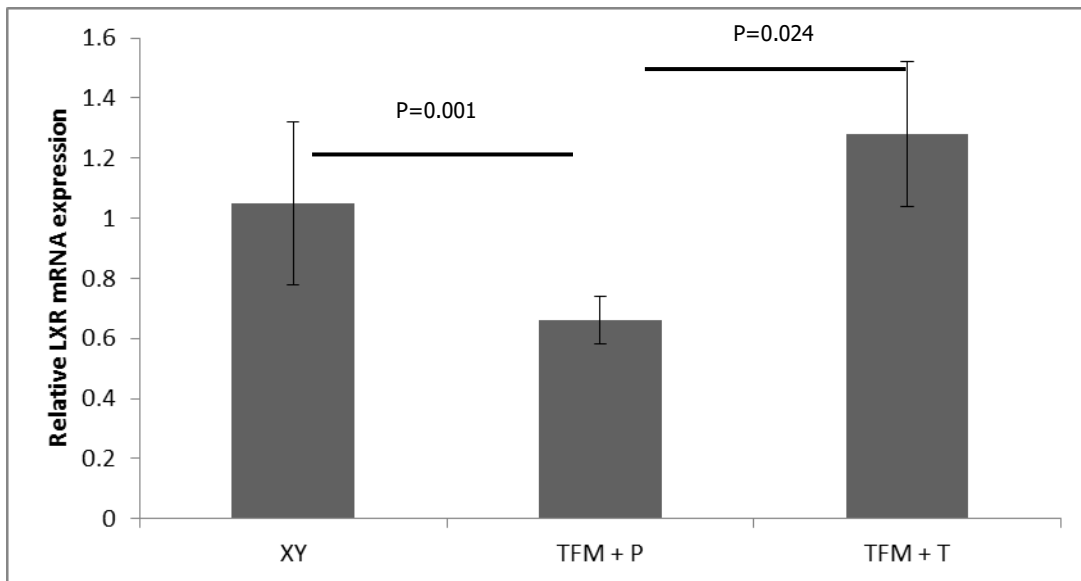
**Figure 6.10** Relative mRNA expressions of master regulators of metabolism in different tissue of Tfm mice comparing to XY littermates

The mean relative mRNA expression of the significant changes is depicted with error bars representing standard error of mean. p values are represented over the corresponding groups

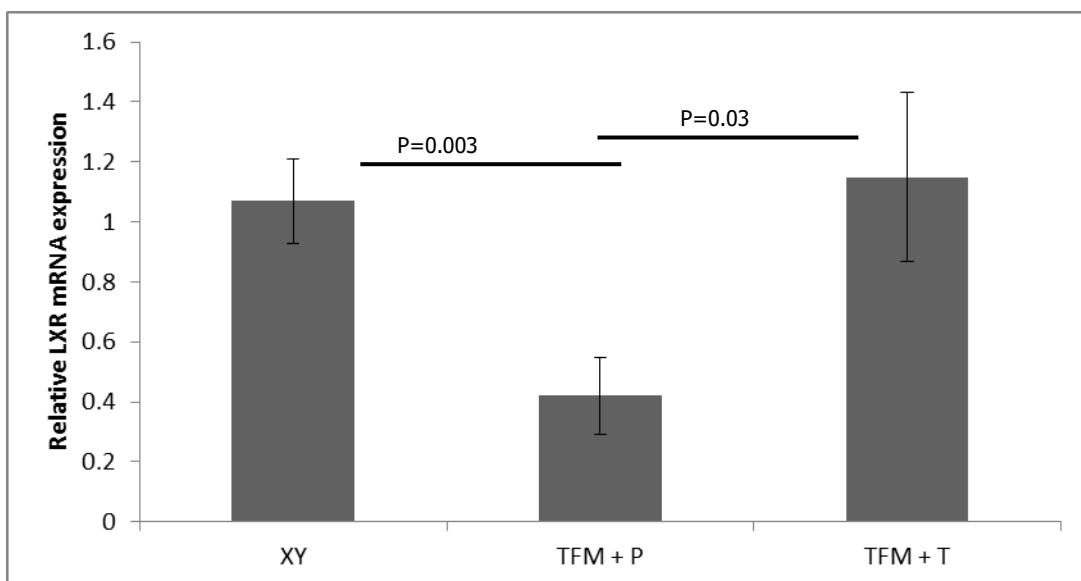
**Figure 6.10.1** Relative mRNA expression of Liver X Receptor alpha in the muscle tissue of Tfm mice



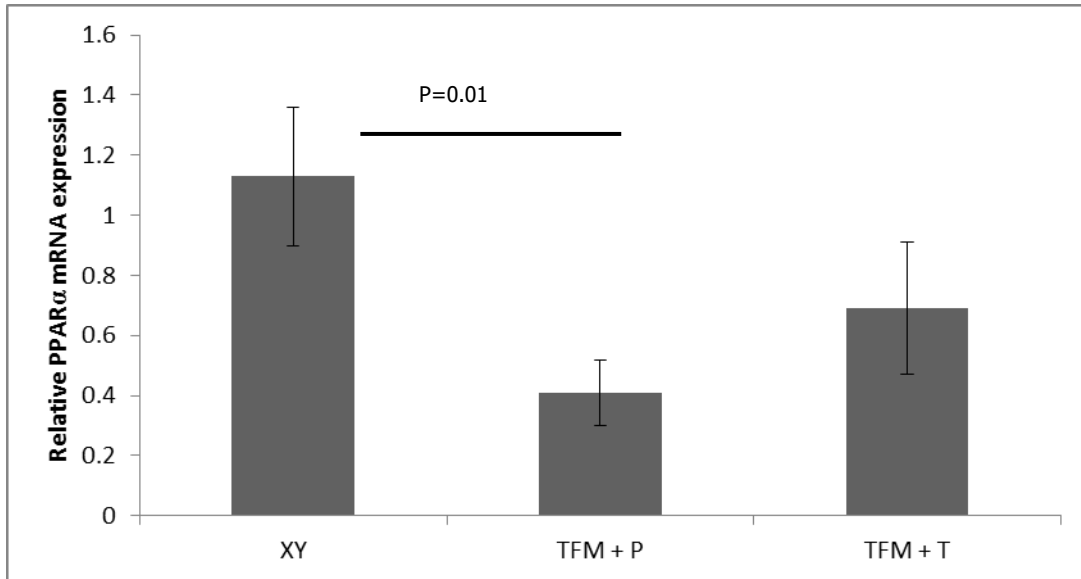
**Figure 6.10.2** Relative mRNA expression of Liver X Receptor alpha in the liver tissue of Tfm mice



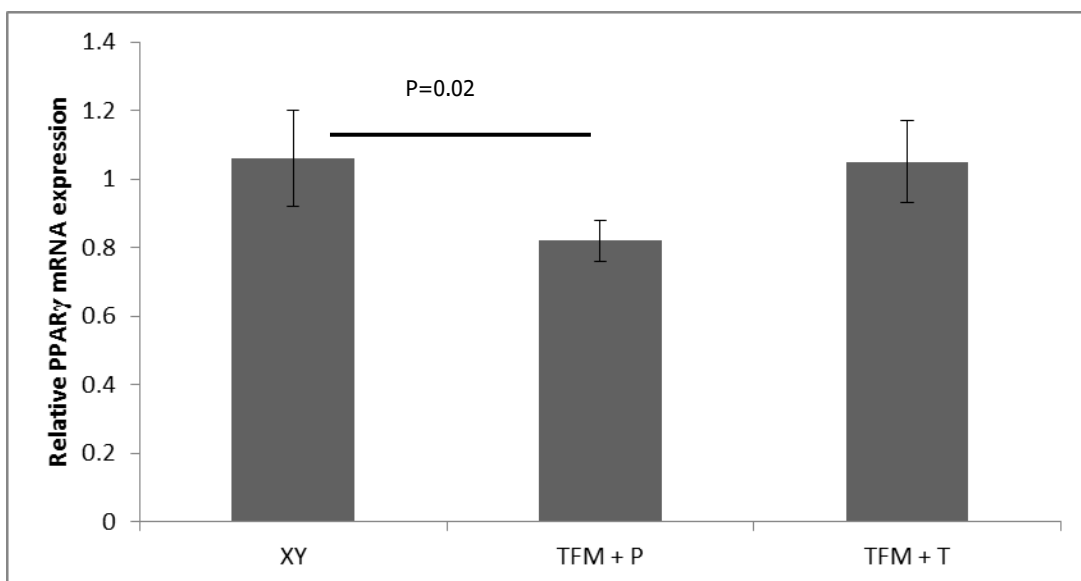
**Figure 6.10.3** Relative mRNA expression of Liver X Receptor alpha in the subcutaneous adipose tissue of Tfm mice



**Figure 6.10.4** Relative mRNA expression of Peroxisome Proliferator-activated Receptor alpha in the subcutaneous adipose tissue of Tfm mice



**Figure 6.10.5** Relative mRNA expression of Peroxisome Proliferator-activated Receptor gamma in the subcutaneous adipose tissue of Tfm mice



## 6.6 Discussion

This study shows testosterone has a significant role in modulating the carbohydrate, lipid metabolism and its actions differ in muscle, liver, subcutaneous adipose tissue and visceral adipose tissue. There is a significant difference in the effect of testosterone on subcutaneous adipose tissue as compared to the visceral adipose tissue.

In the subcutaneous tissue the effects are seen on lipid metabolism (receptors LXR, PPAR $\alpha$ , PPAR $\gamma$  and enzymes SREBPf, SREBPf2 and ApoE), and on carbohydrate metabolism (Enzymes HK2, PFK and GLUT4, receptors, PPAR $\gamma$ ). However the effect on visceral fat is mainly limited to expression of SCD1, which is a rate-limiting step in the synthesis of unsaturated fatty acid.

In the liver, effects of testosterone are seen in the regulation of lipid metabolism (LXR receptor, ApoE, ABCA1, ACACA and FASN enzymes) and carbohydrate metabolism (GCK, PFK and G6PDx) all of which are adversely affected by the low testosterone and defective androgen receptor state.

In the skeletal muscle the main effect is seen in the carbohydrate metabolism (Enzymes HK2, PFK and GLUT4).

Testosterone therapy only partially reversed the metabolic abnormalities noted above. The liver X receptor expression showed significant reversal in the liver, subcutaneous adipose tissue and muscle tissue to that seen in the normal wild type after testosterone therapy. The expressions of ApoE and ABCA1 showed significant improvement in the liver tissues after testosterone therapy.

In the subcutaneous adipose tissue, the expressions of ApoE and SBEBPf1 showed significant

improvement and SREBPf2 showed a trend towards significant improvement after testosterone therapy.

In the carbohydrate metabolism there was a significant improvement in the expression of GCK and a trend towards significant improvement in the expression of G6PDx in liver and improvement the expression HK2 in the subcutaneous adipose tissues after testosterone treatment.

In the visceral fat the testosterone treated group showed restoration of the levels of SCD1 and a significant increase in the expression of LPL and an increased expression of PPAR $\gamma$ , which regulates fatty acid storage in adipose tissue.

#### **6.6.1 Effect of testosterone on carbohydrate metabolism**

This study shows testosterone deficiency state adversely affects the key enzymes of carbohydrate metabolism GCK, PFK and G6PDx HK2, and GLUT4 in a tissue specific manner.

GLUT4 expression is known to correlate positively with insulin responsiveness and defects in expression of GLUT4 have been observed in patients with T2D (Pessin and Saltiel 2000).

Testosterone has previously been shown to increase the expression of GLUT4 in cultured skeletal muscle cells, hepatocytes and adipocytes (Chen, Li et al. 2006, Muthusamy, Murugesan et al. 2009) as well as augmenting membrane translocation and promoting glucose uptake in adipose and skeletal muscle tissue.

The key enzymes involved in glycolysis, phosphofructokinase (PFK) and hexokinase (HK) were significantly reduced in muscle, liver and subcutaneous adipose tissue of Tfm mice.

Previous studies have demonstrated an increase in the activity of PFK and HK in cultured rat skeletal muscle cells and increased hexokinase activity in muscle tissue of castrated rats



following testosterone treatment thus diminishing the raised blood levels of glucose seen in untreated castrated control rats (Bergamini, Bombara et al. 1969, Ramamani, Aruldas et al. 1999, Sato, Iemitsu et al. 2008). This could be clinically important in muscle as this tissue accounts for approximately 75 % of whole-body insulin-stimulated glucose uptake (Shulman, Rothman et al. 1990).

One of the liver's functions is to maintain blood glucose concentrations within a physiological range. Glucokinase (GCK) or hexokinase IV is the main enzyme that regulates the flux and the use of glucose in the liver and GCK activation is expected to increase glycolysis in hepatocytes leading to reduction of hepatic glucose production and increased insulin secretion (Guo, Li et al. 2012). Defects in the GCK gene is one of the most widely known form of monogenic diabetes (MODY-maturity onset of diabetes in the young (Hattersley, Turner et al. 1992). Knock out model studies have shown that mice lacking GCK only in the liver are mildly hyperglycemic but they display pronounced defects in both glycogen synthesis and glucose turnover rates during a hyperglycemic clamp (Postic, Shiota et al. 1999). Our study showed GCK is significantly reduced in the Tfm mouse, and the gene expression normalized in testosterone treated animals. In light of the above mentioned studies it is likely that the testosterone deficient state leads to decrease in GCK in the liver, which contributes to the development of hyperglycemia and diabetes. It also further suggests that the effect on GCK gene expression is independent of androgen receptor.

This study further shows that the mRNA expression of Glucose-6-phosphate dehydrogenase (G6PD), a rate-limiting enzyme of the pentose phosphate pathway, is elevated in the liver of Tfm mice. G6PD in liver is regulated by high carbohydrate and high fat diet, and hormonal signals including insulin and glucocorticoids (Kletzien, Harris et al. 1994). Studies have suggested role of G6PD in diabetic subjects, by altering lipid metabolism in adipocytes (Park,

Rho et al. 2005). However the exact role of hepatic G6PD in the pathogenesis of metabolic syndrome is poorly understood. The results from this study suggest hepatic G6PD is responsive to androgens and might be a factor in carbohydrate and lipid abnormalities seen in androgen deficient state. Furthermore, as the increased expression of G6PD did not completely reverse with the testosterone supplementation androgen receptor is likely to be important in the regulation of hepatic G6PD gene expression.

The pentose phosphate pathway generates NADPH which stimulates the synthesis of fatty acids. Testosterone inhibits the expression of this enzyme which would reduce the fatty acid synthesis. This combined with the inhibition of ACACA and FAS will have greater effect on suppression of conversion of glucose into fat.

### **6.6.2 Effect of testosterone on lipid metabolism**

The combination of persistent excess energy intake in the form of fat and carbohydrate with reduced energy expenditure leads to a rise in free fatty acids and increases the body fat. The excess fatty acids are taken by non-adipose tissues like liver and muscle due to the “spill over” effect (Kelly and Jones 2013). This results in increased insulin resistance and hyperglycaemia. In the liver this results in excess fat and hepatic steatosis. Testosterone deficient state promotes this spillover by its adverse effect on the key enzymes and regulators of metabolic pathways.

This study demonstrates that testosterone deficiency negatively alters the expression of targets of lipid metabolism primarily in liver and subcutaneous adipose tissue and to a lesser extent in the visceral adipose tissue. Decreased lipoprotein lipase (LPL) in Tfm mice with low testosterone may limit the hydrolysis of lipoproteins and the subsequent uptake of free fatty

acid into subcutaneous adipose tissue. Clinical study of hypogonadal men treated with testosterone for 9 months resulted in a marked decrease in both LPL activity and triglyceride uptake in abdominal adipose tissue (Marin, Oden et al. 1995)

Human Stearoyl-CoA desaturase-1 (SCD1) is a critical control point of lipid storage and suppression of the enzyme activating metabolic pathways that promote the burning of fat and decrease lipid synthesis (Dobrzyn, Jazurek et al. 2010). SCD1 also plays an important role in the pathogenesis of lipid-induced insulin resistance with SCD1 deficiency up-regulating insulin-signaling components and glycogen metabolism in insulin-sensitive tissues (Cohen, Ntambi et al. 2003). Expression of SCD1 correlates positively with insulin resistance and therefore a tissue biomarker of insulin resistance. In this study we demonstrated significantly increased SCD1 expression in visceral adipose tissue of Tfm mice. SCD1 expression returned to XY litter mate levels after testosterone treatment in the visceral fat of Tfm mouse. These results suggest that testosterone has the potential to improve lipid metabolism via reducing SCD1 expression in visceral adipose tissue.

Apolipoprotein E is a plasma protein is the transport ligand for low density lipoprotein receptors. It plays an important role in the transport of cholesterol and other lipids among various cells of the body. Familial type III hyperlipoproteinemia, a genetic disorder characterized by elevated plasma cholesterol levels and accelerated coronary artery disease is the result of mutations in ApoE (Mahley 1988). Variations in the expression of alleles of ApoE gene expression is associated with associated with myocardial infarction and mortality from coronary artery disease (Eichner, Kuller et al. 1993). In this study we found lower subcutaneous adipose tissue and liver ApoE expression in testosterone deficient Tfm mice may be indicative of decreased reverse cholesterol transport. This deficiency was reversed

with testosterone replacement.

