TESTOSTERONE DEFICIENCY, LONG-TERM TESTOSTERONE THERAPY, AND CARDIOVASCULAR RISK: A LONGITUDINAL STUDY

A Dissertation

by

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ABSTRACT

Cardiovascular disease is the major cause of morbidity and the leading cause of death globally. As compared to women, men have a higher cardiovascular risk. Recent studies suggest that testosterone as it is the most abundant male hormone plays a critical role in limiting vascular inflammation and maintaining normal lipid and glucose metabolism, and testosterone deficiency in aging men may contribute to the elevated cardiovascular risk in the male gender. Studies investigated the effect of low baseline testosterone level or testosterone measured at one single point in time on cardiovascular risk. However, the association between time-varying testosterone levels or recent testosterone changes (e.g., sudden drop-offs) and cardiovascular events have not yet been studied. Moreover, whether these associations differ between patients with prior cardiovascular events and those without is unknown. Additionally, whether testosterone deficiency is associated with major cardiovascular disease pathways such as inflammation biomarkers is not clear; how long-term testosterone therapy, a treatment to improve testosterone levels, influences cardiovascular disease development, and its longitudinal effect on inflammation, have not been elucidated.

In this study, we used data of 776 hypogonadal men from a registry study in Germany and the National Health and Nutrition Examination Survey (NHANES) data and applied Cox proportional hazards regression models, linear mixed effect models, linear and logistic regression models to investigate the association of time-varying testosterone level and testosterone changes since the last visit and cardiovascular events, stratified by prior cardiovascular event status; the association between testosterone deficiency and inflammation biomarkers (C-reaction protein (C-RP), alanine aminotransferase (ALT), aspartate aminotransferase (AST)); the effect of long-

term testosterone therapy on the risk of cardiovascular events and inflammation biomarkers. We found lower time-varying testosterone level and greater testosterone declines since the last visit were associated with a higher risk of myocardial infarction, and the associations were not different among patients with prior cardiovascular events and those without. Testosterone deficiency was associated with higher levels of C-RP and ALT; long-term testosterone therapy alleviated inflammation and reduced cardiovascular events. Clinicians should be informed of the effect of testosterone on cardiovascular health when assessing male patients' cardiovascular risk and make sure timely treatment is prescribed if needed.

DEDICATION

This dissertation is dedicated to all female scientists who work tirelessly for the love of investing new areas of science.

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CHAPTER I

INTRODUCTION

Cardiovascular disease is the leading cause of death and the most significant health problem worldwide. Atherosclerosis, a pathological process when plaque made up of fat, cholesterol, and calcium forms in the walls of the arteries, is the major cause of cardiovascular disease. Atherosclerosis may start with damage to the endothelium caused by cigarette smoking, increased blood glucose, low-density lipoprotein (LDL) cholesterol, or blood pressure [1], which are now known as major risk factors for cardiovascular disease. More recently, there has been a focus on the role of testosterone in atherosclerosis among men. Testosterone as it is the most abundant male hormone is critical in maintaining normal glucose and lipid metabolism in men. It has been suggested that testosterone might play a role in limiting vascular inflammation and cytokine activity underpinning the atherosclerosis process, and the morphological changes in the walls of the arteries might be due to metabolic syndrome resulting from testosterone deficiency [2,3]. In this chapter, an overview of the global burden and pathophysiology of cardiovascular disease will be introduced; current literature regarding risk factors associated with cardiovascular disease will be reviewed; traditional treatment for cardiovascular disease will be introduced; findings of human studies and animal experiments about the effect of testosterone or testosterone therapy on the risk factors and development of cardiovascular disease will be summarized; limitations and gaps of previous studies and significance of this study will be briefly discussed.

The Global Burden of Cardiovascular Disease

Cardiovascular disease is a disorder of the heart and blood vessels that includes many conditions such as coronary heart disease (also called coronary artery disease, ischemic heart disease), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, deep vein thrombosis and pulmonary embolism, and congenital heart disease [4]. Coronary heart disease is the most common type of cardiovascular disease, which accounts for 370,000 deaths annually, followed by stroke [5]. Nearly 18 million people die from cardiovascular disease each year, an estimated 31% of all deaths worldwide. Roughly 85% of all cardiovascular mortality are due to heart attacks and strokes, which are acute events usually resulting from clots built up of fatty deposits in the blood vessels that prevent blood supply to the heart and brain [4].

Common symptoms of cardiovascular events such as heart attack and stroke include chest pain, shortness of breath, nausea, numbness of one side of the body, difficulty seeing or walking, severe headache, and fainting. A very severe stroke can cause sudden death [6]. Patients experience significant health loss and changes in their ability to complete daily tasks and quality of life. The economic cost is also tremendous. According to a report released by the American Heart Association and the American Stroke Association, cardiovascular disease costs America $555 billion in 2016, including a direct medical cost of $318 billion and an indirect cost of $237 billion. The total cost will skyrocket to $1.1 trillion by 2035 [7]. Individuals age 45-64 face the highest indirect costs due to cardiovascular disease, and coronary heart disease ranks highest for direct medical costs in 2016 and will remain highest by 2035. Strategies for preventing cardiovascular disease are critical to improve population well-being and reduce economic loss around the world.

Pathophysiology of Cardiovascular Disease

Atherosclerosis is the major cause of cardiovascular disease [8]. It is a pathological process where the deposition called plaque made of fats, cholesterol, calcium, and other substances develops in the innermost layer of the endothelium of the arteries that supply oxygen-rich blood for heart and brain. If the plaque becomes unstable and ruptures, a clot begins to form in the artery. As the clot continues to grow, blood flow will be reduced. If the clot blocks the artery (thrombosis), a heart attack or stroke may happen. Atherosclerosis develops slowly over time, and the evolution of atherosclerosis from the initiation of fatty streak development through lesions progression to the plaque rupture and a clinical event is a complicated course. Over the last several decades, there have been numerous hypotheses proposed to explain the complex events associated with atherosclerosis. Although the precise mechanisms of atherosclerosis are not completely understood, there has been evidence suggesting oxidation and inflammation-driven mainly by hyperlipidemia may be involved throughout the process [8].

Oxidative stress is a prominent feature of the atherogenesis, which is involved in many steps of cardiovascular disease progression. The first of many steps towards atherosclerosis is fatty streak development, which is usually induced by elevation in LDL cholesterol levels [9]. The development of the fatty streak contains several components including lipoprotein transport, lipoprotein retention, lipoprotein modification, monocyte adherence, monocyte migration (chemotaxis), monocyte differentiation, and foam cell formation [10]. LDL can be rapidly transported across endothelium and trapped in the matrix of fibers and fibrils secreted by the artery wall cells [11]. Those cells then secrete oxidants that could seed the LDL retained in the matrix of the subendothelial space and initiate LDL oxidation [12]. Oxidants of plausible physiological relevance in cardiovascular disease may originate from both cellular and

extracellular sources, through both enzymatic and nonenzymatic pathways, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nitric oxide synthases (NOSs), myeloperoxidase, xanthine oxidase, lipoxygenase/cyclooxygenase, and mitochondrial respiratory chain/oxidative phosphorylation [13].