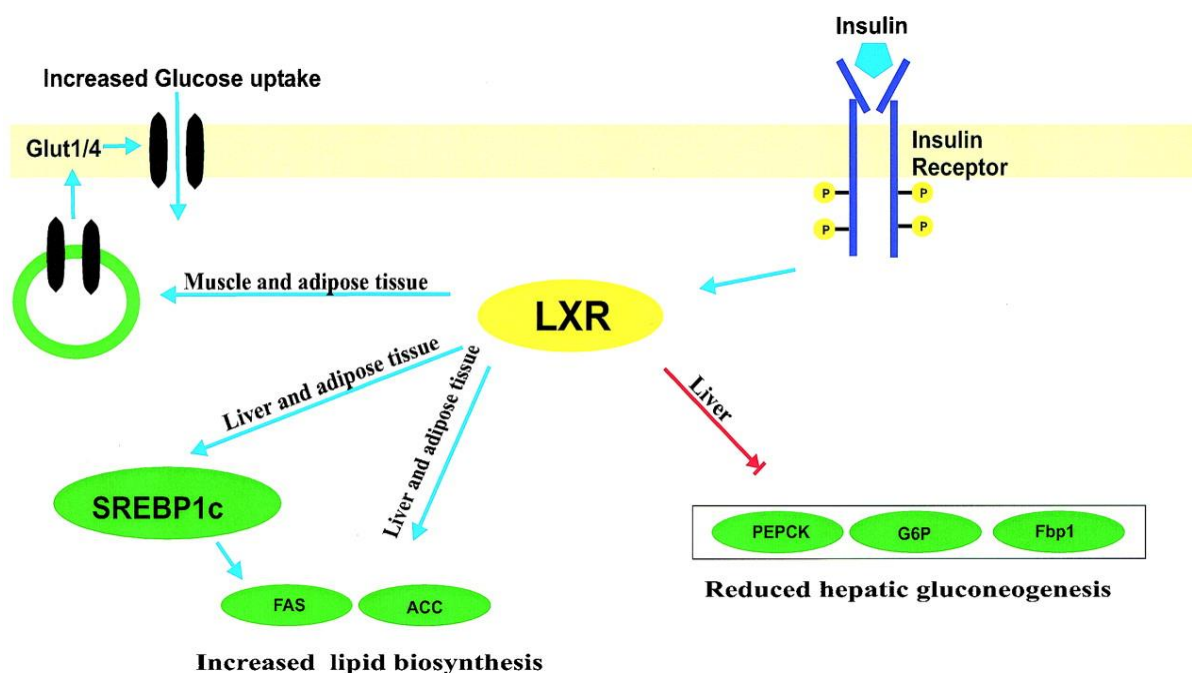
In this study we further demonstrated that mRNA expression of SREBf1 and SREBf2, were significantly decreased in subcutaneous adipose tissue and liver of Tfm mice compared to the wild type mice. In the testosterone treated mice these abnormalities were reversed. SREBf1 and SREBf2 are two are key transcription factors and master regulators on lipogenesis (Xie, Li et al. 2010). Similarly orchietomy significantly reduced hepatic SREBP-1 expression in mice fed a high fat diet with testosterone replacement reversing this abnormality (Senmaru, Fukui et al. 2013). As SREBPs are known to directly induce transcription of many genes needed for uptake and synthesis of cholesterol, fatty acids, triglycerides and phospholipids (Horton, Shimomura et al. 2003). These findings suggest low testosterone state induce metabolic abnormalities by modifying the expression of the key enzymes in the lipid transport and replacement with testosterone restores many of these abnormalities suggesting androgen receptor independent pathways.

Increased liver fat in Tfm mice from the present study is considered partly due to increased de novo lipogenesis and the expression of FASN and ACACA as discussed in the previously reported results from our team (Kelly, Nettleship et al. 2014). The present study additionally indicates that ABCA1 and ApoE involved in cholesterol and lipoprotein efflux are reduced in the testosterone-deficient state in the liver of Tfm mice. Studies have reported that the overexpression of hepatic ABCA1 in transgenic mice results in a marked increase in HDL release, decreased LDL and significantly reduced atherosclerosis when compared with control mice (Joyce, Amar et al. 2002), and increased hepatic cholesterol content was reported in these mice as the level of expression of the ABCA1 transporter decreased (Basso, Freeman et al. 2003). These results suggest the increased hepatic lipid accumulation in our Tfm mice may additionally result from absence of beneficial testosterone effects on lipid transport.

### **6.6.3 Effect of testosterone on master regulators of metabolism**

Testosterone altered the expression of master metabolic regulators suggesting a potential mode of action to influence lipid and glucose regulation through modulation of signaling pathways. Reduced expression of the nuclear receptor, Liver X receptor (LXR), in muscle, liver and subcutaneous adipose tissue of Tfm mice compared to testosterone-replete animals whether with or without AR function suggest that testosterone may increase LXR signaling to exert some of its protective metabolic effects. LXRs are key transcriptional regulators of lipid and carbohydrate metabolism known to control molecular pathways including cholesterol efflux, glucose regulation, fatty acid synthesis and inflammation (Hong and Tontonoz 2014). Some of the metabolic pathways in which Liver X receptor plays a pivotal role are depicted in figure 6.11. In parallel with testosterone-associated changes in LXR expression in the present study, we saw alterations in known LXR target genes: FASN, ApoE, ABCA1, LPL, SREBP1 and additionally, protects against diet-induced hepatic lipid accumulation in this model (Kelly, Nettleship et al. 2014).

**Figure 6.11 Interplay of metabolic effects of insulin and LXR**



SREBP1c -Sterol regulatory element-binding protein 1c, FAS- Fatty acid synthase, ACC- acetyl CoA carboxylase, Glut1/4 – Glucose transporter1/4, LXR- Liver X receptor, PECK- Phosphoenolpyruvate carboxykinase, G6P- Glucose 6 phosphatase Adapted from Steffenson et.al (Steffensen and Gustafsson 2004).

Tfm mice also had reduced subcutaneous adipose tissue and visceral adipose tissue expression of PPAR $\gamma$  mRNA, indicating a potential mechanism by which testosterone deficiency may lead to metabolic dysregulation and adverse fat distribution.

The present study indicates that metabolic actions of testosterone may at least in part, beyond its classical nuclear AR to modulate targets of lipid and glucose metabolism and that these actions are further differentially dependent on the target tissue. Whether the AR-independent effects in this study are via conversion to estradiol and subsequent activation of the oestrogen receptor (ER) was not addressed. We have previously shown that testosterone has additional actions on hepatic and aortic lipid accumulation in Tfm mice even with aromatase inhibition and ER blockade (Nettleship, Jones et al. 2007, Kelly, Nettleship et al. 2014). Further investigation is required to elucidate the AR-independent signaling mechanisms of testosterone action.

## **6.7 Conclusion**

These results suggest that testosterone has tissue-specific metabolic effects in the regulation of gene targets which control glucose utilization in liver, subcutaneous fat and skeletal muscle and lipid metabolism in liver and subcutaneous fat. The study showed low testosterone state adversely affects the metabolic pathways of carbohydrate and lipid metabolism by its differential regulation of target molecules in different tissues. The metabolic abnormalities are partly reverted by supplementation of testosterone. These leads to the conclusion that some of these effects are androgen receptor-independent and may potentially explain some of the observed clinical effects of testosterone in the anthropometric measures, cardiovascular risk profile and mortality in men with type 2 diabetes discussed in the preceding chapters.

## **6.8 Limitations**

This study looked into the mRNA expression of the target gene. Western blotting for determinations of the actual protein will confirm the translation to protein products. However the actual metabolic pathways are more complex with interplay of various other influences including posttranslational modifications, activation of proteins by phosphorylation, and transport to the target site. All these could be variable depending of intracellular milieu. Tfm mouse, being an AR deficient mouse with low testosterone from birth is prone to have other metabolic abnormalities which could affect the gene expression. The testosterone replacement is not completely physiological in that it has constant blood level as opposed to cyclical production in normal physiological state. It is also noted that supra physiological levels in the first few days are apparent with near infra physiologic levels towards the end of the interval (Nettleship, Jones et al. 2007). The effect of oestrogen could not be excluded in

this study. However, as noted above previous studies with oestrogen receptor blockade has suggested role of testosterone in metabolic pathways through non-androgen receptor mediated pathways. Further studies with androgen and oestrogen blockade littermates would confirm the non-androgen receptor mediated mechanisms of androgen action.





## **Chapter seven**

### **Discussion and conclusion**

#### **7.1 Introduction**

In the clinical studies I have described in the diabetic cohort, the relationship between testosterone and mortality, cardiovascular events and risk profile in a 7 year follow up, a cross sectional study looking at cardiovascular risk profile in relation to testosterone, the relationship between SHBG and cardiovascular risk profile and the role of androgen receptor polymorphism in the cardio metabolic effect of testosterone. I have examined and discussed whether or not the ratio of testosterone to AR CAG lengths (T/AR CAG or BioT/ AR CAG) is a more sensitive assessment of androgen status. In the animal study, I described the potential molecular targets for action of testosterone on various metabolic pathways of carbohydrate and lipid metabolism and discussed the possible androgen receptor mediated and non-androgen receptor mediated mechanisms of action of testosterone.

I will describe the above findings in a wider context of hypogonadism and its relationship with diabetes and metabolic syndrome, the mechanism by which testosterone exerts its cardio-metabolic effects and the role of testosterone replacement therapy in hypogonadal men with diabetes and metabolic syndrome.

## **7.2 Testosterone deficiency, metabolic syndrome and mortality**

I have shown low testosterone is a risk factor for all-cause mortality. Although no single underlying cause of death was identified, a sub group analysis showed that men with low testosterone levels had an increased risk of cardiovascular mortality.

Further the study has shown that testosterone treated patients had an improved survival compared to the untreated low testosterone group. The fact that testosterone replacement improves survival suggests that testosterone has a direct impact on the disease state.

This raises further question of why the testosterone is low in these states and what are the mechanisms of action of testosterone responsible for its beneficial effects.

There are a number of studies published recently reporting an increased mortality in men with low testosterone. The mortality study (chapter 2) was the first study in diabetic men to report an increase in mortality with low testosterone levels (Muraleedharan, Marsh et al. 2013).

Subsequent studies both in the general population and specific disease groups have confirmed this link between low testosterone levels and increased mortality. But to date there are no further studies in type 2 diabetic men looking at survival in relation to testosterone. This has been discussed earlier and in the recent meta-analysis (Araujo, Dixon et al. 2011) concluding low testosterone levels are associated with increased mortality in men.

In Chapter 3 I have addressed this question by collecting data on the cardiovascular events and risk profile over a period of 7 years. The study showed that low testosterone has a long term adverse effect on the glycaemic control in diabetic men. There are many studies which demonstrated low testosterone as a risk factor for diabetes as discussed in the recent meta-analysis which reported that baseline testosterone was significantly lower among patients with incident diabetes in comparison with controls and that type 2 diabetes can be considered

independently associated with male hypogonadism (Corona, Monami et al. 2011). However there is lack of published data looking at the long term effect of a low testosterone state on glycaemic control. These results from the longitudinal study suggest that there are adverse cardio-metabolic risks associated with low testosterone level in the long term possibly contributing to the increased mortality seen in the low testosterone state.

In the cross sectional study (chapter 4) I found significant correlation between low testosterone state and adverse cardiovascular risk profile including glycaemic control, anthropometric measurements and carotid stiffness index  $\beta$ . Furthermore I found that SHBG is independently associated with cardiovascular risk profile with higher SHBG predicting a more favourable cardiovascular risk profile. These findings suggest that testosterone and SHBG indeed plays an active role in the modification of cardio-metabolic risk profile. The mechanisms by which testosterone and SHBG interacts with the metabolic pathways are still poorly understood.

To elucidate the mechanism by which testosterone exerts its actions in modifying the cardiovascular risk profile, in chapter 5, I examined the role of AR CAG in relation to cardio-metabolic risk profile. Previously published reports suggest AR CAG repeats have a significant effect on the actions of testosterone (Mariotti, Castellotti et al. 2000). Data from our own study group have shown a link between AR CAG polymorphisms with leptin and obesity (Stanworth, Kapoor et al. 2008). The present study did not show any significant correlation between AR CAG repeats and cardio-metabolic risk profile in the cross sectional study. However we found strong correlation between the ratio of testosterone to AR CAG lengths (T/AR CAG or BioT/ AR CAG) with the cardio metabolic risk profile in men with type 2 diabetes. The evidence I have presented in this thesis support that the ratio of

testosterone to AR CAG (T/AR CAG or BioT/ AR CAG) may have better sensitivity in predicting androgenicity. This is a novel finding. This also leads to the suggestion that the actions of testosterone on cardio-metabolic risk profile are, at least in part, mediated through the androgen receptors.

As discussed earlier, not all actions of testosterone are mediated by the classic androgen receptor. To study more about the possible mechanism of action of testosterone, in chapter 6, I looked into the mRNA expression of target genes for the molecules (both enzymes and receptors) involved in the carbohydrate and lipid metabolism in the testicular feminised mouse (Tfm). Tfm mouse has a non-functioning androgen receptor and low levels of testosterone due different mutations. The results showed low testosterone state adversely affect the gene expression of major enzymes and receptors involved in the metabolism by differential action in muscle, subcutaneous adipose tissue, liver and visceral adipose tissue. The testosterone administration partially reversed some of the adverse gene expressions. One of the major regulators of metabolic pathway is Liver X receptor alpha (LXR) which is key transcriptional regulators of lipid and carbohydrate metabolism. This study showed reduced expression of the LXR in muscle, liver and subcutaneous adipose tissue of Tfm mice compared to testosterone-replete animals. This suggests that testosterone may increase LXR signaling to exert some of its protective metabolic effects potentially by androgen independent pathways. The mRNA expression changes were confirmed using western blotting for the end product proteins (Kelly, Akhtar et al. 2016). These findings would support the concept of the wider role of testosterone as “multi-system player” as discussed in a recent review (Saad 2015), influencing various metabolic pathways in various tissues and that some of the actions of testosterone in metabolic pathways are androgen receptor independent.

### **7.3 Proposed mechanism of action of testosterone on the metabolic pathways**

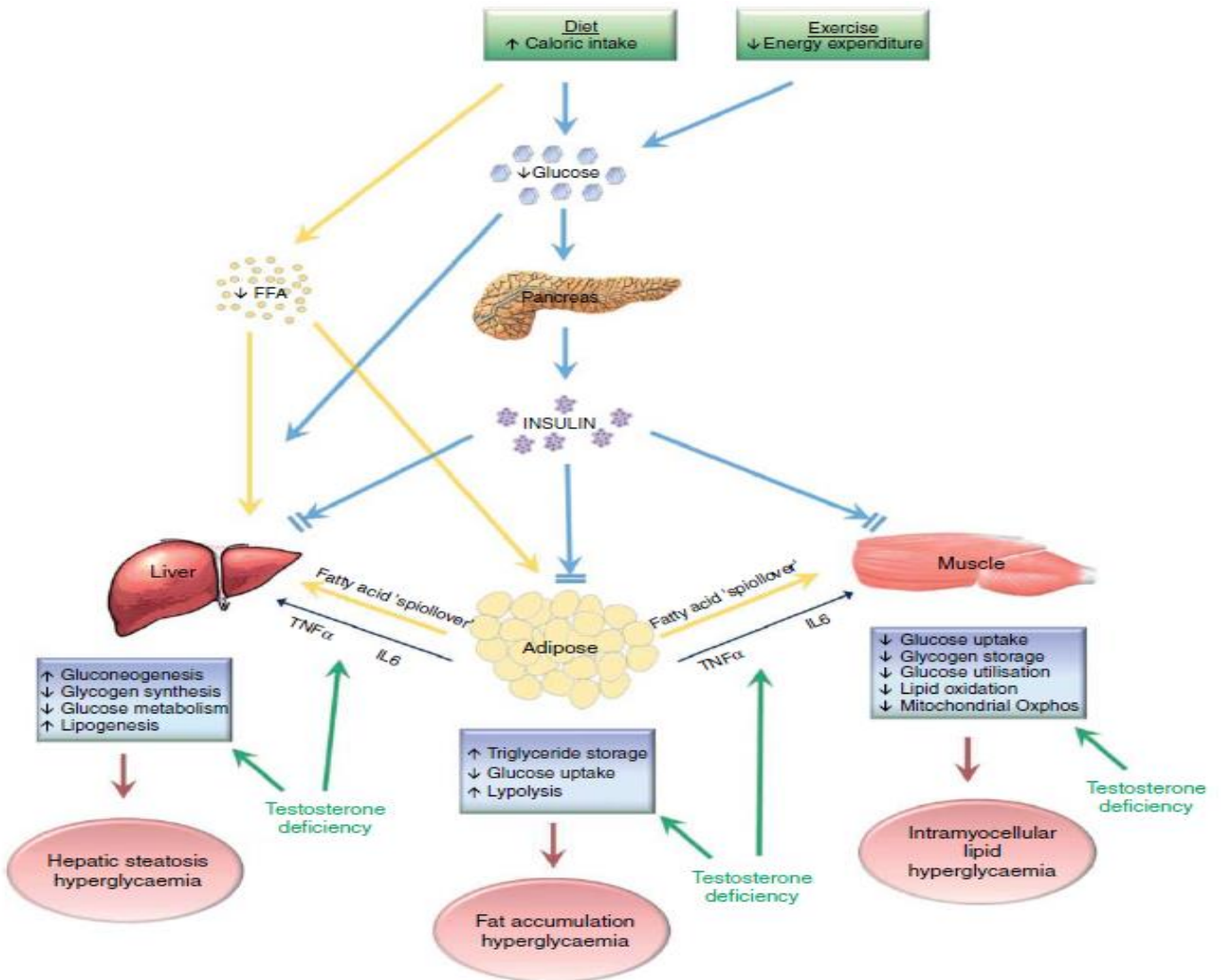
Testosterone is converted to 17 $\beta$  oestradiol (E2) by the enzymatic activity of aromatase in adipose tissue. Hypogonadal–obesity cycle hypothesis by Cohen suggested that with the increasing fatty-tissue accumulation there is an increase of aromatase activity associated with a greater conversion of testosterone to oestradiol. This results in further decrease in testosterone resulting in increased abdominal adiposity which further worsens insulin resistance (Cohen 1999).

The hypogonadal–obesity–adipocytokine hypothesis described in chapter one (Figure 1.3) extends Cohen’s theory to explain the lack of compensatory increase in LH and FSH to increase the testosterone levels (Jones 2007). Excess leptin from the adipocytes renders the hypothalamo-pituitary axis resistant to the feedback from the low testosterone. Leptin also directly inhibits the stimulatory action of gonadotrophins on the Leydig cells of the testis to decrease testosterone production (Isidori, Caprio et al. 1999).

Insulin resistance leads to defects in insulin-stimulated glucose transport activity, impaired insulin-mediated inhibition of hepatic glucose production and stimulation of glycogen synthesis in liver and reduces insulin ability to inhibit lipolysis in adipose tissue. As discussed in the previous section the excess free fatty acid resulting from insulin resistance leads to fat deposition in non-adipose tissue including skeletal muscle and liver occurring as a ‘spillover’ effect (Yu and Ginsberg 2005). The lipid accumulation will in turn lead to insulin resistance and abnormalities of glucose metabolism. The proposed sequence leading to the increased insulin resistance is shown in figure 8.1. This theory is supported by evidence suggesting heterogeneity insulin sensitivity among different tissues (Stumvoll, Jacob et al.

2000). This also suggests that the mechanisms by which testosterone may impart beneficial actions on men with type 2 diabetes or metabolic syndrome are likely to be tissue specific involving targets of lipid and carbohydrate metabolism. This theory is supported by the results from the animal study in the thesis which showed that testosterone has differential action on the key enzymes and receptors involved in the carbohydrate and lipid metabolism in different tissues.

**Figure 7.1 Putative mechanisms of obesity-induced insulin resistance**



Chronic excessive dietary fat and carbohydrate intake coupled with a decrease in energy expenditure leads to a sustained rise in circulating free fatty acids (FFA) and blood glucose concentration. Excess FFA are incorporated into adipocyte triglyceride storage increasing visceral and subcutaneous fat mass. Adipose accumulation promotes the release of FFA into the circulation via lipolysis and these are taken up by muscle and liver in a 'spillover' effect. With accumulation of intra-myocellular lipid, insulin-mediated skeletal muscle glucose uptake and utilisation is impaired along with decreased glycogen synthesis and lipid oxidation. As a result, excess glucose is diverted to the liver. In the liver, increased liver lipid also impairs the ability of insulin to regulate gluconeogenesis and activate glycogen synthesis. Hepatic lipogenesis further increases lipid content and can lead to hepatic steatosis. Impaired insulin action in the adipose tissue allows for increased lipolysis, which additionally promotes re-esterification of lipids in other tissues (such as liver and muscle) and further exacerbates insulin resistance. At the same time, adipose-derived inflammatory mediators contribute to the development of tissue insulin resistance in liver, adipose and muscle tissue and promote inflammation. Adapted from Kelly et al (Kelly and Jones 2013)



#### **7.4 Effect of testosterone therapy on metabolic syndrome, type 2 diabetes and mortality**

There is no universal agreement on the risks and benefits of testosterone replacement. As discussed in a recent review (Morgentaler 2016), concerns regarding cardiovascular risk were based on two deeply flawed retrospective studies and are contradicted by dozens of studies showing cardiovascular benefits of testosterone replacement therapy or higher endogenous testosterone, including placebo-controlled studies in men with known heart disease. The role of testosterone replacement in pituitary and gonadal disease is more widely accepted.

Controversies exist in the treatment of hypogonadism linked to metabolic syndrome and type 2 diabetes. This stems from lack of well-defined long term prospective studies looking into the risks and benefits of testosterone replacement therapy in hypogonadism of various aetiologies. However the majority of the short term studies and long term population based studies show that testosterone replacement is indeed have beneficial effects on mortality and morbidity. In a recent review looking into the effect of testosterone on cardiovascular system the authors concluded that existing evidence suggests men with coronary artery disease have lower levels of endogenous testosterone, more specifically lower levels of bioavailable testosterone. Furthermore low endogenous bioavailable testosterone levels have been shown to be associated with higher rates of all-cause and cardiovascular-related mortality (Oskui, French et al. 2013). The review, after extensively analysing the published studies further concluded that testosterone replacement therapy in men with hypogonadism improves obesity, T2DM, myocardial ischemia, exercise capacity, and QTc length.

In our mortality study of men with type 2 diabetes, we found that mortality was significantly lower in the testosterone treated group compared to that of untreated low testosterone group.

It also showed the mortality rates were similar in those with normal testosterone and those who had treatment. This leads to two conclusions, one testosterone might play a causative role in the disease process causing the mortality and the physiological replacement with testosterone significantly reduces the adverse effects caused by the low testosterone.

In the cross sectional study, the cardiovascular risk profile, again, was worse in those with low testosterone who had no treatment.

Further, the androgen receptor polymorphism in relation to the levels of testosterone, as measured by ratio of testosterone (either total or bioavailable) is shown as better marker of predicating cardiovascular risk profile. This is a novel concept and would explain why some people are more sensitive to the effects of testosterone replacement while other are not at the same level achieved for the testosterone.

We also found a correlation between SHBG levels and the cardiovascular risk profile, with higher SHBG having a more favourable risk profile. Testosterone replacement is known to cause increase in SHBG and could be a contributory factor in the beneficial effect of testosterone on the cardiovascular risk profile. These results further suggest an independent important role of SHBG in predicating cardiovascular risk profile in men with type 2 diabetes.

In the animal studies we found the testosterone treated animals had partial reversal of the molecular abnormalities caused by high fat diet in the context of low testosterone and a non-functioning androgen receptor. This sheds light into the tissue specific molecular mechanisms into the protective action of testosterone in metabolic syndrome and type 2 diabetes.

## 7.5 Conclusion

The thesis has described for the first time the relationship between low testosterone, mortality and cardiovascular risk profile and the likely beneficial effects of testosterone replacement in low testosterone state in men with type 2 diabetes. It has also shown a link between a potentially more sensitive marker of androgenisation (testosterone AR CAG ratio), again for the first time, and the cardiovascular risk profile as well as role of SHBG in the metabolic disease process. We have studied some of the important enzymes and receptors involved in the metabolic pathways to understand the mechanism of action of testosterone and highlighted the potential role of Liver X receptor in the modulation of metabolic pathways. The results also discussed the possible androgen dependent and independent pathways of testosterone action.

In the clinical context low testosterone state leads to a state of insulin resistance which in turn leads to poor glycaemic control as demonstrated in this study. The increased blood glucose would lead to decreased peripheral utilisation of glucose and increase free fatty acid production, which is also contributed by dietary fats. The excess fat leads to the “spill over” of free fatty acid from the storage sites and ectopic deposition of the fat in liver, arterial wall and visceral adipose tissue. This in turn cause increase in BMI, waist circumference, hepatic steatosis and atherosclerosis. This leads to worsening of cardiovascular risk profile and increased mortality as demonstrated in the thesis. Physiological testosterone reverses some of these pathologic states as evident in the clinical and animal study.

The role of SHBG is still poorly understood in relation to the metabolic pathways and its effect on type 2 diabetes, metabolic syndrome and cardiovascular disease. The results from

this study suggest SBGH has an independent role in the cardiovascular risk profile. These effects of SHBG need to be studied further.

The ratio of bioavailable testosterone to AR CAG ratio is a novel concept and this need to be validated in future studies. As this takes into consideration of the level of testosterone as well as the AR CAG polymorphism, this is likely to be more sensitive marker of androgen mediated actions of testosterone. This would also help to identify the actions which may be independent of androgen receptor.

The animal model, Tfm mouse, looking at the actions of testosterone at the molecular level I have demonstrated that low testosterone differentially influence the enzymes and receptors in the metabolic pathways with low testosterone level adversely affecting the metabolic milieu. It also further noted some of these actions are likely to be mediated through alternate pathways than androgen receptor.

As discussed earlier, without a long term prospective placebo controlled study, the issue of testosterone replacement is likely to remain controversial. Unless such evidence is available the beneficial effect, or otherwise, of testosterone replacement in men late onset hypogonadism will continue to be debated with differing views.

## **7.6 Future directions**

Large long term randomised prospective clinical studies to look into the effect of testosterone and mortality will answer some of the controversies in testosterone replacement therapy in men with hypogonadism.

Clinical studies using DEXA and MRI scans to study the changes in fat distribution would more accurately confirm the body fat changes in testosterone deficient state and the effect of testosterone replacement.

The role of SHBG as a bio marker for cardiovascular risk profile needs further investigation, with potential for new therapeutic interventions in metabolic syndrome and type 2 diabetes.

Future animal studies designed to differentiate the effects of testosterone using androgen and oestrogen receptor blockade would help to identify the precise androgen receptor mediated and non-receptor mediated actions of testosterone.

To study the effect of testosterone on different metabolic pathways of carbohydrate and lipid metabolism, isolated cell line culture studies would be needed.



## References

- Abate, N., S. M. Haffner, A. Garg, R. M. Peshock and S. M. Grundy (2002). "Sex Steroid Hormones, Upper Body Obesity, and Insulin Resistance." The Journal of Clinical Endocrinology & Metabolism **87**(10): 4522-4527.
- Agarwal, P. K. and M. G. Oefelein (2005). "Testosterone replacement therapy after primary treatment for prostate cancer." Journal of Urology **173**(2): 533-536.
- Agledahl, I., J. B. Hansen and J. Svartberg (2008). "Impact of testosterone treatment on postprandial triglyceride metabolism in elderly men with subnormal testosterone levels." Scandinavian Journal of Clinical & Laboratory Investigation **68**(7): 641-648.
- Albertelli, M. A., A. Scheller, M. Brogley and D. M. Robins (2006). "Replacing the mouse androgen receptor with human alleles demonstrates glutamine tract length-dependent effects on physiology and tumorigenesis in mice." Molecular Endocrinology **20**(6): 1248-1260.
- Alberti, K. G. M. M., R. H. Eckel, S. M. Grundy, P. Z. Zimmet, J. I. Cleeman, K. A. Donato, J.-C. Fruchart, W. P. T. James, C. M. Loria and S. C. Smith, Jr (2009). "Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity." Circulation **120**(16): 1640-1645.
- Alevizaki, M., A. T. Cimponeriu, M. Garofallaki, H. L. Sarika, C. C. Alevizaki, C. Papamichael, G. Philippou, E. A. Anastasiou, J. P. Lekakis and M. Mavrikakis (2003). "The androgen receptor gene CAG polymorphism is associated with the severity of coronary artery disease in men." Clinical Endocrinology **59**(6): 749-755.
- Alibhai, S. M., M. Duong-Hua, R. Sutradhar, N. E. Fleshner, P. Warde, A. M. Cheung and L. F. Paszat (2009). "Impact of androgen deprivation therapy on cardiovascular disease and diabetes." J Clin Oncol **27**(21): 3452-3458.
- Allan, C. A. and R. I. McLachlan (2004). "Age-related changes in testosterone and the role of replacement therapy in older men." Clinical Endocrinology **60**(6): 653-670.
- Allan, C. A. and R. I. McLachlan (2010). "Androgens and obesity." Current Opinion in Endocrinology Diabetes and Obesity **17**(3): 224-232.
- Anderson, F. H., R. M. Francis and K. Faulkner (1996). "Androgen supplementation in eugonadal men with osteoporosis - Effects of 6 months of treatment on bone mineral density and cardiovascular risk factors." Bone **18**(2): 171-177.
- Andersson, B., P. Marin, L. Lissner, A. Vermeulen and P. Bjorntorp (1994). "Testosterone concentrations in women and men with NIDDM." Diabetes Care **17**(5): 405-411.
- Apostolakis, M., D. Matzelt and K. D. Voigt (1963). "The effect of testosterone propionate on glycolytic and transamination enzyme activities in the liver, biceps muscle and levator ani muscle in rats." Biochemische Zeitschrift **337**(4): 414-&.
- Araujo, A. B., J. M. Dixon, E. A. Suarez, M. H. Murad, L. T. Guey and G. A. Wittert (2011). "Endogenous Testosterone and Mortality in Men: A Systematic Review and Meta-Analysis." Journal of Clinical Endocrinology & Metabolism **96**(10): 3007-3019.
- Araujo, A. B., G. R. Esche, V. Kupelian, A. B. O'Donnell, T. G. Travison, R. E. Williams, R. V. Clark and J. B. McKinlay (2007). "Prevalence of symptomatic androgen deficiency in men." Journal of Clinical Endocrinology & Metabolism **92**(11): 4241-4247.

Araujo, A. B., V. Kupelian, S. T. Page, D. J. Handelsman, W. J. Bremner and J. B. McKinlay (2007). "Sex Steroids and All-Cause and Cause-Specific Mortality in Men. ." Archives of Internal Medicine **167**(12): 1252-1260.

Barrettconnor, E. (1992). "Lower endogenous androgen levels and dyslipidemia in men with non-insulin-dependent diabetes-mellitus." Annals of Internal Medicine **117**(10): 807-811.

Basaria, S., A. D. Coviello, T. G. Travison, T. W. Storer, W. R. Farwell, A. M. Jette, R. Eder, S. Tennstedt, J. Ullor, A. Q. Zhang, K. Choong, K. M. Lakshman, N. A. Mazer, R. Miciek, J. Krasnoff, A. Elmi, P. E. Knapp, B. Brooks, E. Appleman, S. Aggarwal, G. Bhasin, L. Hede-Brierley, A. Bhatia, L. Collins, N. LeBrasseur, L. D. Fiore and S. Bhasin (2010). "Adverse Events Associated with Testosterone Administration." New England Journal of Medicine **363**(2): 109-122.

Basaria, S. and A. S. Dobs (2007). "Testosterone making an entry into the cardiometabolic world." Circulation **116**(23): 2658-2661.

Basaria, S., D. C. Muller, M. A. Carducci, J. Egan and A. S. Dobs (2006). "Hyperglycemia and insulin resistance in men with prostate carcinoma who receive androgen-deprivation therapy." Cancer **106**(3): 581-588.

Basso, F., L. Freeman, C. L. Knapper, A. Remaley, J. Stonik, E. B. Neufeld, T. Tansey, M. J. Amar, J. Fruchart-Najib, N. Duverger, S. Santamarina-Fojo and H. B. Brewer, Jr. (2003). "Role of the hepatic ABCA1 transporter in modulating intrahepatic cholesterol and plasma HDL cholesterol concentrations." J Lipid Res **44**(2): 296-302.

Bello, A. K., P. Stenvinkel, M. Lin, B. Hemmelgarn, R. Thadhani, S. Klarenbach, C. Chan, D. Zimmerman, G. Cembrowski, G. Strippoli, J. J. Carrero and M. Tonelli (2014). "Serum testosterone levels and clinical outcomes in male hemodialysis patients." Am J Kidney Dis **63**(2): 268-275.

Bergamini, E., G. Bombara and C. Pellegrino (1969). "The effect of testosterone on glycogen metabolism in rat levator ani muscle." Biochimica et Biophysica Acta (BBA) - General Subjects **177**(2): 220-234.

Berube, D., G. E. Seralini, R. Gagne and G. L. Hammond (1990). "Localization of the human sex hormone-binding globulin gene (SHBG) to the short arm of chromosome 17 (17p12---p13)." Cytogenet Cell Genet **54**(1-2): 65-67.

Bhasin, S., G. R. Cunningham, F. J. Hayes, A. M. Matsumoto, P. J. Snyder, R. S. Swerdloff and V. M. Montori (2010). "Testosterone Therapy in Men with Androgen Deficiency Syndromes: An Endocrine Society Clinical Practice Guideline." Journal of Clinical Endocrinology & Metabolism **95**(6): 2536-2559.

Birzniece, V., U. J. Meinhardt, D. J. Handelsman and K. K. Y. Ho (2009). "Testosterone stimulates extra-hepatic but not hepatic fat oxidation (Fox): comparison of oral and transdermal testosterone administration in hypopituitary men." Clinical Endocrinology **71**(5): 715-721.

Biswas, M., D. Hampton, R. G. Newcombe and D. A. Rees (2012). "Total and free testosterone concentrations are strongly influenced by age and central obesity in men with type 1 and type 2 diabetes but correlate weakly with symptoms of androgen deficiency and diabetes-related quality of life." Clinical Endocrinology **76**(5): 665-673.

Blouin, K., J. P. Despres, C. Couillard, A. Tremblay, D. Prud'homme, C. Bouchard and A. Tchernof (2005). "Contribution of age and declining androgen levels to features of the metabolic syndrome in men." Metabolism-Clinical and Experimental **54**(8): 1034-1040.