In this oxidative modification hypothesis, the oxidatively modified LDL is atherogenic, but LDL in its native state is not [13]. The trapped LDL oxidation modification process is thought to occur in two phases. In the early phase, LDL is modified to minimally modified LDL by major cells of the arterial wall [10], a process during which a significant proportion of the unsaturated acyl chains of cholesteryl esters and phospholipids in the minimally modified LDL are oxidized to hydroperoxides, isoprostanes, and short-chain aldehydes [14]. However, in this phase, cholesterol is still the predominant sterol, apolipoprotein B (ApoB) (the major protein of LDL) does not change much and still binds to the LDL-receptor, and leukocytes have not yet been recruited. In the second phase, leukocytes (mainly monocytes and T-lymphocytes) are recruited. The minimally modified LDL stimulates the production of monocyte chemotactic protein-1 (MCP-1) that promotes monocyte chemotaxis, resulting in monocyte binding to the endothelium and its subsequent migration into the matrix of the subendothelial space. In this matrix, the minimally modified LDL stimulates the production of monocyte colony stimulating factor that promotes the differentiation and conversion of monocytes into macrophages. The LDL lipids are further modified, with 50% of the cholesterol converting into 7-ketocholesterol and other oxysterols and the structure of ApoB being extensively changed, making the initially minimally modified LDL highly modified. The highly oxidized LDL can hardly be recognized by the LDL receptor but easily identified by the oxidized LDL receptor or scavenger receptor pathways in macrophages such as scavenger receptor-A (SR-A), cluster of differentiation 36

(CD36), and lectin-like oxidized LDL receptor-1 (LOX-1) [15]. The lipid-laden macrophages are deposited underneath the arterial endothelium. These cholesterol-loaded cells are called foam cells because of their foamy cytoplasm. The foam-cell formation is the hallmark of arterial fatty streak---the early atherosclerotic lesion.

Foam cells, along with T-lymphocytes and mast cells, release a variety of growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) and cytokines such as interleukin-1 (IL-1), interferon-gamma (IFN-γ), tumor necrosis factor-beta (TNF-β), and angiotensin II (Ang II) that promote inflammation and production of reactive oxygen species. The growth factors stimulate smooth muscle cell migration into the lesion and secretion of collagen that results in the development of the atheromatous plaque. The reactive oxygen species stimulate the release of matrix metalloproteinases that degrade fibrous wall of the plaque and basement membrane of the endothelial cells, leading to the superficial erosion of the endothelial cells, injury of the micro-vessels in the plaque, or damage of the fibrous cap that exposes the pro-thrombogenic content of the plaque [15].

There are two possible forms of the evolution of plaque: stable and unstable. The stable plaques often have a dense matrix of collagen embedded with smooth muscle cells. They are low in inflammation cell content, and growth factors such as PDGF and vascular endothelial growth factor (VEGF) promote endothelial cell survival. This kind of plaques can be achieved by treatments that could lower lipid in the circulation [16]. The unstable plaques, on the contrary, often have a high inflammation cell content and are subject to excessive inflammation. Inflammation cells produce cytokines such as TNF-β and IFN-γ that digest collagen fibrils and inhibit collagen secretion from smooth muscle cells. The fibrous cap of these plaques is thin and fragile and prone to rupture. When a physical disruption occurs, anti-coagulant pathways may override, and

a healing process takes over. Further accumulation of collagen and smooth muscle proliferation may then turn the fatty atherosclerotic plague to a more fibrous one. Alternatively, the physical disruption may expand the atherosclerotic lesion and trigger thrombosis by activating platelets and the clotting cascade, and cause a pathologic obstruction in the arteries that blocks blood flow, which is the proximate cause of the clinical event [10,15]. Factors that contribute to oxidation and inflammation may accelerate atherosclerosis and proceed to cardiovascular disease.

Risk Factors Associated with Cardiovascular Disease

Decades of epidemiologic research have identified hereditary and acquired cardiovascular risk factors that contribute to cardiovascular disease outcomes. The major cause of cardiovascular disease is usually the presence of a combination of risk factors, such as certain medical conditions (e.g., elevated cholesterol level, hypertension, diabetes, and obesity resulting from various reasons including physical indolence and unhealthy diet), certain behaviors (e.g., cigarette smoking, alcohol consumption), family history, and other characteristics. Most of these risk factors are prevalent in the elderly as consequences of aging, resulting in a much higher cardiovascular disease incidence in the elderly compared with their younger counterparts [17]. Identifying the risk factors and understanding how they contribute to cardiovascular health will help improve the effectiveness of treatment in preventing and managing cardiovascular disease.

Medical Conditions

High Cholesterol

Cholesterol is a fat-like substance synthesized by the liver. There are two types of lipoproteins that carry cholesterol to and from cells: LDL and high-density lipoprotein (HDL). LDL cholesterol contributes to the fatty deposition in arteries, whereas HDL cholesterol takes one-fourth to one-third LDL cholesterol away from the arteries and back to the liver [18].

As discussed previously, abnormally elevated LDL cholesterol due to excess cholesterol-rich or fat-rich food consumption or certain health conditions might contribute to the endothelial dysfunction of the arteries by oxidation and inflammation, leading to clinical cardiovascular events. High LDL cholesterol level activates oxidants secreted from various cellular and extracellular sources, which seed LDL retained in the subendothelial space. The trapped LDL then goes through a two-phase oxidation modification process that makes it hardly be recognized by the regular LDL receptor but easily identified by the scavenger receptors in the macrophages. Those lipid-laden macrophages are then deposited underneath the endothelium of the arteries, forming the so-called foam cells that could produce growth factors and cytokines that stimulate smooth muscle cell migration into the lesion and secretion of collagen that results in the development of the atheromatous plaque. A physical rupture of the plaque may trigger thrombosis and cause a pathologic obstruction in the arteries that blocks blood flow and results in a clinical event.

Hypertension

Hypertension, also known as high blood pressure, is a well-established risk factor for almost all different cardiovascular diseases, including coronary heart disease, left ventricular hypertrophy, valvular heart disease, atrial fibrillation, and stroke [19]. High blood pressure adds extra force against the artery walls, making the arteries stretch more than normal, injuring the endothelium and making it more vulnerable to the plaque buildup associated with atherosclerosis. The damaged and dysfunctional epithelium, along with changes in sodium and calcium concentrations among hypertensive individuals, enhance hypertrophy and proliferation of vascular smooth muscle cells that contribute to atherosclerosis. The hypertrophy would increase the distance for oxygen diffusion and therefore decrease the partial pressure of oxygen

in the blood, which would result in incomplete oxidation and increased levels of free radicals [20]. This oxygen radical formation would destroy endothelium-derived nitric oxide (NO) that helps regulate inflammation leukocyte adhesion, platelet activation, and thrombosis, increase the permeability of arterioles, and enlarge the endothelial lesion. This eventually leads to endothelial dysfunction, the major component of atherosclerosis. There is also evidence from animal models suggesting hypertension is associated with leukocyte adhesion, macrophage accumulation, smooth muscle cell migration, and work synergistically with hyperlipidemia in the progression of atherosclerosis [21].

Diabetes

Of various diabetes-related complications, atherosclerotic cardiovascular disease is the leading cause of death and the most prevalent cause of morbidity in diabetic patients [22]. Diabetic patients may experience changes in vascular structure and endothelial function, and oxidative stress is the initiating event in the development of diabetic vascular pathology [23]. It has been proposed that hyperglycemia or altered glucose utilization in diabetic patients would lead to overproduction of reactive oxygen species by the mitochondrial electron transport chain, NADPH oxidase, xanthine oxidase, or endothelial NOS, which eventually results in impaired endothelial function and the formation of structurally dysfunctional vessels [24]. Among these sources of reactive oxygen species, mitochondrial electron transport chain is a central event that involves several damage pathways including the formation of advanced glycosylation end products (AGEs), a process that glucose, glyceraldehyde, and fructose react non-enzymatically with proteins, lipids, and nucleic acids and is accelerated in the presence of hyperglycemia [25]. The accumulation of AGEs on wall proteins of large and small vessels results in an increase of vascular permeability and a thickened, inelastic vessel walls. The interaction between AGEs and

their receptors induces endothelial cell surface adhesion molecules, which accelerates the progression of vascular disease in diabetic patients and plays an essential role in the pathogenesis of cardiovascular disease [26].