Bo, J. J., C. Zhang, L. H. Zhang, P. Liu, J. J. Sha, J. W. Lv, D. M. Liu, Y. R. Huang and Z. Li (2011). "Androgen deprivation therapy through bilateral orchiectomy: increased metabolic risks." Asian J Androl **13**(6): 833-837.

Bocchi, E. A., V. O. Carvalho and G. V. Guimaraes (2008). "Inverse correlation between testosterone and ventricle ejection fraction, hemodynamics and exercise capacity in heart



- failure patients with erectile dysfunction." Int Braz J Urol **34**(3): 302-310; discussion 310-302.
- Bojesen, A., S. Juul, N. H. Birkebaek and C. H. Gravholt (2006). "Morbidity in Klinefelter Syndrome: A Danish Register Study Based on Hospital Discharge Diagnoses " J Clin Endocrinol Metab **91**(4): 1254-1260.
- Bosco, C., D. Crawley, J. Adolfsson, S. Rudman and M. Van Hemelrijck (2015). "Quantifying the Evidence for the Risk of Metabolic Syndrome and Its Components following Androgen Deprivation Therapy for Prostate Cancer: A Meta-Analysis." PLoS ONE **10**(3): e0117344.
- Boyanov, M. A., Z. Boneva and V. G. Christov (2003). "Testosterone supplementation in men with type 2 diabetes, visceral obesity and partial androgen deficiency." Aging Male **6**(1): 1-7.
- Braga-Basaria, M., A. S. Dobs, D. C. Muller, M. A. Carducci, M. John, J. Egan and S. Basaria (2006). "Metabolic syndrome in men with prostate cancer undergoing long-term androgen-deprivation therapy." J Clin Oncol **24**(24): 3979-3983.
- Brand, J. S., I. van der Tweel, D. E. Grobbee, M. H. Emmelot-Vonk and Y. T. van der Schouw (2011). "Testosterone, sex hormone-binding globulin and the metabolic syndrome: a systematic review and meta-analysis of observational studies." International Journal of Epidemiology **40**(1): 189-207.
- Brown, M. S. and J. L. Goldstein (1999). "A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood." Proceedings of the National Academy of Sciences **96**(20): 11041-11048.
- Calof, O. M., A. B. Singh, M. L. Lee, A. M. Kenny, R. J. Urban, J. L. Tenover and S. Bhasin (2005). "Adverse events associated with testosterone replacement in middle-aged and older men: A meta-analysis of randomized, placebo-controlled trials." Journals of Gerontology Series a-Biological Sciences and Medical Sciences **60**(11): 1451-1457.
- Caminiti, G., M. Volterrani, F. Iellamo, G. Marazzi, R. Massaro, M. Miceli, C. Mammi, M. Piepoli, M. Fini and G. M. C. Rosano (2009). "Effect of Long-Acting Testosterone Treatment on Functional Exercise Capacity, Skeletal Muscle Performance, Insulin Resistance, and Baroreflex Sensitivity in Elderly Patients With Chronic Heart Failure A Double-Blind, Placebo-Controlled, Randomized Study." Journal of the American College of Cardiology **54**(10): 919-927.
- Caronia, L. M., A. A. Dwyer, D. Hayden, F. Amati, N. Pitteloud and F. J. Hayes (2013). "Abrupt decrease in serum testosterone levels after an oral glucose load in men: implications for screening for hypogonadism." Clinical Endocrinology **78**(2): 291-296.
- Carrero, J. J., A. R. Qureshi, A. Nakashima, S. Arver, P. Parini, B. Lindholm, P. Barany, O. Heimbürger and P. Stenvinkel (2011). "Prevalence and clinical implications of testosterone deficiency in men with end-stage renal disease." Nephrology Dialysis Transplantation **26**(1): 184-190.
- Carrero, J. J., A. R. Qureshi, P. Parini, S. Arver, B. Lindholm, P. BÅrÅny, O. HeimbÅrger and P. Stenvinkel (2009). "Low serum testosterone increases mortality risk among male dialysis patients." Journal of the American Society of Nephrology - **20**(- 3): - 620.
- Carson, C. C., III and G. Rosano (2012). "Exogenous Testosterone, Cardiovascular Events, and Cardiovascular Risk Factors in Elderly Men: A Review of Trial Data." Journal of Sexual Medicine **9**(1): 54-67.
- Chamberlain, N. L., E. D. Driver and R. L. Miesfeld (1994). "The Length and Location of CG Trinucleotide Repeats in the Androgen Receptor N-Terminal Domain Affect Transactivation Function." Nucleic Acids Research **22**(15): 3181-3186.

Chandel, A., S. Dhindsa, S. Topiwala, A. Chaudhuri and P. Dandona (2008). "Testosterone Concentration in Young Patients With Diabetes." Diabetes Care **31**(10): 2013-2017.

Chang, C. S., A. Saltzman, S. Y. Yeh, W. J. Young, E. Keller, H. J. Lee, C. H. Wang and A. Mizokami (1995). "Androgen receptor - an overview." Critical Reviews in Eukaryotic Gene Expression **5**(2): 97-125.

Charest, N. J., Z. X. Zhou, D. B. Lubahn, K. L. Olsen, E. M. Wilson and F. S. French (1991). "A Frameshift Mutation Destabilizes Androgen Receptor Messenger-Rna In The Tfm Mouse." Molecular Endocrinology **5**(4): 573-581.

Chen, B. H., K. Brennan, A. Goto, Y. Song, N. Aziz, N. C. You, M. F. Wellons, J. E. Manson, D. L. White, A. W. Butch and S. Liu (2012). "Sex hormone-binding globulin and risk of clinical diabetes in American black, Hispanic, and Asian/Pacific Islander postmenopausal women." Clin Chem **58**(10): 1457-1466.

Chen, X., X. Li, H. Y. Huang, X. Li and J. F. Lin (2006). "[Effects of testosterone on insulin receptor substrate-1 and glucose transporter 4 expression in cells sensitive to insulin]." Zhonghua Yi Xue Za Zhi **86**(21): 1474-1477.

Chow, J. D. Y., M. E. E. Jones, K. Prella, E. R. Simpson and W. C. Boon (2011). "A selective estrogen receptor  $\alpha$  agonist ameliorates hepatic steatosis in the male aromatase knockout mouse." Journal of Endocrinology **210**(3): 323-334.

Chubb, S. A. P., Z. Hyde, O. P. Almeida, L. Flicker, P. E. Norman, K. Jamrozik, G. J. Hankey and B. B. Yeap (2008). "Lower sex hormone-binding globulin is more strongly associated with metabolic syndrome than lower total testosterone in older men: the Health in Men Study." European Journal of Endocrinology **158**(6): 785-792.

Cohen, P., J. M. Ntambi and J. M. Friedman (2003). "Stearoyl-CoA desaturase-1 and the metabolic syndrome." Curr Drug Targets Immune Endocr Metabol Disord **3**(4): 271-280.

Cohen, P. G. (1999). "The hypogonadal-obesity cycle: role of aromatase in modulating the testosterone-estradiol shunt - a major factor in the genesis of morbid obesity." Medical Hypotheses **52**(1): 49-51.

Corona, G., E. Mannucci, G. Forti and M. Maggi (2009). "Hypogonadism, ED, metabolic syndrome and obesity: a pathological link supporting cardiovascular diseases." International Journal of Andrology **32**(6): 587-598.

Corona, G., E. Mannucci, C. Schulman, L. Petrone, R. Mansani, A. Cilotti, G. Balercia, V. Chiarini, G. Forti and M. Maggi (2006). "Psychobiologic Correlates of the Metabolic Syndrome and Associated Sexual Dysfunction." European Urology **50**(3): 595-604.

Corona, G., M. Monami, V. Boddi, M. Cameron-Smith, A. D. Fisher, G. de Vita, C. Melani, D. Balzi, A. Sforza, G. Forti, E. Mannucci and M. Maggi (2010). "Low Testosterone is Associated with an Increased Risk of MACE Lethality in Subjects with Erectile Dysfunction." Journal of Sexual Medicine **7**(4): 1557-1564.

Corona, G., M. Monami, G. Rastrelli, A. Aversa, A. Sforza, A. Lenzi, G. Forti, E. Mannucci and M. Maggi (2011). "Type 2 diabetes mellitus and testosterone: a meta-analysis study." International Journal of Andrology **34**(6pt1): 528-540.

Corrales, J. J., M. Almeida, R. Burgo, M. T. Mories, J. M. Miralles and A. Orfao (2006). "Androgen-replacement therapy depresses the ex vivo production of inflammatory cytokines by circulating antigen-presenting cells in aging type-2 diabetic men with partial androgen deficiency." Journal of Endocrinology **189**(3): 595-604.

Couillard, C., J. Gagnon, J. Bergeron, A. S. Leon, D. C. Rao, J. S. Skinner, J. H. Wilmore, J. P. Despres and C. Bouchard (2000). "Contribution of body fatness and adipose tissue distribution to the age variation in plasma steroid hormone concentrations in men: The HERITAGE family study." Journal of Clinical Endocrinology & Metabolism **85**(3): 1026-1031.

Couse, J. F. and K. S. Korach (1999). "Estrogen receptor null mice: what have we learned and where will they lead us? (vol 20, pg 358, 1999)." Endocrine Reviews **20**(4): 459-459.

D'Agostino, P., S. Milano, C. Barbera, G. Di Bella, M. La Rosa, V. Ferlazzo, R. Farruggio, D. M. Miceli, M. Miele, L. Castagnetta and E. Cillari (1999). Sex hormones modulate inflammatory mediators produced by macrophages. Neuroendocrine Immune Basis of the Rheumatic Diseases. M. Cutolo, A. T. Masi, J. W. J. Bijlsma et al. New York, New York Acad Sciences. **876**: 426-429.

De Pergola, G. (2000). "The adipose tissue metabolism: role of testosterone and dehydroepiandrosterone." International Journal of Obesity **24**: S59-S63.

De Pergola, G., N. Pannacciulli, M. Ciccone, M. Tartagni, P. Rizzon, R. Giorgino, J. Svartberg, D. Von Muhlen, E. Mathiesen, O. Joakimsen, K. H. Bonna, E. Stensland-Bugge, M. M. D. Fukui, Y. M. D. Kitagawa, N. M. D. Nakamura, M. M. D. Kadono, S. M. D. Mogami, C. M. D. Hirata, N. M. D. Ichio, K. M. D. Wada, G. M. D. Hasegawa, T. M. D. Yoshikawa, L. Fu, Q.-P. Gao, J.-X. Shen, M. M. D. P. Muller, A. W. M. D. P. van den Beld, M. L. M. D. P. Bots, D. E. M. D. P. Grobbee, S. W. J. M. D. P. Lamberts and Y. T. P. van der Schouw (2003). "Free testosterone plasma levels are negatively associated with the intima-media thickness of the common carotid artery in overweight and obese glucose-tolerant young adult men  
Low testosterone levels are associated with carotid atherosclerosis in men  
Association Between Serum Testosterone Concentration and Carotid Atherosclerosis in Men With Type 2 Diabetes  
Relationship between testosterone and indexes indicating endothelial function in male coronary heart disease patients  
Endogenous Sex Hormones and Progression of Carotid Atherosclerosis in Elderly Men." International Journal of Obesity July **27**(7): 803-807.

de Ronde, W. and F. H. de Jong (2011). "Aromatase inhibitors in men: effects and therapeutic options." Reproductive Biology and Endocrinology : RB&E **9**: 93-93.

De Ronde, W., Y. T. Van der Schouw, H. A. P. Pols, L. J. G. Gooren, M. Muller, D. E. Grobbee and F. H. De Jong (2006). "Calculation of bioavailable and free testosterone in men: A comparison of 5 published algorithms." Clinical Chemistry **52**(9): 1777-1784.

Demirbag, R., R. Yilmaz, A. Ulucay and D. Unlu (2005). "The inverse relationship between thoracic aortic intima media thickness and testosterone level." Endocrine Research **31**(4): 335-344.

Derweesh, I. H., C. J. DiBlasio, M. C. Kincade, J. B. Malcolm, K. D. Lamar, A. L. Patterson, A. E. Kitabchi and R. W. Wake (2007). "Risk of new-onset diabetes mellitus and worsening glycaemic variables for established diabetes in men undergoing androgen-deprivation therapy for prostate cancer." BJU International **100**(5): 1060-1065.

Dhindsa, S., H. Ghanim, M. Batra, N. D. Kuhadiya, S. Abuaysheh, S. Sandhu, K. Green, A. Makdissi, J. Hejna, A. Chaudhuri, M. Punyanitya and P. Dandona (2016). "Insulin Resistance and Inflammation in Hypogonadotropic Hypogonadism and Their Reduction After Testosterone Replacement in Men With Type 2 Diabetes." Diabetes Care **39**(1): 82-91.

Dhindsa, S., S. Prabhakar, M. Sethi, A. Bandyopadhyay, A. Chaudhuri and P. Dandona (2004). "Frequent occurrence of hypogonadotropic hypogonadism in type 2 diabetes." Journal of Clinical Endocrinology & Metabolism **89**(11): 5462-5468.

Dicker, A., M. Rydén, E. Näslund, I. E. Muehlen, M. Wirén, M. Lafontan and P. Arner (2004). "Effect of testosterone on lipolysis in human pre-adipocytes from different fat depots." Diabetologia **47**(3): 420-428.

Ding, E. L., Y. Song, V. S. Malik and S. Liu (2006). "Sex differences of endogenous sex hormones and risk of type 2 diabetes: a systematic review and meta-analysis.[see comment]. [Review] [129 refs]."

JAMA. 295(11):1288-99, 2006 Mar 15.

Dobrzyn, P., M. Jazurek and A. Dobrzyn (2010). "Stearoyl-CoA desaturase and insulin signaling--what is the molecular switch?" Biochim Biophys Acta **1797**(6-7): 1189-1194.

Eckel, R. H. (1989). "Lipoprotein Lipase." New England Journal of Medicine **320**(16): 1060-1068.

Edmunds, S. E., A. P. Stubbs, A. A. Santos and M. L. Wilkinson (1990). "Estrogen and androgen regulation of sex hormone binding globulin secretion by a human liver cell line." J Steroid Biochem Mol Biol **37**(5): 733-739.

Eichner, J. E., L. H. Kuller, T. J. Orchard, G. A. Grandits, L. M. McCallum, R. E. Ferrell and J. D. Neaton (1993). "Relation of apolipoprotein E phenotype to myocardial infarction and mortality from coronary artery disease." Am J Cardiol **71**(2): 160-165.

Elisabeth Hak, A., J. C. M. Witteman, F. H. De Jong, M. I. Geerlings, A. Hofman and H. A. P. Pols (2002). "Low levels of endogenous androgens increase the risk of atherosclerosis in elderly men: The Rotterdam Study." Journal of Clinical Endocrinology and Metabolism **87**(8): 3632-3639.

English, K. M., R. P. Steeds, T. H. Jones, M. J. Diver and K. S. Channer (2000). "Low-dose transdermal testosterone therapy improves angina threshold in men with chronic stable angina - A randomized, double-blind, placebo-controlled study." Circulation **102**(16): 1906-1911.

Fan, A. Z. and J. H. Dwyer (2007). "Sex differences in the relation of HDL cholesterol to progression of carotid intima-media thickness: the Los Angeles Atherosclerosis Study." Atherosclerosis **195**(1): e191-196.

Faris, J. E. and M. R. Smith (2010). "Metabolic sequelae associated with androgen deprivation therapy for prostate cancer." Current Opinion in Endocrinology Diabetes and Obesity **17**(3): 240-246.

Feldman, H. A., C. Longcope, C. A. Derby, C. B. Johannes, A. B. Araujo, A. D. Coviello, W. J. Bremner and J. B. McKinlay (2002). "Age trends in the level of serum testosterone and other hormones in middle-aged men: Longitudinal results from the Massachusetts Male Aging Study." Journal of Clinical Endocrinology and Metabolism **87**(2): 589-598.

Finkle, W. D., S. Greenland, G. K. Ridgeway, J. L. Adams, M. A. Frasco, M. B. Cook, J. F. Fraumeni, Jr. and R. N. Hoover (2014). "Increased risk of non-fatal myocardial infarction following testosterone therapy prescription in men." PLoS One **9**(1): e85805.

Fu, L., Q.-P. Gao and J.-X. Shen (2008). "Relationship between testosterone and indexes indicating endothelial function in male coronary heart disease patients. [Article]." Asian Journal of Andrology March 2008;10(2):214-218.

Fukui, M., Y. Kitagawa, N. Nakamura, M. Kadono, S. Mogami, C. Hirata, N. Ichio, K. Wada, G. Hasegawa and T. Yoshikawa (2003). "Association between serum testosterone concentration and carotid atherosclerosis in men with type 2 diabetes." Diabetes Care **26**(6): 1869-1873.

Gapstur, S. M., P. H. Gann, P. Kopp, L. Colangelo, C. Longcope and K. Liu (2002). "Serum androgen concentrations in young men: A longitudinal analysis of associations with age, obesity, and race. The CARDIA Male Hormone Study." Cancer Epidemiology Biomarkers & Prevention **11**(10): 1041-1047.

Garaulet, M., F. Perex-Llamas, T. Fuente, S. Zamora and F. Tebar (2000). "Anthropometric, computed tomography and fat cell data in an obese population: relationship with insulin, leptin, tumor necrosis factor-alpha, sex hormone-binding globulin and sex hormones." European Journal of Endocrinology **143**(5): 657-666.