Obesity

There are numerous adverse effects of obesity in general, especially hemodynamic alterations and cardiovascular structural and functional changes [27]. Experiments demonstrate the potential link between obesity and atherosclerosis. Although these two are distinct disease conditions, the pathophysiological mechanisms of obesity recapitulate many features of the inflammatory processes in atherosclerosis [28]. Lipid accumulation, inflammation cell infiltration, cytokine production, apoptosis, and oxidation stress are involved in both conditions. The various adipokines secreted by adipose tissue can induce insulin resistance, endothelial vasomotor dysfunction, hypercoagulability, and dyslipidemia, all of which potentially promote atherosclerotic process [29].

Behavior

Cigarette Smoking

Cigarette smoking can influence one's cardiovascular risk by affecting vasodilatory dysfunction, inflammation, and lipid modification, the components that precede the structural and clinical manifestations of atherosclerosis [30,31]. Vasodilatory dysfunction is among the earliest manifestations of atherosclerosis.S Multiple human studies, animal models, and in vitro experiments demonstrated that cigarette smoke exposure was associated with a decreased NO availability [32], a vasoregulatory molecule that is primarily responsible for the vasodilatory function of the endothelium. Additionally, since NO helps regulate inflammation leukocyte adhesion, platelet activation, and thrombosis, changes in NO availability will inevitably affect

atherogenesis and thrombotic events [33]. Besides, cigarette smoking has been reported to be associate with elevated levels of pro-inflammatory cytokines among smokers including interleukin-6 (IL-6) and tumor necrosis-alpha (TNF-α) that trigger the expression of inflammatory molecules (e.g., vascular cell adhesion molecule-1 (VCAM-1)), and cause 20% to 25% increase in the peripheral blood leukocyte count, which promotes the inflammation in the blood and at the vessel walls [31,34].

Alcohol Use

The association between alcohol consumption and cardiovascular risk or events is complex because the dose-response relationship is non-linear or monotonic [35].

Low-to-moderate alcohol consumption has been cardioprotective. Evidence shows that low-to-moderate alcohol consumption increases serum HDL level by stimulating the expression of apolipoprotein A1 and A2 (ApoA1 and ApoA2), the major protein components of HDL [36]. HDL recycles one-fourth to one-third LDL back to the liver for processing, removes LDL from macrophages and prevents the formation of foam cells, and reduces inflammation by isolating oxidized LDL in the vessel wall that prevents the attraction of immune cells [18]. Other studies suggest that moderate alcohol consumption is associated with improved glucose metabolism and insulin sensitivity, which are also factors associated with cardiovascular disease [37].

While low-to-moderate alcohol consumption has some benefits on the myocardium and the vasculature, acute and chronic heavy consumption clearly has a deleterious effect on the cardiovascular system [18]. High concentrations of alcohol and its metabolite acetaldehyde act as direct toxins to the cardiac myocytes, causing oxidative stress, mitochondrial damage, and cardiac fibrosis that results in contractile dysfunction. Animal models show that heavy alcohol consumption increases the activity of the rein-angiotensin aldosterone system and the

sympathetic nervous system, which promotes the retention of sodium and water, increases intravascular pressure and cardiac preload, resulting in nonischemic dilated cardiomyopathy and heart failure [38].

Other Characteristics

Aging

Low-grade systemic inflammation and oxidative stress resulting from the age-related accumulation of cellular damage and reduced activity of protective stress response pathways are thought to be responsible for the development of various age-related chronic diseases including cardiovascular disease [39,40].

Aging is a process that involves structural and functional changes in the arteries resulting from phenotypic alterations of endothelial cells, smooth muscle cells, and pericytes [41]. The organic-specific morphological changes include vascular wall thickening, collagen deposition, perivascular fibrosis, and vessel dilatation, with vascular wall thickening being a key hallmark of the aged vasculature promoting arterial stiffness [42]. The vascular permeability and inflammation are increased because of the impaired endothelium-dependent vasorelaxation in the aged vessels [43]. Moreover, the less laminar intraluminal blood flow and increased number of sites for lipid deposition due to the formation of more heterogeneous endothelial cells in the aging process would promote the development of atherosclerotic plaques [44].

Family History of Cardiovascular Disease

Family history of cardiovascular disease is a well-recognized independent risk factor for cardiovascular disease, and it is associated with both short-term and long-term cardiovascular risks [45]. It represents a combination of genetic, environmental, behavioral factors, and the interactions between them. The strength of the association between family history and

cardiovascular disease is greatest with earlier age of presentation of cardiovascular events in the family members, also known as premature family history [46]. In the general population, a history of premature atherosclerotic cardiovascular disease in one parent confers about three times increase in cardiovascular risk to offspring [47]. Premature cardiovascular events likely have a greater genetic component, while late cardiovascular events reflect a larger contribution of environmental and behavioral factors that are less heritable [45].

Ethnicity/Race Disparities

In the US, blacks continue to face a greater cardiovascular risk and mortality rate than other race groups, resulting from a disproportionally higher burden of hypertension, diabetes, and obesity in black populations [48]. In addition to the relatively lower socioeconomic status that affects access to healthy food, safe places to exercise, and high-quality health care, a genetic predisposition to retaining more sodium and therefore conserving more water that results in an increase in blood volume and blood pressure may account for their higher cardiovascular risk [49]. Besides, exposure to racial discrimination, a psychosocial stressor and nontraditional risk factor, may also play a role in cardiovascular risk disparities [50].

Sex/Gender Differences

Male gender has also been considered a strong risk factor for cardiovascular disease [51]. Studies show that coronary heart disease develops 7-10 years earlier in men compared to women in western countries. Acute coronary syndrome and ST-elevation or non-ST-elevation myocardial infarction occurs 3-4 times more often in men than in women before age 60 [52]. In addition to the differences in behavioral characteristics, influences of the environment, and disparities in gene expression and its impacts on vascular function between males and females, recently there has been a focus on the contributing role of testosterone decline over the aging

process in the development of cardiovascular disease in men [3]. Testosterone is responsible for the principal male sex differentiation and maintenance of libido, and closely associated with lipid and glucose metabolism. Low testosterone level has been associated with insulin resistance, hyperlipidemia, and obesity, which are strong predictors of cardiovascular disease. The role of male sex hormone in disease progression will be discussed more extensively in a later session of this chapter.

Traditional Treatment and Prevention Strategies for Cardiovascular Disease

Cardiovascular disease is traditionally treated through medications, surgeries, and lifestyle modifications. Medications such as anti-coagulants, antiplatelet agents, Ang II receptor blockers, beta blockers, calcium channel blockers, diuretics, vasodilators, and statins are used to decrease the coagulating ability of the blood, lower blood cholesterol levels and keep blood clots from forming or becoming larger in the vessels, dilate and relax vessels, decrease cardiac output, and relieve heart's workload [53]. Besides medications, common cardiac surgeries or procedures including coronary angioplasty and coronary artery bypass graft surgery may also be administered for certain cases. These procedures or surgeries can open, reconstruct, or replace plaque-blocked arteries and recover blood flow and supply of blood and oxygen to the heart [54]. Lifestyle modifications, such as maintaining a healthy weight, quitting smoke and limiting alcohol, being physically active, having a healthy diet to control blood pressure, blood glucose, and cholesterol are also extremely important to reduce the risk of recurrence for all cardiovascular patients. The overall treatment strategy is to remove or modify risk factors, relieve symptoms, reduce the risk of recurrence, and prevent complications.