Garaulet, M., F. Perez-Llamas, T. Fuente, S. Zamora and F. J. Tebar (2000). "Anthropometric, computed tomography and fat cell data in an obese population:

relationship with insulin, leptin, tumor factor-alpha, sex hormone-binding globulin and sex hormones." European Journal of Endocrinology **143**(5): 657-666.

Gornstein, R. A., C. A. Lapp, S. M. Bustos-Valdes and P. Zamorano (1999). "Androgens modulate interleukin-6 production by gingival fibroblasts in vitro." Journal of Periodontology **70**(6): 604-609.

Grossmann, M., E. J. Gianatti and J. D. Zajac (2010). "Testosterone and type 2 diabetes." Curr Opin Endocrinol Diabetes Obes **17**(3): 247-256.

Gruen, R., E. Hietanen and M. R. C. Greenwood "Increased adipose tissue lipoprotein lipase activity during the development of the genetically obese rat (fa/fa)." Metabolism - Clinical and Experimental **27**(12): 1955-1966.

Gruenewald, D. A. and A. M. Matsumoto (2003). "Testosterone supplementation therapy for older men: Potential benefits and risks." Journal of the American Geriatrics Society December 2004;**52**(12):2077-2081 **51**(1): 101-115

Gungor, O., F. Kircelli, J. J. Carrero, G. Ascii, H. Toz, E. Tatar, E. Hur, M. S. Sever, T. Arinsoy and E. Ok (2010). "Endogenous testosterone and mortality in male hemodialysis patients: Is it the result of aging?" Clinical Journal of the American Society of Nephrology **5**(11): 2018-2023.

Guo, X., H. Li, H. Xu, S. Woo, H. Dong, F. Lu, A. J. Lange and C. Wu (2012). "Glycolysis in the control of blood glucose homeostasis." Acta Pharmaceutica Sinica B **2**(4): 358-367.

Hackett, G., A. H. Heald, A. Sinclair, P. W. Jones, R. C. Strange and S. Ramachandran (2016). "Serum testosterone, testosterone replacement therapy and all-cause mortality in men with type 2 diabetes: retrospective consideration of the impact of PDE5 inhibitors and statins." Int J Clin Pract **70**(3): 244-253.

Hackett, G. I. (2016). "Testosterone Replacement Therapy and Mortality in Older Men." Drug Safety **39**(2): 117-130.

Haddad, R. M., C. C. Kennedy, S. M. Caples, M. J. Tracz, E. R. Bolona, K. Sideras, M. V. Uraga, P. J. Erwin and V. M. Montori (2007). "Testosterone and cardiovascular risk in men: A systematic review and meta-analysis of randomized placebo-controlled trials." Mayo Clinic Proceedings **82**(1): 29-39.

Haffner, S. M., P. Karhapaa, L. Mykkanen and M. Laakso (1994). "Insulin-resistance, body-fat distribution, and sex-hormones in men." Diabetes **43**(2): 212-219.

Haffner, S. M., L. Mykkanen, R. A. Valdez and M. S. Katz (1993). "Relationship of sex-hormones to lipids and lipoproteins in nondiabetic men." Journal of Clinical Endocrinology & Metabolism **77**(6): 1610-1615.

Haffner, S. M., J. Shaten, M. P. Stern, G. D. Smith and L. Kuller (1996). "Low levels of sex hormone-binding globulin and testosterone predict the development of non-insulin-dependent diabetes mellitus in men." American Journal of Epidemiology **143**(9): 889-897.

Haffner, S. M., R. A. Valdez, M. P. Stern and M. S. Katz (1993). "Obesity, body-fat distribution and sex-hormones in men." International Journal of Obesity **17**(11): 643-649.

Hall, D. W. R., W. E. Hoven, N. J. Noordzij-Kamermans and K. D. Jaitly "Hormonal control of hamster ear sebaceous gland lipogenesis." Archives of Dermatological Research **275**(1): 1-7.

Hall, J., R. D. Jones, T. H. Jones, K. S. Channer and C. Peers (2006). "Selective inhibition of L-type Ca<sup>2+</sup> channels in A7r5 cells by physiological levels of testosterone." Endocrinology **147**(6): 2675-2680.

Hamm, L. (1942). "Testosterone propionate in the treatment of angina pectoris." Journal of Clinical Endocrinology **2**(5): 325-328.

Handelsman, D. J. (2013). "Mendelian randomization: loosening the Gordian knot of testosterone and male ageing." Asian Journal of Andrology **15**(5): 579-581.

Haring, R., M. Nauck, H. Volzke, K. Endlich, U. Lendeckel, N. Friedrich, M. Dorr, R. Rettig, H. K. Kroemer and H. Wallaschofski (2011). "Low Serum Testosterone Is Associated with Increased Mortality in Men with Stage 3 or Greater Nephropathy." American Journal of Nephrology **33**(3): 209-217.

Haring, R., Z. Teng, V. Xanthakis, A. Coviello, L. Sullivan, S. Bhasin, J. M. Murabito, H. Wallaschofski and R. S. Vasan (2013). "Association of sex steroids, gonadotrophins, and their trajectories with clinical cardiovascular disease and all-cause mortality in elderly men from the Framingham Heart Study." Clinical Endocrinology **78**(4): 629-634.

Haring, R., H. Volzke, A. Steveling, A. Krebs, S. B. Felix, C. Schofl, M. Dorr, M. Nauck and H. Wallaschofski (2010). "Low serum testosterone levels are associated with increased risk of mortality in a population-based cohort of men aged 20-79." European Heart Journal **31**(12): 1494-1501.

Harman, S. M., E. J. Metter, J. D. Tobin, J. Pearson and M. R. Blackman (2001). "Longitudinal Effects of Aging on Serum Total and Free Testosterone Levels in Healthy Men." Journal of Clinical Endocrinology & Metabolism February **86**(2): 724-731.

Hatakeyama, H., M. Nishizawa, A. Nakagawa, S. Nakano, T. Kigoshi and K. Uchida (2002). "Testosterone inhibits tumor necrosis factor-alpha-induced vascular cell adhesion molecule-1 expression in human aortic endothelial cells." FEBS Letters **530**(1-3): 129-132.

Hattersley, A. T., R. C. Turner, M. A. Permutt, P. Patel, Y. Tanizawa, K. C. Chiu, S. O'Rahilly, P. J. Watkins and J. S. Wainscoat (1992). "Linkage of type 2 diabetes to the glucokinase gene." Lancet **339**(8805): 1307-1310.

Hayes, F. J., S. Decruz, S. B. Seminara, P. A. Boepple and W. F. Crowley (2001). "Differential regulation of gonadotropin secretion by testosterone in the human male: Absence of a negative feedback effect of testosterone on follicle-stimulating hormone secretion." Journal of Clinical Endocrinology & Metabolism **86**(1): 53-58.

Hayes, F. J., S. B. Seminara, S. Decruz, P. A. Boepple and W. F. Crowley (2000). "Aromatase inhibition in the human male reveals a hypothalamic site of estrogen feedback." Journal of Clinical Endocrinology & Metabolism **85**(9): 3027-3035.

He, W. W., M. V. Kumar and D. J. Tindall (1991). "A Frame-Shift Mutation in the Androgen Receptor Gene Causes Complete Androgen Insensitivity in the Testicular-Feminized Mouse." Nucleic Acids Research **19**(9): 2373-2378.

Heemers, H., B. Maes, F. Fougelle, W. Heyns, G. Verhoeven and J. V. Swinnen (2001). "Androgens Stimulate Lipogenic Gene Expression in Prostate Cancer Cells by Activation of the Sterol Regulatory Element-Binding Protein Cleavage Activating Protein/Sterol Regulatory Element-Binding Protein Pathway." Molecular Endocrinology **15**(10): 1817-1828.

Heufelder, A. E., F. Saad, M. C. Bunck and L. Gooren (2009). "Fifty-two-Week Treatment With Diet and Exercise Plus Transdermal Testosterone Reverses the Metabolic Syndrome and Improves Glycemic Control in Men With Newly Diagnosed Type 2 Diabetes and Subnormal Plasma Testosterone." Journal of Andrology **30**(6): 726-733.

Hill, S., V. Arutchelvam and R. Quinton (2009). "Enclomiphene, an estrogen receptor antagonist for the treatment of testosterone deficiency in men." IDrugs **12**(2): 109-119.

Hiort, O., G. H. G. Sinnecker, P. M. Holterhus, E. M. Nitsche and K. Kruse (1996). "The clinical and molecular spectrum of androgen insensitivity syndromes." American Journal of Medical Genetics **63**(1): 218-222.

Hofbauer, L. C., R. M. Ten and S. Khosla (1999). "The anti-androgen hydroxyflutamide and androgens inhibit interleukin-6 production by an androgen-responsive human osteoblastic cell line." Journal of Bone and Mineral Research **14**(8): 1330-1337.

Hong, C. and P. Tontonoz (2014). "Liver X receptors in lipid metabolism: opportunities for drug discovery." Nat Rev Drug Discov **13**(6): 433-444.

Horton, J. D., I. Shimomura, S. Ikemoto, Y. Bashmakov and R. E. Hammer (2003). "Overexpression of sterol regulatory element-binding protein-1a in mouse adipose tissue produces adipocyte hypertrophy, increased fatty acid secretion, and fatty liver." J Biol Chem **278**(38): 36652-36660.

Hsing, A. W., Y. T. Gao, G. Wu, X. Wang, J. Deng, Y. L. Chen, I. A. Sesterhenn, F. K. Mostofi, J. Benichou and C. S. Chang (2000). "Polymorphic CAG and GGN repeat lengths in the androgen receptor gene and prostate cancer risk: A population-based case-control study in China." Cancer Research **60**(18): 5111-5116.

Hsing, A. W., L. Tsao and S. S. Devesa (2000). "International trends and patterns of prostate cancer incidence and mortality." International Journal of Cancer **85**(1): 60-67.

Huang, B., B. L. Rodriguez, C. M. Burchfiel, P. H. Chyou, J. D. Curb and D. S. Sharp (1997). "Associations of adiposity with prevalent coronary heart disease among elderly men: The Honolulu heart program." International Journal of Obesity **21**(5): 340-348.

Hyde, Z., P. E. Norman, L. Flicker, G. J. Hankey, O. P. Almeida, K. A. McCaul, S. A. Chubb and B. B. Yeap (2012). "Low free testosterone predicts mortality from cardiovascular disease but not other causes: the Health in Men Study." J Clin Endocrinol Metab **97**(1): 179-189.

Isidori, A. M., M. Caprio, F. Strollo, C. Moretti, G. Frajese, A. Isidori and A. Fabbri (1999). "Leptin and androgens in male obesity: evidence for leptin contribution to reduced androgen levels." J Clin Endocrinol Metab **84**(10): 3673-3680.

Isidori, A. M., E. Giannetta, E. A. Greco, D. Gianfrilli, V. Bonifacio, A. Isidori, A. Lenzi and A. Fabbri (2005). "Effects of testosterone on body composition, bone metabolism and serum lipid profile in middle-aged men: a meta-analysis." Clin Endocrinol (Oxf) **63**(3): 280-293.

Isidori, A. M. and A. Lenzi (2007). "Testosterone replacement therapy: What we know is not yet enough." Mayo Clinic Proceedings **82**(1): 11-13

Itani, S. I., N. B. Ruderman, F. Schmedier and G. Boden (2002). "Lipid-Induced Insulin Resistance in Human Muscle Is Associated With Changes in Diacylglycerol, Protein Kinase C, and I $\kappa$ B- $\alpha$ ." Diabetes **51**(7): 2005-2011.

Jenster, G., J. Vanderkorput, J. Trapman and A. O. Brinkmann (1992). "Functional domains of the human androgen receptor." Journal of Steroid Biochemistry and Molecular Biology **41**(3-8): 671-675.

Johnsen, S. H., E. B. Mathiesen, E. Fosse, O. Joakimsen, E. Stensland-Bugge, I. Njolstad and E. Arnesen (2005). "Elevated high-density lipoprotein cholesterol levels are protective against plaque progression - A follow-up study of 1952 persons with carotid atherosclerosis - The Tromso study." Circulation **112**(4): 498-504.

Jones, R. D., J. E. Nettleship, D. Kapoor, H. T. Jones and K. S. Channer (2005). "Testosterone and atherosclerosis in aging men: purported association and clinical implications." Am J Cardiovasc Drugs **5**(3): 141-154.

Jones, R. D., P. J. Pugh, J. Hall, K. S. Channer and T. H. Jones (2003). "Altered circulating hormone levels, endothelial function and vascular reactivity in the testicular feminised mouse." European Journal of Endocrinology **148**(1): 111-120.

Jones, T. H. (2007). "Testosterone associations with erectile dysfunction, diabetes, and the metabolic syndrome." European Urology Supplements **6**(16): 847-857.

Jones, T. H. (2009). "Late onset hypogonadism." BMJ **338**.

Jones, T. H. (2010). "Testosterone deficiency: A risk factor for cardiovascular disease?" Trends in Endocrinology and Metabolism **21**(8): 496-503.

Jones, T. H. (2011). "Cardiovascular risk during androgen deprivation therapy for prostate cancer." BMJ **342**.

Jones, T. H., S. Arver, H. M. Behre, J. Buvat, E. Meuleman, I. Moncada, A. M. Morales, M. Volterrani, A. Yellowlees, J. D. Howell, K. S. Channer and T. Investigators (2011). "Testosterone Replacement in Hypogonadal Men With Type 2 Diabetes and/or Metabolic Syndrome (the TIMES2 Study)." Diabetes Care **34**(4): 828-837.

Jones, T. H., R. D. Jones and K. S. Channer (2003). Testosterone and cardiovascular disorders. Recent Research Developments in Endocrinology and Metabolism. Kerala, India, Transworld Research Network. **1**: 143-167.

Jones, T. H. and F. Saad (2009). "The effects of testosterone on risk factors for, and the mediators of, the atherosclerotic process." Atherosclerosis **207**(2): 318-327.

Joyce, C. W., M. J. Amar, G. Lambert, B. L. Vaisman, B. Paigen, J. Najib-Fruchart, R. F. Hoyt, Jr., E. D. Neufeld, A. T. Remaley, D. S. Fredrickson, H. B. Brewer, Jr. and S. Santamarina-Fojo (2002). "The ATP binding cassette transporter A1 (ABCA1) modulates the development of aortic atherosclerosis in C57BL/6 and apoE-knockout mice." Proc Natl Acad Sci U S A **99**(1): 407-412.

Kalinchenko, S. I., L. O. Vorslov, N. L. Aglamazian and L. I. Morgunov (2007). "Efficacy and safety of hormonal therapy with androgens (androgel) in men with erectile dysfunction, partial androgen deficiency of aging male and cardiovascular diseases." Urologiia (Moscow, Russia) (1): 57, 59-61

Kalinchenko, S. Y., Y. A. Tishova, G. J. Mskhalaya, L. J. G. Gooren, E. J. Giltay and F. Saad (2010). "Effects of testosterone supplementation on markers of the metabolic syndrome and inflammation in hypogonadal men with the metabolic syndrome: the double-blinded placebo-controlled Moscow study." Clinical Endocrinology **73**(5): 602-612.

Kapoor, D., H. Aldred, S. Clark, K. S. Channer and T. H. Jones (2007). "Clinical and biochemical assessment of hypogonadism in men with type 2 diabetes: correlations with bioavailable testosterone and visceral adiposity." Diabetes Care. **30**(4):911-7, 2007 Apr.

Kapoor, D., S. Clarke, K. S. Channer and T. H. Jones (2007). "Erectile dysfunction is associated with low bioactive testosterone levels and visceral adiposity in men with type 2 diabetes." Int J Androl **30**(6): 500-507.

Kapoor, D., S. Clarke, R. Stanworth, K. S. Channer and T. H. Jones (2007). "The effect of testosterone replacement therapy on adipocytokines and C-reactive protein in hypogonadal men with type 2 diabetes." Eur J Endocrinol **156**(5): 595-602.

Kapoor, D., E. Goodwin, K. S. Channer and T. H. Jones (2006). "Testosterone replacement therapy improves insulin resistance, glycaemic control, visceral adiposity and hypercholesterolaemia in hypogonadal men with type 2 diabetes." Eur J Endocrinol **154**(6): 899-906.

Kapoor, D., C. J. Malkin, K. S. Channer and T. H. Jones (2005). "Androgens, insulin resistance and vascular disease in men." Clin Endocrinol (Oxf) **63**(3): 239-250.

Kaufman, J. M. and A. Vermeulen (2005). "The decline of androgen levels in elderly men and its clinical and therapeutic implications." Endocrine Reviews **26**(6): 833-876.

Kazemiesfarjani, P., M. A. Trifiro and L. Pinsky (1995). "Evidence for a Repressive Function of the Long Polyglutamine Tract in the Human Androgen Receptor - Possible Pathogenetic Relevance for the (CAG)(N)-Expanded Neuronopathies." Human Molecular Genetics **4**(4): 523-527.

Keating, N. L., A. J. O'Malley, S. J. Freedland and M. R. Smith (2010). "Diabetes and cardiovascular disease during androgen deprivation therapy: observational study of veterans with prostate cancer." J Natl Cancer Inst **102**(1): 39-46.

Keating, N. L., A. J. O'Malley and M. R. Smith (2006). "Diabetes and Cardiovascular Disease During Androgen Deprivation Therapy for Prostate Cancer. [Report]." Journal of Clinical Oncology September 20, 2006;**24**(27):4448-4456.