Testosterone Deficiency, Testosterone Therapy, and Cardiovascular Disease

Recent studies have focused on the role of testosterone deficiency in the development of cardiovascular disease in men; pronounced testosterone declines during the aging process have been considered a potential contributing factor for the excess risk of cardiovascular disease in men [51].

Testosterone is the principal male sex hormone responsible for the maturation of sexual organs and secondary sexual characteristics. It is also an important metabolic hormone for maintaining the overall physiological function, including carbohydrate, protein, and lipid metabolism in men [55]. In men's testes, luteinizing hormone (LH) release stimulated by gonadotropin-releasing hormone (GnRH) further stimulates testosterone synthesis by Leydig cells. A large majority of the total circulating testosterone is bound to sex hormone-binding globulin (SHBG) (roughly 50%) or albumin (about 50%), and only a small fraction (0.5%-3.0%) is in a biologically active and free form that binds to the androgen receptor and is present in the cytosol of most tissues [56].

A deficiency of testosterone secretion from the Leydig cells is called male hypogonadism, which can be classified as primary hypogonadism (testicular failure due to radiation therapy, trauma, torsion-induced ischemia, or infection) and secondary hypogonadism (central suppression of the hypothalamus and pituitary) [57]. A milder form of hypogonadism associated with aging also exists and has become more recognized in recent years. Testosterone levels increase during puberty and adolescence, reaching their peak in young to middle adulthood and then gradually decreasing when males enter their later adulthood. There is a natural decline of testosterone levels with advancing age, typically 1% - 3% per year [58]. On average, this change is small, and the levels remain within the normal range in most men, which

is different from the pronounced sex hormone changes in women at menopause [59]. However, a small proportion of men develop mild or severe testosterone deficiency during the aging process. Approximately 25% of men over 65 years of age will have low total testosterone levels [57]. A condition of suppressed circulating testosterone levels and physical and psychological symptoms in aging men is called late-onset hypogonadism (LOH) [59,60].

Hypogonadism has been associated with obesity, greater waist circumference, diabetes, hypertension, and lower HDL cholesterol, which are strong predictors of cardiovascular disease [57,61]. Testosterone inhibits adipocytes lipoprotein lipase activity, and low testosterone promotes increasing adipocytes and fat deposition, which results in increased insulin resistance and poor glycemic control [62]. Animal models have demonstrated an anti-atherogenic action of testosterone in males, whereas testosterone deficiency promotes the early stages of atherogenesis, the first step towards coronary heart disease [63,64]. Prior cohort studies have reported the association between serum testosterone level and cardiovascular risk, though the findings have been inconsistent due to various study designs, different statistical methods applied, and different lengths of follow-up and sample sizes [51]. Additionally, the authors only looked at the effect of baseline serum testosterone level or testosterone level measured at one single visit, which may not be relevant to the cardiovascular events that occurred many years later, given the dynamic changes of testosterone level over time. Moreover, participants in prior studies were restricted to men with the same prior cardiovascular disease history; surprisingly few investigated whether the effects of testosterone level on the risk of cardiovascular events were different among patients with prior cardiovascular events and those without, given the discrepancies in comorbidity conditions in the two populations may potentially modify this association. It might

be worth exploring how time-varying testosterone level affects the risk of cardiovascular events, stratified by patients' prior cardiovascular event status.

In addition to the absolute testosterone level, remarkable changes of testosterone levels may also have an acute effect on the development of cardiovascular disease. In animal studies, wild-type mice that went through castration experienced a pronounced testosterone decline within hours. Sudden testosterone declines induced macrophage infiltration and elevated inflammation biomarkers such as IL-6 and interleukin-1 beta (IL-1β), and lipid profiles such as cholesterol and triglyceride remarkably increased in the castrated mice as compared to the sham-operated mice [65]. Given the critical role that testosterone plays in maintaining the normal physiological function, it may also be important to know whether recent pronounced testosterone declines influence the risk of cardiovascular events, especially for those who already experienced testosterone deficiency that posed a baseline higher risk for many chronic conditions including cardiovascular disease. If remarkable testosterone declines are confirmed to be associated with cardiovascular risk, monitoring of testosterone levels might be necessary to deliver timely interventions when pronounced drop-offs of testosterone are detected to maintain a normal metabolic function and prevent cardiovascular events.

Testosterone therapy, a treatment to restore testosterone levels to normal physiological ranges, is used to relieve the hypogonadism-related symptoms. Reductions in fat mass, fasting glucose, fasting insulin, and triglycerides have been associated with testosterone therapy [57]. Although low testosterone level has been associated with many predictors of cardiovascular disease, and growing evidence has demonstrated the benefits of testosterone therapy on metabolic profiles that are closely related to atherosclerosis and cardiovascular health, controversies remain about the effects of testosterone therapy on the risk of cardiovascular

events. One of the earliest randomized controlled trials, the Testosterone in Older Men (TOM) trial, was terminated because of the higher number of cardiovascular events in the intervention group compared to the placebo group [66]. However, study subjects in this trial were only men aged 65 years or older with a high prevalence of chronic diseases and "with no standard indication for testosterone therapy" [67]. Participating men represented the older and immobile population and received a high dose of testosterone that made it difficult to extrapolate the findings to other doses of testosterone, or to the male population who do not have this many chronic conditions or mobility issues [57]. A recent study reviewed seven systematic reviews that included a total of 94 randomized controlled trials and investigated the association between exogenous testosterone and cardiovascular events [68]. Six of them showed no association, with summary estimates ranging from 1.07 to 1.82 and relatively imprecise confidence intervals (CIs) (e.g., odds ratio (OR)=1.07 (95%CI: 0.69, 1.65); OR=1.82 (95%CI: 0.78, 4.23); one showed an increased risk for cardiovascular disease associated with exogenous testosterone administration, with a summary estimate of 1.54 (95% CI: 1.09-2.18). However, because of limited sample sizes and short follow-up duration (2 weeks to 3 years), these trials may have been underpowered to detect a true difference in cardiovascular risk between treatment and control groups. While randomized controlled trials are considered the gold standard in medical research, its highly strict inclusion and exclusion criteria and controlled environments may make its results difficult to carry over to the uncontrolled setting. Real-world evidence from large observational studies with cardiovascular events as a primary endpoint, longer follow-up, participants with wide age range, sufficient information on participants' comorbidity status, a clear indication for testosterone therapy, and reasonable statistical methods is needed to provide more reliable evidence in determining the effect of testosterone therapy on the risk of cardiovascular events. As mentioned

by Elagizi *et al.*, "a major research initiative is needed to explore the possible cardioprotective effects of testosterone therapy" [51].

In addition to its effect on metabolic profiles, animal studies have also demonstrated that testosterone deficiency was associated with elevated blood-brain-barrier permeability accompanied by the up-regulation of inflammatory molecules [69], which may trigger arterial inflammation that causes arterial hyperplasia even when the traditional risk factors were absent [70]. Inflammation is the major pathway of cardiovascular disease, and almost all risk factors contribute to the onset or progression of cardiovascular disease through this pathway. Multiple inflammation markers elevated years before the first cardiovascular event and were highly predictive of future cardiovascular risk in otherwise health populations [71]. In an experiment investigating the mechanism through which testosterone deficiency-induced endothelial dysfunction in rats, the authors found higher levels of IL-6, nuclear factor kappa B (NF-κB), NADPH oxidase-1, and NADPH oxidase-4 in castrated rats compared to castrated rats supplemented with testosterone and sham rats (p-value<0.01), indicating elevated oxidative stress and inflammation in the rats undergoing testosterone deficiency and potential beneficial effects of testosterone therapy in attenuating this result [72]. However, to our knowledge, studies using large nationally representative samples of human subjects to evaluate the association between testosterone deficiency and inflammation are scarce. Besides, whether testosterone therapy reduces cardiovascular events by alleviating inflammation and how it affects inflammation over time in human subjects have not been elucidated.

In this study, we used data of 776 hypogonadal men from a registry study in Germany and the National Health and Nutrition Examination Survey (NHANES) data and applied Cox proportional hazards regression models, linear mixed effect models, linear regression models,

and logistic regression models to investigate the association of time-varying testosterone level and testosterone changes since the last visit with the risk of cardiovascular events, stratified by patients' prior cardiovascular event status; the association between testosterone deficiency and inflammation biomarkers; and the effect of long-term testosterone therapy on the risk of cardiovascular events and inflammation biomarkers. The findings of this study provide insights into the etiologic role of testosterone in cardiovascular disease development and clinical importance of testosterone therapy in preventing cardiovascular events among hypogonadal men.

CHAPTER II

TIME-VARYING TESTOSTERONE LEVEL AND RISK OF MYOCARDIAL INFARCTION AND STROKE AMONG HYPOGONADAL MEN

Introduction

Cardiovascular disease remains a major cause of premature death and chronic disability worldwide. Compared to women, men have a higher risk of acute coronary syndromes, ST-elevation myocardial infarction before age 60, and on average develop coronary heart disease, the most common type of cardiovascular disease, 7-10 years earlier than women do [52].

Cardiovascular disease is closely related to atherosclerosis, a pathological process when plaque made up of fat, cholesterol, and calcium forms in the walls of the arteries [5]. Rupture of atherosclerotic plaques may lead to thrombotic events, such as myocardial infarction and stroke, which can cause sudden death or significant morbidity[73]. There has been a recent focus on the role of testosterone deficiency in atherosclerosis among men, which was thought to contribute to the higher risk of cardiovascular disease in male gender [3].

Testosterone is responsible for the principal male sex differentiation and maintenance of libido, and closely associated with lipid and glucose metabolism that have been linked to cardiovascular health. Testosterone levels increase during puberty and adolescence, reaching their peak in young to middle adulthood and then gradually decreasing when males enter their later adulthood. There is a natural decline of testosterone levels with advancing age, typically 1% - 3% per year[58]. On average, this change is small, and the levels remain within the normal range in most men, which is different from the pronounced sex hormone changes in women at menopause [59]. However, a small proportion of men develop mild or severe testosterone

deficiency during the aging process [57]. A condition of suppressed circulating testosterone levels (below healthy adult male reference range) and physical and psychological symptoms in aging men is called late-onset hypogonadism (LOH) [59,60]. This aging-related testosterone decline has been associated with an increase in atherosclerosis risk[57]. Animal models have consistently demonstrated an anti-atherogenic action of testosterone in males, whereas testosterone deficiency promotes the early stages of atherogenesis[63,64]. Studies have also shown that low testosterone levels were associated with higher carotid intima media thickness [74–76], which is a surrogate marker of atherosclerosis that is closely related to abnormal glucose metabolism and lipid profile as well as a strong predictor of future clinical ischemic cardiac and cerebrovascular events [77–79].

Although testosterone deficiency has been associated with atherosclerosis and predictors of cardiovascular disease, inconsistent findings were reported regarding the role of testosterone in cardiovascular risk [80]. In addition to the different demographic makeup of the study populations, various statistical methods applied, and insufficient length of follow-up and sample sizes, another possible explanation is that prior studies were using testosterone level measured at one single point in time as the exposure, such as baseline testosterone level, which might not be relevant for the cardiovascular event that occurred years later, given the dynamic changes of serum testosterone level over time and the possible long time period in between baseline measurement and disease occurrence later on [80–82]. It might make more sense to use testosterone levels close to the time when cardiovascular events occurred as the exposure when investigating the association between testosterone levels and the cardiovascular risk. Additionally, participants of these studies were normally restricted to those who had the same prior cardiovascular event status (either free of prior cardiovascular events or had prior cardiovascular events) [51]; whether testosterone level affects differently among patients with and without prior cardiovascular events

is unknown. As compared to patients without prior cardiovascular history, those with prior cardiovascular events are more likely to have comorbidities and pathological changes (e.g., atherogenesis) in their blood vessels, and therefore are hypothesized to be more susceptible to cardiovascular events when exposed to risk factors.

In this study, we used data from 376 hypogonadal men in a registry-based study in Germany that contain serum testosterone levels measured at each visit and corresponding cardiovascular event status at each visit. We treated testosterone level as a time-varying variable and fitted data using Cox proportional hazards regression models to investigate the effect of time-varying testosterone level on cardiovascular risk, which took into account the dynamic changes of testosterone level over time. For comparison, we also evaluated the association between baseline testosterone level and cardiovascular risk. All the analyses were conducted stratified by patients' prior cardiovascular event status.

Materials and Methods

Study Population

We used de-identified data from a registry study in Germany. Seven hundred and seventy-six hypogonadal men were recruited from one urology center in Bremerhaven, Germany from 2004 to 2016. Hypogonadism diagnosis was confirmed if they had total testosterone level≤12.1 nmol/L and symptoms such as decreased libido, erectile dysfunction, depression, and fatigue, as assessed by the Aging Males' Symptoms scale (AMS). The threshold of 12.1 nmol/L was selected based on clinical experience and confirmed by Bhasin *et al.* [83]. Ethical guidelines formulated by the German Ärztekammer (German Medical Association) for observational studies in patients receiving standard treatment were followed. After receiving an explanation about the nature and the purpose of the study, all patients provided written consent to be included

in the registry and have their data analyzed. Eligible participants without contraindications were given option of testosterone therapy at study entry. Four hundred of them decided to receive testosterone therapy, whereas three hundred and seventy-six opted against testosterone therapy for various reasons including the concern about the testosterone therapy safety issues. This study only used data from the participants who did not take treatment to avoid the influence of exogenous testosterone administration.

Testosterone Measurement and Cardiovascular Outcome Ascertainment

Participants were followed semi-annually for updates in serum testosterone levels. Cardiovascular events (i.e., myocardial infarction and stroke) occurring during follow-up were recorded. Cardiovascular events were partly reported in the form of "physician letters" from the hospital or the cardiologist/neurologist/family physician, and partly by patients themselves or relatives. The latter usually occurred when already scheduled patient visits had to be postponed due to an event.

Statistical Analysis

In the descriptive analysis, characteristics among patients who experienced cardiovascular events and those who did not during the study period were presented.

Cox proportional hazards regression models were fitted to the data to investigate the association of time-varying testosterone as well as baseline testosterone levels and the risk of cardiovascular events. Time to event was the duration from study entry to the time when the cardiovascular event was recorded or the end of the study. Event is denoted 1 if a cardiovascular event was present during the follow-up period, or 0 if not (censored). When using time-varying testosterone level as the exposure, we modeled the association between testosterone level measured at the current visit and the risk of cardiovascular events at this visit; when using

baseline testosterone level as the exposure, we modeled the association between testosterone level measured at baseline and the risk of cardiovascular events that occurred during the study period. Covariates that are closely related to the risk of cardiovascular risk including age at study entry, baseline smoking/drinking status, baseline comorbidities (type 2 diabetes, hypertension, dyslipidemia), body mass index (BMI) and family history of coronary heart disease were included in the model. Cardiovascular risk was presented in a hazard ratio (HR) and its 95% confidence interval (CI).