Kelly, D. M., S. Akhtar, D. J. Sellers, V. Muraleedharan, K. S. Channer and T. H. Jones (2016). "Testosterone differentially regulates targets of lipid and glucose metabolism in liver, muscle and adipose tissues of the testicular feminised mouse." Endocrine.

Kelly DM, A. S., Brooke JC, Muraleedharan V, Channer KS & Jones TH (2012). "Testosterone increases hepatic liver X receptor and ApoE expression and improves lipid metabolism in the testicular feminized mouse: a potential protective mechanism against atherosclerosis and fatty liver disease. ." Endocrine Reviews **33** OR22–OR25.

Kelly, D. M. and T. H. Jones (2013). "Testosterone: a metabolic hormone in health and disease." Journal of Endocrinology **217**(3): R25-R45.

Kelly, D. M. and T. H. Jones (2015). "Testosterone and obesity." Obesity Reviews **16**(7): 581-606.

Kelly, D. M., J. E. Nettleship, S. Akhtar, V. Muraleedharan, D. J. Sellers, J. C. Brooke, D. S. McLaren, K. S. Channer and T. H. Jones (2014). "Testosterone suppresses the expression of regulatory enzymes of fatty acid synthesis and protects against hepatic steatosis in cholesterol-fed androgen deficient mice." Life Sci **109**(2): 95-103.

Kelly, D. M., D. J. Sellers, M. N. Woodroffe, T. H. Jones and K. S. Channer (2013). "Effect of Testosterone on Inflammatory Markers in the Development of Early Atherogenesis in the Testicular-Feminized Mouse Model." Endocrine Research **38**(3): 125-138.

Kennedy, W. R., M. Alter and J. H. Sung (1968). "Progressive Proximal Spinal and Bulbar Muscular Atrophy of Late Onset - a Sex-Linked Recessive Trait." Neurology **18**(7): 671-&.

Kenny, A. M., C. A. Gruman, G. Fabregas, B. Biskup and G. Mansoor (2001). "Effects of testosterone on lipids and vascular reactivity in older men." Gerontologist **41**: 185-185.

Khaw, K.-T., M. Dowsett, E. Folkard, S. Bingham, N. Wareham, R. Luben, A. Welch and N. Day (2007). "Endogenous Testosterone and Mortality Due to All Causes, Cardiovascular Disease, and Cancer in Men: European Prospective Investigation Into Cancer in Norfolk (EPIC-Norfolk) Prospective Population Study " Circulation **116**(23): 2694-2701.

Khaw, K. T. and E. Barrett-Connor (1992). "Lower endogenous androgens predict central adiposity in men." Ann Epidemiol **2**(5): 675-682.

Khaw, K. T. and E. Barrettconnor (1988). "Blood-pressure and endogenous testosterone in men - an inverse relationship." Journal of Hypertension **6**(4): 329-332.

Khurana, K. K., S. D. Navaneethan, S. Arrigain, J. D. Schold, J. V. Nally Jr and D. A. Shoskes (2014). "Serum Testosterone Levels and Mortality in Men With CKD Stages 3-4." American Journal of Kidney Diseases **64**(3): 367-374.

Kittles, R. A., D. Young, S. Weinrich, J. Hudson, G. Argyropoulos, F. Ukoli, L. Adams-Campbell and G. M. Dunston (2001). "Extent of linkage disequilibrium between the androgen receptor gene CAG and GGC repeats in human populations: implications for prostate cancer risk." Human Genetics **109**(3): 253-261.

Kletzien, R. F., P. K. Harris and L. A. Foellmi (1994). "Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress." Faseb j **8**(2): 174-181.

Kley, H. K., E. Nieschlag, W. Wiegelmann, H. G. Solbach and H. L. Kruskemper (1975). "Steroid-hormones and their binding in plasma of male patients with fatty liver, chronic hepatitis and liver cirrhosis." Acta Endocrinologica **79**(2): 275-285.

Klocker, H., I. E. Eder, Z. Culig and G. Bartsch (2004). "[Clinical consequences of androgen receptor malfunction]." Verh Dtsch Ges Pathol **88**: 122-129.

Kontoleon, P. E., M. I. Anastasiou-Nana, P. D. Papapetrou, G. Alexopoulos, V. Ktenas, A. C. Rapti, E. P. Tsagalou and J. N. Nanas (2003). "Hormonal profile in patients with congestive heart failure." Int J Cardiol **87**(2-3): 179-183.

Kupelian, V., S. T. Page, A. B. Araujo, T. G. Travison, W. J. Bremner and J. B. McKinlay (2006). "Low sex hormone-binding globulin, total testosterone, and symptomatic androgen

deficiency are associated with development of the metabolic syndrome in nonobese men." Journal of Clinical Endocrinology & Metabolism **91**(3): 843-850.

Kylin, E. (1923). "Studien über das Hypertonie-Hyperglykämie-Hyperurikämiesyndrom." Zentrabl f innere Med Leipz **181**: 105-127.

Kyriazis, J., I. Tzanakis, K. Stylianou, I. Katsipi, D. Moisiadis, A. Papadaki, V. Mavroeydi, S. Kagia, N. Karkavitsas and E. Daphnis (2011). "Low serum testosterone, arterial stiffness and mortality in male haemodialysis patients." Nephrology Dialysis Transplantation **26**(9): 2971-2977.

La Spada, A. R., E. M. Wilson, D. B. Lubahn, A. E. Harding and K. H. Fischbeck (1991). "Androgen Receptor Gene Mutations In X-Linked Spinal and Bulbar Muscular Atrophy." Nature (London) **352**(6330): 77-79.

Laaksonen, D. E., L. Niskanen, K. Punnonen, K. Nyysönen, T. P. Tuomainen, R. Salonen, R. Rauramaa and J. T. Salonen (2003). "Sex hormones, inflammation and the metabolic syndrome: a population-based study." European Journal of Endocrinology **149**(6): 601-608.

Laaksonen, D. E., L. Niskanen, K. Punnonen, K. Nyysönen, T. P. Tuomainen, V. P. Valkonen, R. Salonen and J. T. Salonen (2004). "Testosterone and sex hormone-binding globulin predict the metabolic syndrome and diabetes in middle-aged men." Diabetes Care **27**(5): 1036-1041.

Lage, M. J., B. L. Barber and R. A. Markus (2007). "Association between androgen-deprivation therapy and incidence of diabetes among males with prostate cancer." Urology **70**(6): 1104-1108.

Lakshman, K. M., S. Bhasin and A. B. Araujo (2010). "Sex hormone-binding globulin as an independent predictor of incident type 2 diabetes mellitus in men." J Gerontol A Biol Sci Med Sci **65**(5): 503-509.

Larsson, B., K. Svardsudd, L. Welin, L. Wilhelmsen, P. Bjorntorp and G. Tibblin (1984). "Abdominal adipose-tissue distribution, obesity, and risk of cardiovascular-disease and death - 13 year follow up of participants in the study of men born in 1913." British Medical Journal **288**(6428): 1401-1404.

Larsson, B. O., J. Seidell, K. Svardsudd, L. Welin, G. Tibblin, L. Wilhelmsen and P. Bjorntorp (1989). "Obesity, adipose-tissue distribution and health in men - the study of men born in 1913." Appetite **13**(1): 37-44.

Laughlin, G. A., E. Barrett-Connor and J. Bergstrom (2008). "Low Serum Testosterone and Mortality in Older Men " J Clin Endocrinol Metab **93**(1): 68-75.

Le, T. N., J. E. Nestler, J. F. Strauss and E. P. Wickham (2012). "Sex Hormone-Binding Globulin and Type 2 Diabetes Mellitus." Trends in Endocrinology and Metabolism **23**(1): 32-40.

Legoascogne, C., N. Sananes, M. Guezou, E. E. Baulieu and P. Robel (1993). "Suppressed expression of the cytochrome p450(17-alpha) protein in the testicular feminized (tfm) mouse testes." Journal of Endocrinology **139**(1): 127-130.

Lehtonen, A., R. Huupponen, J. Tuomilehto, S. Lavonius, S. Arve, H. Isoaho, I. Huhtaniemi and R. Tilvis (2008). "Serum testosterone but not leptin predicts mortality in elderly men." Age and Ageing **37**(4): 461-464.

Leonard, S. L. (1952). "The effect of castration and testosterone propionate injection on glycogen storage in skeletal muscle." Endocrinology **51**(4): 293-297.

Lerchbaum, E., S. Pilz, B. O. Boehm, T. B. Grammer, B. Obermayer-Pietsch and W. Maerz (2012). "Combination of low free testosterone and low vitamin D predicts mortality in older men referred for coronary angiography." Clinical Endocrinology **77**(3): 475-483.

Lesser, M. A. (1946). "Testosterone propionate therapy in 100 cases of angina pectoris." Journal of Clinical Endocrinology & Metabolism **6**(8): 549-557.

Levine, G. N., A. V. D'Amico, P. Berger, P. E. Clark, R. H. Eckel, N. L. Keating, R. V. Milani, A. I. Sagalowsky, M. R. Smith, N. Zakai, A. Amer Heart, S. Amer Canc and A. Amer Urological (2010). "Androgen-Deprivation Therapy in Prostate Cancer and Cardiovascular Risk A Science Advisory From the American Heart Association, American Cancer Society, and American Urological Association Endorsed by the American Society for Radiation Oncology." Circulation **121**(6): 833-840.

Li, Z. G., V. A. Danis and P. M. Brooks (1993). "Effect of gonadal-steroids on the production of il-1 and il-6 by blood mononuclear-cells invitro." Clinical and Experimental Rheumatology **11**(2): 157-162.

Lim, S., K. R. Son, I. C. Song, H. S. Park, C. J. Jin, H. C. Jang, K. S. Park, Y.-B. Kim and H. K. Lee (2009). "Fat in Liver/Muscle Correlates More Strongly With Insulin Sensitivity in Rats Than Abdominal Fat." Obesity **17**(1): 188-195.

Lin, H.-Y., I. C. Yu, R.-S. Wang, Y.-T. Chen, N.-C. Liu, S. Altuwaijri, C.-L. Hsu, W.-L. Ma, J. Jokinen, J. D. Sparks, S. Yeh and C. Chang (2008). "Increased hepatic steatosis and insulin resistance in mice lacking hepatic androgen receptor." Hepatology **47**(6): 1924-1935.

Lin, J.-W., J.-K. Lee, C.-K. Wu, J. L. Caffrey, M.-H. Chang, J.-J. Hwang, N. Dowling and Y.-S. Lin (2011). "Metabolic Syndrome, Testosterone, and Cardiovascular Mortality in Men." Journal of Sexual Medicine **8**(8): 2350-2360.

Liu, P. Y., Swerdloff, R. , Veldhuis, J.D. (2004). "The rationale, efficacy and safety of androgen therapy in older men: Future research and current practice recommendations." Journal of Clinical Endocrinology and Metabolism **89**(10): 4789-4796

Liva, S. M. and R. R. Voskuhl (2001). "Testosterone acts directly on CD4(+) T lymphocytes to increase IL-10 production." Journal of Immunology **167**(4): 2060-2067.

Lubahn, D. B., D. R. Joseph, M. Sar, J. A. Tan, H. N. Higgs, R. E. Larson, F. S. French and E. M. Wilson (1988). "The human androgen receptor - complementary deoxyribonucleic-acid cloning, sequence-analysis and gene-expression in prostate." Molecular Endocrinology **2**(12): 1265-1275.

MacDonald, A. A., G. P. Herbison, M. Showell and C. M. Farquhar (2010). "The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis." Human Reproduction Update **16**(3): 293-311.

Macotela, Y., J. Boucher, T. T. Tran and C. R. Kahn (2009). "Sex and Depot Differences in Adipocyte Insulin Sensitivity and Glucose Metabolism." Diabetes **58**(4): 803-812.

Maffei, L., Y. Murata, V. Rochira, G. Tubert, C. Aranda, M. Vazquez, C. D. Clyne, S. Davis, E. R. Simpson and C. Carani (2004). "Dysmetabolic syndrome in a man with a novel mutation of the aromatase gene: Effects of testosterone, alendronate, and estradiol treatment." Journal of Clinical Endocrinology & Metabolism **89**(1): 61-70.

Mahley, R. W. (1988). "Apolipoprotein E: cholesterol transport protein with expanding role in cell biology." Science **240**(4852): 622-630.

Makinen, J., M. J. Jarvisalo, P. Pollanen, A. Perheentupa, K. Irjala, M. Koskenvuo, J. Makinen, I. Huhtaniemi and O. T. Raitakari (2005). "Increased carotid atherosclerosis in andropausal middle-aged men." J Am Coll Cardiol **45**(10): 1603-1608.

Malkin, C. J., T. H. Jones and K. S. Channer (2007). "The effect of testosterone on insulin sensitivity in men with heart failure." Eur J Heart Fail **9**(1): 44-50.

Malkin, C. J., P. J. Pugh, R. D. Jones, D. Kapoor, K. S. Channer and T. H. Jones (2004). "The Effect of Testosterone Replacement on Endogenous Inflammatory Cytokines and Lipid Profiles in Hypogonadal Men  
10.1210/jc.2003-031069." J Clin Endocrinol Metab **89**(7): 3313-3318.

Malkin, C. J., P. J. Pugh, T. H. Jones and K. S. Channer (2003). "Testosterone for secondary prevention in men with ischaemic heart disease?" Qjm **96**(7): 521-529.

Malkin, C. J., P. J. Pugh, P. D. Morris, S. Asif, T. H. Jones and K. S. Channer (2010). "Low serum testosterone and increased mortality in men with coronary heart disease." Heart **96**(22): 1821-1825.

Malkin, C. J., P. J. Pugh, J. N. West, E. J. van Beek, T. H. Jones and K. S. Channer (2006). "Testosterone therapy in men with moderate severity heart failure: a double-blind randomized placebo controlled trial." Eur Heart J **27**(1): 57-64.

Maravelias, C., A. Dona, M. Stefanidou and C. Spiliopoulou (2005). "Adverse effects of anabolic steroids in athletes - A constant threat." Toxicology Letters **158**(3): 167-175.

Marin, P., S. Holmang, L. Jonsson, L. Sjostrom, H. Kvist, G. Holm, G. Lindstedt and P. Bjorntorp (1992). "The effects of testosterone treatment on body composition and metabolism in middle-aged obese men." International Journal of Obesity April **2006;30**(4):711-714 **16**(12): 991-997

Marin, P., L. Lonn, B. Andersson, B. Oden, L. Olbe, B. A. Bengtsson and P. Bjorntorp (1996). "Assimilation of triglycerides in subcutaneous and intraabdominal adipose tissues in vivo in men: Effects of testosterone." Journal of Clinical Endocrinology & Metabolism **81**(3): 1018-1022.

Marin, P., B. Oden and P. Bjorntorp (1995). "Assimilation and mobilization of triglycerides in subcutaneous abdominal and femoral adipose-tissue in-vivo in men - effects of androgens." Journal of Clinical Endocrinology & Metabolism **80**(1): 239-243.

Mariotti, C., B. Castellotti, D. Pareyson, D. Testa, M. Eoli, C. Antozzi, V. Silani, R. Marconi, F. Tezzon, G. Siciliano, C. Marchini, C. Gellera and S. D. Donato (2000). "Phenotypic manifestations associated with CAG-repeat expansion in the androgen receptor gene in male patients and heterozygous females: a clinical and molecular study of 30 families." Neuromuscul Disord **10**(6): 391-397.

Mathur, A., C. Malkin, B. Saeed, R. Muthusamy, T. H. Jones and K. Channer (2009). "Long-term benefits of testosterone replacement therapy on angina threshold and atheroma in men." European Journal of Endocrinology **161**(3): 443-449.

Mauras, N., V. Hayes, S. Welch, A. Rini, K. Helgeson, M. Dokler, J. D. Veldhuis and R. J. Urban (1998). "Testosterone deficiency in young men: Marked alterations in whole body protein kinetics, strength, and adiposity." Journal of Clinical Endocrinology & Metabolism **83**(6): 1886-1892.

Max, S. R. (1984). "Androgen-estrogen synergy in rat levator ani muscle: glucose-6-phosphate dehydrogenase." Molecular and Cellular Endocrinology **38**(2): 103-107.

Max, S. R. and J. F. Knudsen (1980). "Effect of sex hormones on glucose-6-phosphate dehydrogenase in rat levator ani muscle." Molecular and Cellular Endocrinology **17**(2): 111-118.

McLaren D, K. D., Akhtar S, Channer K & Jones T ( 2012). Low testosterone is associated with decreased expression of glut-4 and hexokinase 2 in muscle of the testicular feminised mouse. Endocrine Abstracts. **29**: P559.

Menke, A., E. Guallar, S. Rohmann, W. G. Nelson, N. Rifai, N. Kanarek, M. Feinleib, E. D. Michos, A. Dobs and E. A. Platz (2010). "Sex Steroid Hormone Concentrations and Risk of Death in US Men." American Journal of Epidemiology **171**(5): 583-592.

Merce Fernandez-Balsells, M., M. H. Murad, M. Lane, J. F. Lampropulos, F. Albuquerque, R. J. Mullan, N. Agrwal, M. B. Elamin, J. F. Gallegos-Orozco, A. T. Wang, P. J. Erwin, S. Bhasin and V. M. Montori (2010). "Adverse Effects of Testosterone Therapy in Adult Men: A Systematic Review and Meta-Analysis." Journal of Clinical Endocrinology & Metabolism **95**(6): 2560-2575.