Analyses were stratified by prior cardiovascular event status; those with prior cardiovascular events and those without prior cardiovascular events. The significance of the interaction term between time-varying testosterone level or baseline testosterone level and prior cardiovascular event status was also tested to examine whether the effects of testosterone level on cardiovascular events were different in the two strata. Outcome variables were myocardial infarction, stroke, or any cardiovascular events (myocardial infarction or stroke).

All analyses were performed with Stata/MP 14.0.

Results

As shown in **Table 1**, the characteristics were in general different in participants who experienced cardiovascular events and who did not during the study period. As compared to the participants who did not experience cardiovascular events, those who had cardiovascular events during the study period had higher BMI, were more likely to be a smoker or an alcohol user, have hypertension, diabetes, family history of coronary heart disease, and prior cardiovascular events, and had longer follow-up (p-value<0.05). There was no difference in age at study entry, baseline testosterone level, and the proportions of patients with dyslipidemia in the two groups (p-value>0.05).

Table 1 Characteristics of participants, by endpoint outcome

Characteristics	Had CV events (n=79)		Did not have CV events (n=297)		*p*-value [a]
	Mean ± SD N (%)	Range	Mean ± SD N (%)	Range	
Age at study entry (years)	63.49 ± 4.75	49-73	64.06 ± 4.66	45-74	0.3456
Baseline testosterone (nmol/L)	9.49 ± 1.22	5.89-12.13	9.75 ± 1.12	5.89-11.79	0.0941
BMI (kg/m^2)	31.86 ± 4.77	23.20-46.02	29.66 ± 3.94	22.15-46.98	0.0003
Smoker	42 (53.16)	-	96 (32.32)	-	0.001
Alcohol user	50 (63.29)	-	135 (45.45)	-	0.005
Hypertension	68 (86.08)	-	216 (72.73)	-	0.014
Diabetes	46 (58.23)	-	107 (36.03)	-	<0.001
Dyslipidemia	55 (69.62)	-	179 (60.27)	-	0.128
Family history of coronary heart disease	37 (46.84)	-	62 (20.88)	-	<0.001
Prior CV event	34 (43.04)	-	73 (24.58)	-	0.001
Follow-up (years)	7.82 ± 1.51	4-10	7.35 ± 1.79	2-11	0.0181

[a] *p*-value for t-test if the variable is continuous; *p*-value for Chi-Square test if the variable is categorical
CV: cardiovascular
SD: standard deviation
BMI: body mass index

Table 2 presents the results of Cox proportional hazards regression of cardiovascular events on baseline testosterone levels, stratified by patients' prior cardiovascular event status. Among patients without prior cardiovascular events, the adjusted HR for any cardiovascular event, myocardial infarction, and stroke associated with 1 nmol/L increase in baseline testosterone level was 0.76 (95%CI: 0.61, 0.96), 0.69 (95%CI: 0.53, 0.89), and 0.78 (95%CI: 0.56, 1.09), respectively. Among patients with prior cardiovascular events, the adjusted HRs for any cardiovascular event, myocardial infarction, and stroke associated with 1 nmol/L increase in baseline testosterone level were 1.06 (95%CI: 0.83, 1.36), 1.15 (95%CI: 0.79, 1.66), and 1.02 (95%CI: 0.75, 1.39), respectively.

Table 2 Cox proportional hazards regression of cardiovascular events on baseline testosterone levels, by prior cardiovascular event status

	Without prior CV events (N=269)			With prior CV events (N=107)		
	HR (95%CI)			HR (95%CI)		
	Any CV event	MI	Stroke	Any CV event	MI	Stroke
Baseline testosterone (nmol/L)	0.76 (0.61, 0.96)	0.69 (0.53, 0.89)	0.78 (0.56, 1.09)	1.06 (0.83, 1.36)	1.15 (0.79, 1.66)	1.02 (0.75, 1.39)
Age at study entry (years)	1.02 (0.95, 1.09)	0.99 (0.91, 1.07)	1.04 (0.94, 1.14)	0.99 (0.91, 1.06)	0.95 (0.87, 1.03)	0.97 (0.89, 1.07)
BMI (kg/m^2)	1.15 (1.03, 1.28)	1.17 (1.03, 1.33)	1.06 (0.92, 1.22)	0.99 (0.92, 1.06)	0.92 (0.83, 1.03)	1.04 (0.95, 1.15)
Smoker	1.74 (0.92, 3.32)	1.42 (0.64, 3.15)	2.05 (0.82, 5.12)	1.62 (0.78, 3.37)	1.16 (0.41, 3.24)	1.84 (0.73, 4.66)
Alcohol user	1.11 (0.60, 2.04)	0.94 (0.44, 2.01)	1.07 (0.44, 2.61)	1.71 (0.79, 3.71)	2.12 (0.67, 6.66)	1.06 (0.43, 2.60)
Type 2 diabetes	1.18 (0.59, 2.36)	1.12 (0.48, 2.60)	1.95 (0.72, 5.28)	2.04 (0.98, 4.22)	4.02 (1.27, 12.72)	0.87 (0.34, 2.23)
Hypertension	1.56 (0.75, 3.25)	1.12 (0.48, 2.61)	1.84 (0.59, 5.68)	1.67 (0.21, 13.29)	-	1.52 (0.18, 13.12)
Dyslipidemia	1.05 (0.39, 2.81)	1.16 (0.33, 4.07)	1.52 (0.36, 6.44)	5.18 (1.11, 24.20)	2.24 (0.41, 12.40)	5.76 (0.69, 47.97)
Family history of coronary heart disease	2.01 (0.96, 4.20)	1.63 (0.66, 4.00)	2.35 (0.82, 6.68)	2.98 (1.39, 6.37)	3.85 (1.27, 11.64)	3.10 (1.21, 7.95)

CV: cardiovascular
HR: hazard ratio
CI: confidence interval
MI: myocardial infarction
BMI: body mass index

By including testosterone as a time-varying covariate, as shown in **Table 3**, among patients without prior cardiovascular events, the adjusted HRs for any cardiovascular event, myocardial infarction, and stroke associated with 1 nmol/L increase in baseline testosterone level were 0.75 (95%CI: 0.60, 0.94), 0.64 (95%CI: 0.49, 0.84), and 0.79 (95%CI: 0.57, 1.10), respectively. Among patients with prior cardiovascular events, the adjusted HRs for any cardiovascular event, myocardial infarction, and stroke associated with 1 nmol/L increase in baseline testosterone level were 0.93 (95%CI: 0.74, 1.17), 1.02 (95%CI: 0.73, 1.43), and 0.96 (95%CI: 0.70, 1.33), respectively.

Table 3 Cox proportional hazards regression of cardiovascular events on time-varying testosterone levels, by prior cardiovascular event status

	Without prior CV events (N=269)			With prior CV events (N=107)		
	HR (95%CI)			HR (95%CI)		
	Any CV event	MI	Stroke	Any CV event	MI	Stroke
Time-varying testosterone (nmol/L)	0.75 (0.60, 0.94)	0.64 (0.49, 0.84)	0.79 (0.57, 1.10)	0.93 (0.74, 1.17)	1.02 (0.73, 1.43)	0.96 (0.70, 1.33)
Age at study entry (years)	1.02 (0.95, 1.08)	0.99 (0.91, 1.06)	1.03 (0.94, 1.14)	0.98 (0.91, 1.06)	0.95 (0.87, 1.03)	0.98 (0.89, 1.07)
BMI (kg/m^2)	1.13 (1.02, 1.25)	1.15 (1.01, 1.30)	1.04 (0.90, 1.19)	0.99 (0.92, 1.06)	0.93 (0.84, 1.04)	1.04 (0.94, 1.15)
Smoker	1.78 (0.94, 3.37)	1.44 (0.66, 3.17)	2.05 (0.83, 5.10)	1.60 (0.77, 3.30)	1.17 (0.42, 3.28)	1.81 (0.72, 4.56)
Alcohol user	1.12 (0.61, 2.06)	0.94 (0.44, 2.01)	1.11 (0.45, 2.74)	1.81 (0.84, 3.90)	2.25 (0.72, 7.00)	1.09 (0.45, 2.65)
Type 2 diabetes	1.13 (0.57, 2.25)	1.07 (0.46, 2.48)	1.80 (0.67, 4.80)	1.94 (0.92, 4.08)	4.00 (1.26, 12.75)	0.86 (0.34, 2.22)
Hypertension	1.63 (0.78, 3.41)	1.19 (0.50, 2.79)	1.91 (0.62, 5.91)	1.69 (0.21, 13.49)	-	1.51 (0.18, 12.95)
Dyslipidemia	0.96 (0.36, 2.58)	0.99 (0.28, 3.48)	1.40 (0.33, 5.94)	4.81 (1.04, 22.29)	2.04 (0.38, 11.05)	5.65 (0.68, 46.71)
Family history of coronary heart disease	1.90 (0.91, 3.95)	1.45 (0.60, 3.52)	2.27 (0.80, 6.46)	2.83 (1.33, 6.03)	3.54 (1.19, 10.52)	3.08 (1.20, 7.89)

CV: cardiovascular
HR: hazard ratio
CI: confidence interval
MI: myocardial infarction
BMI: body mass index

Though stratum-specific HRs for cardiovascular events seemed different among participants with prior cardiovascular events and those without, the interaction terms between time-varying testosterone level or baseline testosterone level and patients' prior cardiovascular event status were not different from 0 (p-value>0.05; results not shown), indicating the effects of testosterone level (either time-varying or baseline) on cardiovascular risk were not different in the two populations based on formal statistical tests. Thus, collapsed tables describing the associations between time-varying testosterone level or baseline testosterone and cardiovascular events, regardless of participants' prior cardiovascular event status were presented.

Table 4 Cox proportional hazards regression of cardiovascular events on baseline testosterone levels

	Total (N=376)		
	HR (95%CI)		
	Any CV event	**MI**	**Stroke**
Baseline testosterone (nmol/L)	0.91 (0.77, 1.07)	0.85 (0.69, 1.05)	0.90 (0.72, 1.13)
Age at study entry (years)	1.00 (0.95, 1.05)	0.97 (0.92, 1.03)	1.01 (0.94, 1.08)
BMI (kg/m^2)	1.05 (0.98, 1.11)	1.02 (0.95, 1.11)	1.06 (0.98, 1.14)
Smoker	1.78 (1.12, 2.84)	1.43 (0.78, 2.62)	1.84 (0.99, 3.44)
Alcohol user	1.42 (0.90, 2.23)	1.36 (0.76, 2.43)	1.10 (0.60, 2.03)
Type 2 diabetes	1.59 (0.98, 2.57)	1.98 (1.05, 3.75)	1.28 (0.66, 2.51)
Hypertension	1.77 (0.90, 3.49)	1.35 (0.60, 3.06)	1.88 (0.70, 5.05)
Dyslipidemia	2.00 (0.92, 4.36)	1.74 (0.66, 4.56)	2.33 (0.75, 7.24)
Family history of coronary heart disease	2.34 (1.42, 3.85)	2.08 (1.09, 3.99)	2.60 (1.34, 5.05)
Prior CV event	1.59 (0.96, 2.64)	1.28 (0.66, 2.51)	2.04 (1.06, 3.94)

HR: hazard ratio
CI: confidence interval
CV: cardiovascular
MI: myocardial infarction
BMI: body mass index

As shown in **Table 4**, after adjustment, no association was found between baseline testosterone level and the risk of any cardiovascular event, myocardial infarction, or stroke. As shown in **Table 5**, by including testosterone as a time-varying covariate, the adjusted HR for any cardiovascular event associated with 1 nmol/L increase in testosterone level was 0.85 (95%CI: 0.72, 0.99); the adjusted HR for myocardial infarction associated with 1 nmol/L increase in testosterone level was 0.78 (95%CI: 0.64, 0.97); no association between time-varying testosterone level and stroke was found. This result matched our *post hoc* analysis results using stratified proportional hazards models (results not shown).

Table 5 Cox proportional hazards regression of cardiovascular events on time-varying testosterone levels

	Total (N=376)		
	HR (95%CI)		
	Any CV event	MI	Stroke
Time-varying testosterone (nmol/L)	0.85 (0.72, 0.99)	0.78 (0.64, 0.97)	0.90 (0.72, 1.13)
Age at study entry (years)	1.00 (0.95, 1.05)	0.97 (0.92, 1.03)	1.01 (0.94, 1.08)
BMI (kg/m^2)	1.04 (0.98, 1.10)	1.01 (0.94, 1.09)	1.05 (0.97, 1.13)
Smoker	1.76 (1.10, 2.80)	1.40 (0.76, 2.58)	1.83 (0.98, 3.43)
Alcohol user	1.42 (0.91, 2.24)	1.34 (0.75, 2.39)	1.10 (0.60, 2.03)
Type 2 diabetes	1.52 (0.94, 2.46)	1.89 (1.00, 3.58)	1.22 (0.63, 2.37)
Hypertension	1.82 (0.93, 3.56)	1.39 (0.62, 3.13)	1.94 (0.73, 5.19)
Dyslipidemia	1.96 (0.90, 4.28)	1.64 (0.62, 4.33)	2.35 (0.76, 7.31)
Family history of coronary heart disease	2.29 (1.39, 3.77)	1.99 (1.04, 3.82)	2.59 (1.34, 5.03)
Prior CV event	1.58 (0.95, 2.61)	1.26 (0.64, 2.46)	2.07 (1.07, 4.00))

HR: hazard ratio
CI: confidence interval
CV: cardiovascular
MI: myocardial infarction
BMI: body mass index

Discussion

We conducted a cohort study and followed 376 hypogonadal men for up to 11 years to investigate the association of time-varying testosterone level and baseline testosterone level with the risk of cardiovascular events, stratified by patients' prior cardiovascular event status. We did not find any difference in the effects of testosterone level (either baseline or time-varying) on the risk of cardiovascular events among patients with prior cardiovascular events and those without, based on formal statistical tests. Regardless of patients' prior cardiovascular event status, a lower time-varying testosterone level was associated with a higher risk of myocardial infarction, but

not of stroke; no association was found between baseline testosterone level and the risk of cardiovascular events.