Messerli, F. H., G. E. Garavaglia, R. E. Schmieder, K. Sundgaardriise, B. D. Nunez and C. Amodio (1987). "Disparate cardiovascular findings in men and women with essential-hypertension." Annals of Internal Medicine **107**(2): 158-161.

Militaru, C., I. Donoiu, O. Dracea and D. D. Ionescu (2010). "Serum testosterone and short-term mortality in men with acute myocardial infarction." Cardiology Journal **17**(3): 249-253.

Moffat, S. D., A. B. Zonderman, E. J. Metter, C. Kawas, M. R. Blackman, S. M. Harman and S. M. Resnick (2004). "Free testosterone and risk for Alzheimer disease in older men." Neurology **62**(2): 188-193.

Morgentaler, A. (2016). "Controversies and Advances With Testosterone Therapy: A 40-Year Perspective." Urology **89**: 27-32.

Morris, P. D., C. J. Malkin, K. S. Channer and T. H. Jones (2004). "A mathematical comparison of techniques to predict biologically available testosterone in a cohort of 1072 men." European Journal of Endocrinology **151**(2): 241-249.

Mottram, D. R. and A. J. George (2000). "Anabolic steroids." Best Practice & Research Clinical Endocrinology & Metabolism **14**(1): 55-69.

Mrin, P. and S. Arver (1998). "Androgens and abdominal obesity." Baillière's Clinical Endocrinology and Metabolism **12**(3): 441-451.

Muller, M., A. W. van den Beld, M. L. Bots, D. E. Grobbee, S. W. Lamberts and Y. T. van der Schouw (2004). "Endogenous sex hormones and progression of carotid atherosclerosis in elderly men." Circulation **109**(17): 2074-2079.

Muraleedharan, V. and T. H. Jones (2014). "Testosterone and mortality." Clinical Endocrinology **81**(4): 477-487.

Muraleedharan, V., H. Marsh, D. Kapoor, K. S. Channer and T. H. Jones (2013). "Testosterone deficiency is associated with increased risk of mortality and testosterone replacement improves survival in men with type 2 diabetes." Eur J Endocrinol **169**(6): 725-733.

Murphy, L. and P. J. Oshaughnessy (1991). "Testicular Steroidogenesis In The Testicular Feminized (Tfm) Mouse - Loss Of 17-Alpha-Hydroxylase Activity." Journal of Endocrinology **131**(3): 443-449.

Muthusamy, T., P. Murugesan and K. Balasubramanian (2009). "Sex steroids deficiency impairs glucose transporter 4 expression and its translocation through defective Akt phosphorylation in target tissues of adult male rat." Metabolism - Clinical and Experimental **58**(11): 1581-1592.

Muthusamy, T., P. Murugesan, C. Srinivasan and K. Balasubramanian (2011). "Sex steroids influence glucose oxidation through modulation of insulin receptor expression and IRS-1 serine phosphorylation in target tissues of adult male rat." Molecular and Cellular Biochemistry **352**(1): 35-45.

Nettleship, J. E., R. D. Jones, K. S. Channer and T. H. Jones (2009). "Testosterone and coronary artery disease." Frontiers of Hormone Research **37**: 107.

Nettleship, J. E., T. H. Jones, K. S. Channer and R. D. Jones (2007). "Physiological testosterone replacement therapy attenuates fatty streak formation and improves high-density lipoprotein cholesterol in the Tfm mouse: An effect that is independent of the classic androgen receptor." Circulation **116**(21): 2427-2434.

Nettleship, J. E., Pugh, P.J., Channer, K.S., Jones, T. and R. D. Jones (2007). "Inverse Relationship between Serum Levels of Interleukin-1 $\beta$  and Testosterone in Men with Stable Coronary Artery Disease." Hormone and Metabolic Research **39**(5): 366-371.

Nielsen, T. L., C. Hagen, K. Wraae, K. Brixen, P. H. Petersen, E. Haug, R. Larsen and M. Andersen (2007). "Visceral and Subcutaneous Adipose Tissue Assessed by Magnetic Resonance Imaging in Relation to Circulating Androgens, Sex Hormone-Binding Globulin, and Luteinizing Hormone in Young Men." The Journal of Clinical Endocrinology & Metabolism **92**(7): 2696-2705.

Oh, J. Y., E. Barrett-Connor, N. M. Wedick and D. L. Wingard (2002). "Endogenous sex hormones and the development of type 2 diabetes in older men and women: the Rancho Bernardo Study." Diabetes Care **25**(1): 55-60.

Oskui, P. M., W. J. French, M. J. Herring, G. S. Mayeda, S. Burstein and R. A. Kloner (2013). "Testosterone and the Cardiovascular System: A Comprehensive Review of the Clinical Literature." Journal of the American Heart Association **2**(6).

Page, S. T., V. Kupelian, W. J. Bremner and J. B. McKinlay (2006). "The androgen receptor gene CAG repeat polymorphism does not predict increased risk of heart disease: longitudinal results from the Massachusetts Male Ageing Study." Clinical Endocrinology **65**(3): 333-339.

Pardridge, W. M. (1986). "Serum Bioavailability of Sex Steroid- Hormones." Clinics in Endocrinology and Metabolism **15**(2): 259-278.

Park, J., H. K. Rho, K. H. Kim, S. S. Choe, Y. S. Lee and J. B. Kim (2005). "Overexpression of Glucose-6-Phosphate Dehydrogenase Is Associated with Lipid Dysregulation and Insulin Resistance in Obesity." Molecular and Cellular Biology **25**(12): 5146-5157.

Parthasarathy, C., V. N. Renuka and K. Balasubramanian (2009). "Sex steroids enhance insulin receptors and glucose oxidation in Chang liver cells." Clinica Chimica Acta **399**(1-2): 49-53.

Pasquali, R., F. Casimirri, V. Balestra, R. Flaminia, N. Melchionda, R. Fabbri and L. Barbara (1991). "The relative contribution of androgens and insulin in determining abdominal body-fat distribution in premenopausal women." Journal of Endocrinological Investigation **14**(10): 839-846.

Pessin, J. E. and A. R. Saltiel "Signaling pathways in insulin action: molecular targets of insulin resistance." The Journal of Clinical Investigation **106**(2): 165-169.

Pessin, J. E. and A. R. Saltiel (2000). "Signaling pathways in insulin action: molecular targets of insulin resistance." J Clin Invest **106**(2): 165-169.

Phillips, G. B. "Relationship between serum sex hormones and the glucose-insulin-lipid defect in men with obesity." Metabolism - Clinical and Experimental **42**(1): 116-120.

Phillips, G. B., T. Y. Jing, L. M. Resnick, M. Barbagallo, J. H. Laragh and J. E. Sealey (1993). "Sex hormones and hemostatic risk factors for coronary heart disease in men with hypertension." J Hypertens **11**(7): 699-702.

Pitteloud, N., V. K. Mootha, A. A. Dwyer, M. Hardin, H. Lee, K. F. Eriksson, D. J. Tripathy, M. Yialamas, L. Groop, D. Elahi and F. J. Hayes (2005). "Relationship between testosterone levels, insulin sensitivity, and mitochondrial function in men." Diabetes Care **28**(7): 1636-1642.

Ponikowska, B., E. A. Jankowska, J. Maj, K. Wegrzynowska-Teodorczyk, B. Biel, K. Reczuch, L. Borodulin-Nadziejka, W. Banasiak and P. Ponikowski (2010). "Gonadal and adrenal androgen deficiencies as independent predictors of increased cardiovascular mortality in men with type II diabetes mellitus and stable coronary artery disease." International Journal of Cardiology **143**(3): 343-348.

Postic, C., M. Shiota, K. D. Niswender, T. L. Jetton, Y. Chen, J. M. Moates, K. D. Shelton, J. Lindner, A. D. Cherrington and M. A. Magnuson (1999). "Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase." J Biol Chem **274**(1): 305-315.

Pratt, W. B. and D. O. Toft (1997). "Steroid receptor interactions with heat shock protein and immunophilin chaperones." Endocrine Reviews **18**(3): 306-360.

Pugh, P. J., K. S. Channer, H. Parry, T. Downes and T. H. Jones (2002). "Bio-available testosterone levels fall acutely following myocardial infarction in men: Association with fibrinolytic factors." Endocrine Research **28**(3): 161-173.

Pye, S. R., I. T. Huhtaniemi, J. D. Finn, D. M. Lee, T. W. O'Neill, A. Tajar, G. Bartfai, S. Boonen, F. F. Casanueva, G. Forti, A. Giwercman, T. S. Han, K. Kula, M. E. Lean, N. Pendleton, M. Punab, M. K. Rutter, D. Vanderschueren and F. C. Wu (2014). "Late-onset hypogonadism and mortality in aging men." J Clin Endocrinol Metab **99**(4): 1357-1366.

Ramamani, A., M. M. Aruldas and P. Govindarajulu (1999). "Differential response of rat skeletal muscle glycogen metabolism to testosterone and estradiol." Canadian Journal of Physiology and Pharmacology **77**(4): 300-304.

Ramirez, M. E., M. P. McMurry, G. A. Wiebke, K. J. Felten, K. Ren, A. W. Meikle and P.-H. Iverius "Evidence for sex steroid inhibition of lipoprotein lipase in men: Comparison of abdominal and femoral adipose tissue." Metabolism - Clinical and Experimental **46**(2): 179-185.

Rask-Madsen, C. and C. R. Kahn (2012). "Tissue-specific insulin signaling, metabolic syndrome, and cardiovascular disease." Arterioscler Thromb Vasc Biol **32**(9): 2052-2059.

Reaven, G. M. (1988). "Role of insulin resistance in human-disease." Diabetes **37**(12): 1595-1607.

Rebuffescribe, M., P. Marin and P. Bjorntorp (1991). "Effect of testosterone on abdominal adipose-tissue in men." International Journal of Obesity **15**(11): 791-795.

Reis, C., S. Liberman, A. C. Pompeo, M. Srougi, A. Halpern and W. Jacob Filho (2009). "Body composition alterations, energy expenditure and fat oxidation in elderly males suffering from prostate cancer, pre and post orchiectomy." Clinics **64**: 781-784.

Rodriguez, A., D. C. Muller, E. J. Metter, M. Maggio, S. M. Harman, M. R. Blackman and R. Andres (2007). "Aging, androgens, and the metabolic syndrome in a longitudinal study of aging." Journal of Clinical Endocrinology and Metabolism **92**(9): 3568-3572.

Rosignoli, C., J. C. Nicolas, A. Jomard and S. Michel (2003). "Involvement of the SREBP pathway in the mode of action of androgens in sebaceous glands in vivo." Experimental Dermatology **12**(4): 480-489.

Rosner, W., D. J. Hryb, M. S. Khan, A. M. Nakhla and N. A. Romas (1998). "Androgens, estrogens, and second messengers." Steroids **63**(5-6): 278-281.

Saad, F. (2015). "The emancipation of testosterone from niche hormone to multi-system player." Asian Journal of Andrology **17**(1): 58-60.

Saad, F., L. Gooren, A. Haider and A. Yassin (2007). "An exploratory study of the effects of 12 month administration of the novel long-acting testosterone undecanoate on measures of sexual function and the metabolic syndrome." Archives of Andrology **53**(6): 353-357.

Saad, F., L. J. Gooren, A. Haider and A. Yassin (2008). "A dose-response study of testosterone on sexual dysfunction and features of the metabolic syndrome using testosterone gel and parenteral testosterone undecanoate." Journal of Andrology **29**(1): 102-105.

Salehzadeh, F., A. Rune, M. Osler and L. Al-Khalili (2011). "Testosterone or 17 $\beta$ -estradiol exposure reveals sex-specific effects on glucose and lipid metabolism in human myotubes." Journal of Endocrinology **210**(2): 219-229.

Sato, K., M. Iemitsu, K. Aizawa and R. Ajisaka (2008). "Testosterone and DHEA activate the glucose metabolism-related signaling pathway in skeletal muscle." American Journal of Physiology - Endocrinology and Metabolism **294**(5): E961-E968.

Schöttker, B., R. Jorde, A. Peasey, B. Thorand, E. H. J. M. Jansen, L. d. Groot, M. Streppel, J. Gardiner, J. M. Ordóñez-Mena, L. Perna, T. Wilsgaard, W. Rathmann, E. Feskens, E. Kampman, G. Siganos, I. Njølstad, E. B. Mathiesen, R. Kubínová, A. Pająk, R. Topor-Madry, A. Tamosiunas, M. Hughes, F. Kee, M. Bobak, A. Trichopoulou, P. Boffetta and H. Brenner (2014). "Vitamin D and mortality: meta-analysis of individual participant data from a large consortium of cohort studies from Europe and the United States." BMJ : British Medical Journal **348**.

Schulster, M., A. M. Bernie and R. Ramasamy (2016). "The role of estradiol in male reproductive function." Asian Journal of Andrology **18**(3): 435-440.

Schwartz, A., B. P. Patel, S. Vien, B. W. McCrindle, G. H. Anderson and J. Hamilton (2015). "Acute decrease in serum testosterone after a mixed glucose and protein beverage in obese peripubertal boys." Clinical Endocrinology **83**(3): 332-338.

Scragg, J. L., M. L. Dallas and C. Peers (2007). "Molecular requirements for L-type Ca<sup>2+</sup> channel blockade by testosterone." Cell Calcium **42**(1): 11-15.

Scragg, J. L., R. D. Jones, T. H. Jones and C. Peers (2004). "Testosterone is a potent inhibitor of L-type Ca<sup>2+</sup> channels." Biochemical and Biophysical Research Communications **318**(2): 503-506.

Seidell, J. C., P. Bjorntorp, L. Sjostrom, H. Kvist and R. Sannerstedt (1990). "Visceral fat accumulation in men is positively associated with insulin, glucose, and c-peptide levels, but negatively with testosterone levels." Metabolism-Clinical and Experimental **39**(9): 897-901.

Seidell, J. C., P. Björntorp, L. Sjöström, H. Kvist and R. Sannerstedt "Visceral fat accumulation in men is positively associated with insulin, glucose, and C-peptide levels, but negatively with testosterone levels." Metabolism - Clinical and Experimental **39**(9): 897-901.

Selvin, E., W. G. Nelson, M. Feinleib, A. Dobs, L. Zhang, S. Basaria, S. Rohrmann, S. H. Golden, N. Rifai and E. A. Platz (2007). "Androgens and diabetes in men - Results from the Third National Health and Nutrition Examination Survey (NHANES III)." Diabetes Care **30**(2): 234-238.

Senmaru, T., M. Fukui, H. Okada, Y. Mineoka, M. Yamazaki, M. Tsujikawa, G. Hasegawa, J. Kitawaki, H. Obayashi and N. Nakamura (2013). "Testosterone deficiency induces markedly decreased serum triglycerides, increased small dense LDL, and hepatic steatosis mediated by dysregulation of lipid assembly and secretion in mice fed a high-fat diet." Metabolism **62**(6): 851-860.

Shores, M. M., A. M. Matsumoto, K. L. Sloan and D. R. Kivlahan (2006). "Low Serum Testosterone and Mortality in Male Veterans " Arch Intern Med **166**(15): 1660-1665.

Shores, M. M., N. L. Smith, C. W. Forsberg, B. D. Anawalt and A. M. Matsumoto (2012). "Testosterone treatment and mortality in men with low testosterone levels." J Clin Endocrinol Metab **97**(6): 2050-2058.

Shulman, G. I., D. L. Rothman, T. Jue, P. Stein, R. A. DeFronzo and R. G. Shulman (1990). "Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by <sup>13</sup>C nuclear magnetic resonance spectroscopy." N Engl J Med **322**(4): 223-228.

Simon, D., M. A. Charles, K. Nahoul, G. Orssaud, J. Kremiski, V. Hully, E. Joubert, L. Papoz and E. Eschwege (1997). "Association between plasma total testosterone and cardiovascular risk factors in healthy adult men: The telecom study." Journal of Clinical Endocrinology and Metabolism **82**(2): 682-685.

Simon, D., P. Preziosi, E. Barrettconnor, M. Roger, M. Saintpaul, K. Nahoul and L. Papoz (1992). "Interrelation between plasma testosterone and plasma-insulin in healthy adult men - the telecom study." Diabetologia **35**(2): 173-177.

Sinclair, M., M. Grossmann, P. W. Angus, R. Hoermann, P. Hey, T. Scodellaro and P. J. Gow (2016). "Low testosterone as a better predictor of mortality than sarcopenia in men with advanced liver disease." J Gastroenterol Hepatol **31**(3): 661-667.

Singh, R., J. N. Artaza, W. E. Taylor, N. F. Gonzalez-Cadavid and S. Bhasin (2003). "Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway." Endocrinology **144**(11): 5081-5088.



Smith, A. M., K. M. English, C. J. Malkin, R. D. Jones, T. H. Jones and K. S. Channer (2005). "Testosterone does not adversely affect fibrinogen or tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) levels in 46 men with chronic stable angina." European Journal of Endocrinology **152**(2): 285-291.

Smith, E. P., J. Boyd, G. R. Frank, H. Takahashi, R. M. Cohen, B. Specker, T. C. Williams, D. B. Lubahn and K. S. Korach (1994). "Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man." N Engl J Med **331**(16): 1056-1061.

Smith, G. D., Y. Ben-Shlomo, A. Beswick, J. Yarnell, S. Lightman and P. Elwood (2005). "Cortisol, Testosterone, and Coronary Heart Disease: Prospective Evidence From the Caerphilly Study. [Article]." Circulation July 19, 2005;112(3):332-340.

Smith, J. C., S. Bennett, L. M. Evans, H. G. Kynaston, M. Parmar, M. D. Mason, J. R. Cockcroft, M. F. Scanlon and J. S. Davies (2001). "The Effects of Induced Hypogonadism on Arterial Stiffness, Body Composition, and Metabolic Parameters in Males with Prostate Cancer." J Clin Endocrinol Metab **86**(9): 4261-4267.

Srikanthan, P. and A. S. Karlamangla (2011). "Relative Muscle Mass Is Inversely Associated with Insulin Resistance and Prediabetes. Findings from The Third National Health and Nutrition Examination Survey." The Journal of Clinical Endocrinology & Metabolism **96**(9): 2898-2903.

Srinivas-Shankar, U., S. A. Roberts, M. J. Connolly, M. D. L. O. Connell, J. E. Adams, J. A. Oldham and F. C. W. Wu (2010). "Effects of Testosterone on Muscle Strength, Physical Function, Body Composition, and Quality of Life in Intermediate-Frail and Frail Elderly Men: A Randomized, Double-Blind, Placebo-Controlled Study." Journal of Clinical Endocrinology & Metabolism **95**(2): 639-650.

Stanworth, R., D. Kapoor, K. Channer and T. Jones (2008). "Androgen receptor CAG repeat polymorphism is associated with serum testosterone levels, obesity and serum leptin in men with type 2 diabetes 10.1530/EJE-08-0266." Eur J Endocrinol: EJE-08-0266.

Stanworth, R. D., S. Akhtar, K. S. Channer and T. H. Jones (2014). "The role of androgen receptor CAG repeat polymorphism and other factors which affect the clinical response to testosterone replacement in metabolic syndrome and type 2 diabetes: TIMES2 sub-study." Eur J Endocrinol **170**(2): 193-200.

Stanworth, R. D., D. Kapoor, K. S. Channer and T. H. Jones (2011). "Dyslipidaemia is associated with testosterone, oestradiol and androgen receptor CAG repeat polymorphism in men with type 2 diabetes." Clin Endocrinol (Oxf) **74**(5): 624-630.

Stanworth RD, K. D., Channer KS, et al. . (2007). "Testosterone levels correlate positively with HDL cholesterol levels in men with type 2 diabetes." Endocrine Abstracts **14**: P628.

Steffensen, K. R. and J.-Å. Gustafsson (2004). "Putative Metabolic Effects of the Liver X Receptor (LXR)." Diabetes **53**(suppl 1): S36-S42.

Stellato, R. K., H. A. Feldman, O. Hamdy, E. S. Horton and J. B. McKinlay (2000). "Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men - Prospective results from the Massachusetts Male Aging Study." Diabetes Care **23**(4): 490-494.

Stratford, S., K. L. Hoehn, F. Liu and S. A. Summers (2004). "Regulation of insulin action by ceramide: Dual mechanisms linking ceramide accumulation to the inhibition of akt/protein kinase b." Journal of Biological Chemistry **279**(35): 36608-36615.

Stumvoll, M., S. Jacob, H. G. Wahl, B. Hauer, K. Loblein, P. Grauer, R. Becker, M. Nielsen, W. Renn and H. Haring (2000). "Suppression of systemic, intramuscular, and subcutaneous adipose tissue lipolysis by insulin in humans." Journal of Clinical Endocrinology & Metabolism **85**(10): 3740-3745.

Sullivan, M. L., C. M. Martinez, P. Gennis and E. J. Gallagher (1998). "The cardiac toxicity of anabolic steroids." Progress in Cardiovascular Diseases **41**(1): 1-15.

Svartberg, J., U. Aasebo, A. Hjalmsen, J. Sundsfjord and R. Jorde (2004). "Testosterone treatment improves body composition and sexual function in men with COPD, in a 6-month randomized controlled trial." Respir Med **98**(9): 906-913.

Svartberg, J., D. Von Muhlen, E. Mathiesen, O. Joakimsen, K. H. Bonna and E. Stensland-Bugge (2006). "Low testosterone levels are associated with carotid atherosclerosis in men." Journal of Internal Medicine June **259**(6): 576-582.

Svartberg, J., D. von Muhlen, H. Schirmer, E. Barrett-Connor, J. Sundsfjord and R. Jorde (2004). "Association of endogenous testosterone with blood pressure and left ventricular mass in men. The Tromso Study." European Journal of Endocrinology **150**(1): 65-71.

Tajar, A., I. T. Huhtaniemi, T. W. O'Neill, J. D. Finn, S. R. Pye, D. M. Lee, G. Bartfai, S. Boonen, F. F. F. Casanueva, G. Forti, A. Giwercman, T. S. Han, K. Kula, F. Labrie, M. E. J. Lean, N. Pendleton, M. Punab, D. Vanderschueren, F. C. W. Wu and E. Grp (2012). "Characteristics of Androgen Deficiency in Late-Onset Hypogonadism: Results from the European Male Aging Study (EMAS)." Journal of Clinical Endocrinology & Metabolism **97**(5): 1508-1516.

Tappler, B. and M. Katz (1979). "Pituitary-gonadal dysfunction in low-output cardiac failure." Clin Endocrinol (Oxf) **10**(3): 219-226.

Tibblin, G., A. Adlerberth, G. Lindstedt and P. Bjorntorp (1996). "The pituitary-gonadal axis and health in elderly men: a study of men born in 1913." Diabetes **45**(11): 1605-1609.

Tint, A. N., R. Hoermann, H. Wong, E. I. Ekinici, R. J. MacIsaac, G. Jerums, J. D. Zajac and M. Grossmann (2016). "Association of sex hormone-binding globulin and free testosterone with mortality in men with type 2 diabetes mellitus." European Journal of Endocrinology **174**(1): 59-68.

Tivesten, A., L. Vandenput, F. Labrie, M. K. Karlsson, O. Ljunggren, D. Mellstrom and C. Ohlsson (2009). "Low Serum Testosterone and Estradiol Predict Mortality in Elderly Men." Journal of Clinical Endocrinology & Metabolism **94**(7): 2482-2488.

Tomlinson, J. W., N. Holden, R. K. Hills, K. Wheatley, R. N. Clayton, A. S. Bates, M. C. Sheppard, P. M. Stewart and W. M. P. Hypopituita (2001). "Association between premature mortality and hypopituitarism." Lancet **357**(9254): 425-431.

Traish, A. M., R. J. Feeley and A. Guay (2009). "Mechanisms of obesity and related pathologies: Androgen deficiency and endothelial dysfunction may be the link between obesity and erectile dysfunction." FEBS Journal **276**(20): 5755-5767.

Tsai, E. C., E. J. Boyko, D. L. Leonetti and W. Y. Fujimoto (2000). "Low serum testosterone level as a predictor of increased visceral fat in Japanese-American men." International Journal of Obesity **24**(4): 485-491.

Tsai, E. C., W. Y. Fujimoto, A. M. Matsumoto and E. J. Boyko (2004). "Association of bioavailable, free, and total testosterone with insulin resistance - Influence of sex hormone-binding globulin and body fat." Diabetes Care **27**(4): 861-868.

Tsigos, C., D. A. Papanicolaou, I. Kyrou, S. A. Raptis and G. P. Chrousos (1999). "Dose-dependent effects of recombinant human interleukin-6 on the pituitary-testicular axis." Journal of Interferon and Cytokine Research **19**(11): 1271-1276.

Vague, J. (1947). "Sexual differentiation. A factor affecting the forms of obesity." Presse Med **30**: 339-340.

van den Beld, A. W., M. L. Bots, J. A. Janssen, H. A. Pols, S. W. Lamberts and D. E. Grobbee (2003). "Endogenous hormones and carotid atherosclerosis in elderly men." Am J Epidemiol **157**(1): 25-31.

van den Beld, A. W., F. H. de Jong, D. E. Grobbee, H. A. P. Pols and S. W. J. Lamberts (2000). "Measures of bioavailable serum testosterone and estradiol and their relationships

with muscle strength, bone density, and body composition in elderly men." Journal of Clinical Endocrinology & Metabolism **85**(9): 3276-3282.

Van Pottelbergh, I., L. Braeckman, D. De Bacquer, G. De Backer and J. M. Kaufman (2003). "Differential contribution of testosterone and estradiol in the determination of cholesterol and lipoprotein profile in healthy middle-aged men." Atherosclerosis **166**(1): 95-102.

Vermeulen, A., S. Goemaere and J. M. Kaufman (1999). "Testosterone, body composition and aging." Journal of Endocrinological Investigation **22**(5 SUPPL.): 110-116.

Vermeulen, A., J. M. Kaufman, S. Goemaere and I. van Pottelberg (2002). "Estradiol in elderly men." Aging Male **5**(2): 98-102.

Vermeulen, A., L. Verdonck and J. M. Kaufman (1999). "A critical evaluation of simple methods for the estimation of free testosterone in serum." Journal of Clinical Endocrinology & Metabolism **84**(10): 3666-3672.

Vigen, R., C. I. O'Donnell, A. E. Barón and et al. (2013). "Association of testosterone therapy with mortality, myocardial infarction, and stroke in men with low testosterone levels." JAMA **310**(17): 1829-1836.

Vikan, T., S. H. Johnsen, H. Schirmer, I. Njolstad and J. Svartberg (2009). "Endogenous testosterone and the prospective association with carotid atherosclerosis in men: the Tromso study." European Journal of Epidemiology **24**(6): 289-295.

Vikan, T., H. Schirmer, I. Njolstad and J. Svartberg (2009). "Endogenous sex hormones and the prospective association with cardiovascular disease and mortality in men: the Tromso Study." European Journal of Endocrinology **161**(3): 435-442.

Völzke, H., N. Aumann, A. Krebs, M. Nauck, A. Steveling, M. M. Lerch, D. Roszkopf and H. Wallaschofski (2010). "Hepatic steatosis is associated with low serum testosterone and high serum DHEAS levels in men." International Journal of Andrology **33**(1): 45-53.

Wallace, I. R., M. C. McKinley, P. M. Bell and S. J. Hunter (2013). "Sex hormone binding globulin and insulin resistance." Clin Endocrinol (Oxf) **78**(3): 321-329.

Wang, C., G. Jackson, T. H. Jones, A. M. Matsumoto, A. Nehra, M. A. Perelman, R. S. Swerdloff, A. Traish, M. Zitzmann and G. Cunningham (2011). "Low Testosterone Associated With Obesity and the Metabolic Syndrome Contributes to Sexual Dysfunction and Cardiovascular Disease Risk in Men With Type 2 Diabetes." Diabetes Care **34**(7): 1669-1675.

Wang, C., E. Nieschlag, R. Swerdloff, H. M. Behre, W. J. Hellstrom, L. J. Gooren, J. M. Kaufman, J. J. Legros, B. Lunenfeld, A. Morales, J. E. Morley, C. Schulman, I. M. Thompson, W. Weidner and F. C. Wu (2009). "Investigation, treatment and monitoring of late-onset hypogonadism in males." Int J Androl **32**(1): 1-10.

Wang, Q., A. J. Kangas, P. Soininen, M. Tiainen, T. Tynkkynen, K. Puukka, A. Ruokonen, J. Viikari, M. Kahonen, T. Lehtimäki, V. Salomaa, M. Perola, G. Davey Smith, O. T. Raitakari, M. R. Jarvelin, P. Wurtz, J. Kettunen and M. Ala-Korpela (2015). "Sex hormone-binding globulin associations with circulating lipids and metabolites and the risk for type 2 diabetes: observational and causal effect estimates." Int J Epidemiol **44**(2): 623-637.

Wang, X.-F., J.-H. Wang and J.-Y. Li (2010). "Androgen deficiency in elderly men with systolic chronic heart failure." Journal of Geriatric Cardiology **7**(3-4): 138-142.

Wehr, E., S. Pilz, B. O. Boehm, W. März, T. Grammer and B. Obermayer-Pietsch (2011). "Low free testosterone is associated with heart failure mortality in older men referred for coronary angiography." European Journal of Heart Failure **13**(5): 482-488.

Whitsel, E. A., E. J. Boyko, A. M. Matsumoto, B. D. Anawalt and D. S. Siscovick (2001). "Intramuscular testosterone esters and plasma lipids in hypogonadal men: A meta-analysis." American Journal of Medicine **111**(4): 261-269.

Writing Group for the Women's Health Initiative, I. (2002). "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the women's health initiative randomized controlled trial." JAMA **288**(3): 321-333.

Wu, F. C. W., A. Tajar, J. M. Beynon, S. R. Pye, A. J. Silman, J. D. Finn, T. W. O'Neill, G. Bartfai, F. F. Casanueva, G. Forti, A. Giwercman, T. S. Han, K. Kula, M. E. J. Lean, N. Pendleton, M. Punab, S. Boonen, D. Vanderschueren, F. Labrie and I. T. Huhtaniemi (2010). "Identification of Late-Onset Hypogonadism in Middle-Aged and Elderly Men." New England Journal of Medicine **363**(2): 123-135.

Xie, Z., H. Li, K. Wang, J. Lin, Q. Wang, G. Zhao, W. Jia and Q. Zhang (2010). "Analysis of transcriptome and metabolome profiles alterations in fatty liver induced by high-fat diet in rat." Metabolism **59**(4): 554-560.

Yanase, T., W. Fan, K. Kyoya, L. Min, R. Takayanagi, S. Kato and H. Nawata (2008). "Androgens and metabolic syndrome: Lessons from androgen receptor knock out (ARKO) mice." Journal of Steroid Biochemistry and Molecular Biology **109**(3-5): 254-257.

Yeap, B. B. (2009). "Are declining testosterone levels a major risk factor for ill-health in aging men?" International Journal of Impotence Research **21**(1): 24-36.

Yeap, B. B., H. Alfonso, S. A. P. Chubb, D. J. Handelsman, G. J. Hankey, O. P. Almeida, J. Golledge, P. E. Norman and L. Flicker (2014). "In Older Men an Optimal Plasma Testosterone Is Associated With Reduced All-Cause Mortality and Higher Dihydrotestosterone With Reduced Ischemic Heart Disease Mortality, While Estradiol Levels Do Not Predict Mortality." Journal of Clinical Endocrinology & Metabolism **99**(1): E9-E18.

Yeap, B. B., Z. Hyde, O. P. Almeida, P. E. Norman, S. A. P. Chubb, K. Jamrozik, L. Flicker and G. J. Hankey (2009). "Lower testosterone levels predict incident stroke and transient ischemic attack in older men." Journal of Clinical Endocrinology and Metabolism **94**(7): 2353-2359.

Yu, Y. H. and H. N. Ginsberg (2005). "Adipocyte signaling and lipid homeostasis - Sequelae of insulin-resistant adipose tissue." Circulation Research **96**(10): 1042-1052.

Zarotsky, V., M. Y. Huang, W. Carman, A. Morgentaler, P. K. Singhal, D. Coffin and T. H. Jones (2014). "Systematic literature review of the risk factors, comorbidities, and consequences of hypogonadism in men." Andrology **2**(6): 819-834.

Zitzmann, M., M. Brune, B. Kornmann, J. Gromoll, S. von Eckardstein, A. von Eckardstein and E. Nieschlag (2001). "The CAG repeat polymorphism in the AR gene affects high density lipoprotein cholesterol and arterial vasoreactivity." Journal of Clinical Endocrinology & Metabolism **86**(10): 4867-4873.

Zitzmann, M., M. Depenbusch, J. Gromoll and E. Nieschlag (2003). "Prostate volume and growth in testosterone-substituted hypogonadal men are dependent on the CAG repeat polymorphism of the androgen receptor gene: a longitudinal pharmacogenetic study." J Clin Endocrinol Metab **88**(5): 2049-2054.

Zitzmann, M., M. Depenbusch, J. Gromoll and E. Nieschlag (2004). "X-chromosome inactivation patterns and androgen receptor functionality influence phenotype and social characteristics as well as pharmacogenetics of testosterone therapy in Klinefelter patients." J Clin Endocrinol Metab **89**(12): 6208-6217.

Zitzmann, M., J. Gromoll, A. von Eckardstein and E. Nieschlag (2003). "The CAG repeat polymorphism in the androgen receptor gene modulates body fat mass and serum concentrations of leptin and insulin in men." Diabetologia **46**(1): 31-39.

Zitzmann, M. and E. Nieschlag (2003). "The CAG repeat polymorphism within the androgen receptor gene and maleness." International Journal of Andrology **26**(2): 76-83.

Zitzmann, M. and E. Nieschlag (2007). "Androgen receptor gene CAG repeat length and body mass index modulate the safety of long-term intramuscular testosterone undecanoate

therapy in hypogonadal men." Journal of Clinical Endocrinology and Metabolism **92**(10): 3844-3853.

Zitzmann, M., E. Vorona, M. Wenk, F. Saad and E. Nieschlag (2008). "Testosterone administration decreases carotid artery intima media thickness as indicator of vascular damage in middle-aged overweight men." Journal of Andrology: 84.

Zumoff, B., G. W. Strain, L. K. Miller, W. Rosner, R. Senie, D. S. Seres and R. S. Rosenfeld (1990). "Plasma-free and non-sex-hormone-binding-globulin-bound testosterone are decreased in obese men in proportion to their degree of obesity." Journal of Clinical Endocrinology & Metabolism **71**(4): 929-931.