Srinath *et al.* investigated the association between baseline endogenous plasma testosterone level and incident clinical cardiovascular events using data of 1,558 male participants from the Atherosclerosis Risk in Communities (ARIC) study [81]. Although low baseline testosterone level was associated with cardiovascular risk factors such as higher BMI and greater waist circumference at baseline, no association was found between baseline testosterone level and cardiovascular event incidence, which was consistent with our findings. This is possibly because the baseline testosterone level might be irrelevant for the cardiovascular events that occurred many years later. In a recent study, Gagliano-Jucá *et al.* reviewed large prospective cohort studies that assessed cardiovascular outcomes and used reliable assays for the measurement of testosterone levels; inconsistent findings were reported. Yeap *et al.*, Ohlsson *et al.*, Soisson *et al.*, and Magnani *et al.* found a negative association between testosterone level and the risk of adverse cardiovascular outcomes including transient ischemic attack, myocardial infarction, unstable angina, and atrial fibrillation, whereas Ärnlöv *et al.*, Abbott *et al.*, Vikan *et al.*, Haring *et al.*, and Shores *et al.* did not find any association between testosterone and the cardiovascular risk. In addition to the different study population characteristics and the small number of events, which might contribute to the discrepancies in their study findings, Gagliano-Jucá *et al.* also highlighted some limitations of these studies, including one single testosterone measurement, which was suboptimal given the variation in serum testosterone levels over time[80].

In our study, besides baseline testosterone level, we also assessed the association between time-varying testosterone level and the risk of cardiovascular events. We found lower time-varying testosterone level was associated with a higher risk of myocardial infarction. A large

population-based retrospective matched-cohort study of men aged 66 years or older with a median follow-up of 5 years conducted in Canada assessed the effect of testosterone therapy exposure duration on all-cause mortality and a composite cardiovascular outcome (consisting of myocardial infarction, cerebrovascular accident, and venous thromboembolic event). Unlike our analysis, where continuous time-varying exposure (testosterone level) was added to the model to evaluate the effect of testosterone level at each visit and the risk of cardiovascular events, the authors used the time-varying testosterone therapy exposure and assessed the cumulative effect of testosterone therapy on mortality. They found that the mortality was progressively lower with increasing exposure to testosterone, with a decreased risk for patients with the highest tertile of exposure (HR=0.60, 95%CI: 0.45, 0.80). Both their study and ours considered the dynamic changes of exposure and applied advanced statistical techniques to address that, which avoids imprecise estimates due to overly simplistic exposure definition. Another two cohort studies that investigated the treatment effect of testosterone on the stroke risk also mentioned including testosterone treatment as a time-varying variable. However, only testosterone therapy exposure at baseline was used in the analysis, and therefore no "time-varying" factor was actually accounted for in the analysis [67]. In our study, no patients switched to the treatment group, and therefore only testosterone level was included as a time-varying variable, not the treatment status.

The effects of testosterone might be different among patients with prior cardiovascular events and those without, given the different health conditions in the two populations. Patients with prior cardiovascular events might be more likely to have comorbidities and pathological changes of their arteries, and therefore more vulnerable to cardiovascular events when exposed to risk factors such as testosterone deficiency. Srinath *et al.* investigated the association between

endogenous testosterone level and cardiovascular event occurrence among patients without prior cardiovascular events and did not find any association [81]. Collet *et al.* assessed whether testosterone level and sex hormone–binding globulin (SHBG) affected the risk of incident cardiovascular events among patients free of prior cardiovascular events and did not find any association [82]. Chmiel *et al.* found an increased risk of major cardiovascular events associated with low testosterone levels among patients with prior cardiovascular events [84]. However, there is no easy way to compare these associations as they were assessed in different source populations. In this study, we used the same source population from a registry study in Germany and compared the effects of testosterone level on the risk of cardiovascular events among patients with and without prior cardiovascular events. However, due to the limited number of participants in the stratified analysis, we were underpowered to detect the potential difference. Future studies are warranted to identify the more susceptible population and provide targeted intervention if necessary.

There is growing literature on the importance of normal lipid panel and glucose level in maintaining cardiovascular health, with little known about the role of testosterone. The relationship between testosterone deficiency and cardiovascular disease is complex and remains controversial. It has been suggested that testosterone deficiency modulates lipid profiles and glucose metabolism and contributes to atherosclerosis development or progression; androgen deprivation therapy that is previously used to treat prostate cancer increases the risk of coronary heart disease, diabetes, and cardiovascular mortality [85]. Animal experiments have demonstrated that the impaired lipid and glucose profile due to testosterone deficiency or androgen deprivation could be reversed by testosterone therapy. A research team reported that low testosterone in the testicular feminized mice had increased lipid deposition in the aortic root and liver when fed a

high-cholesterol diet; testosterone therapy to return levels to those in wild-type counterparts reduced aortic fatty streaks and hepatic lipid accumulation[86]. In clinical trials, testosterone administration increased coronary artery diameter and flow, improved cardiac ischemia, and reduced peripheral vascular resistance in chronic heart failure. This might be explained by testosterone being an L-calcium channel blocker and inducing potassium channel activation in vascular smooth muscle, though the precise mechanism of the action of testosterone on vessels has not been fully understood [63].

This study has several strengths. First, the follow-up duration is longer than most prior studies, which should add to the validity of our results since more observations contribute to a higher precision of our estimates. Second, the repeated measurement of testosterone levels enables us to investigate the association of time-varying testosterone level at each visit with the risk of cardiovascular events, which might be more relevant as compared to the baseline testosterone level. Limitations should also be noted. First, the study results are specific to the population studied and may not be generalizable. Second, because of the nature of observational study design, residual confounding cannot be entirely ruled out. Third, there are several types of strokes, among which ischemic stroke is more closely related to atherosclerosis and excess lipid accumulation that testosterone deficiency may contribute to. However, in this dataset, we did not have information on the specific type of stroke that the patient had, and it could be the reason why we did not find any association between testosterone and stroke in this study. Last, it is possible that no direct relationship exists between testosterone and cardiovascular risk, and that testosterone level is only a marker of illness and comorbidities, or that both low testosterone level and the development of cardiovascular disease may occur concurrently as part of the aging

process [80,81]. However, without direct measurements before and after treatment to various comorbidities, there is no clear way to assess these relationships.

In conclusion, in this cohort study with extended follow-up, we found lower time-varying testosterone level was associated with a higher risk of myocardial infarction, but not of stroke. We did not find any association between baseline testosterone level and cardiovascular risk. The associations between testosterone level and cardiovascular events were not different among patients with prior cardiovascular events and those without.

CHAPTER III

RECENT TESTOSTERONE DROP-OFF AND RISK OF CARDIOVASCULAR EVENTS

Introduction

Testosterone is the principal male sex hormone responsible for the maturation of sexual organs and secondary sexual characteristics. It is also an important metabolic hormone for maintaining the overall physiological function, including carbohydrate, protein, and lipid metabolism in men [55]. Low testosterone level has been consistently associated with risk factors for cardiovascular disease such as insulin resistance, hyperlipidemia, and metabolic syndrome. Animal models have demonstrated an anti-atherogenic action of testosterone in males, whereas testosterone deficiency promotes the early stages of atherogenesis, the first step of coronary heart disease [63,64]. Studies have also shown that low testosterone levels were associated with higher carotid intima media thickness [74–76], which is a surrogate marker of atherosclerosis and a strong predictor of future clinical ischemic cardiac and cerebrovascular events [77–79]. In a prospective cohort study of 3,443 community-dwelling men seventy years and older, Yeap *et al.* reported that testosterone in the lowest quartile (<11.7 nmol/L) was associated with an increased stroke or transient ischemic attack incidence (hazard ratio (HR)=1.69, 95%CI: 1.15, 2.48) [87].

In addition to the absolute testosterone level, remarkable changes in testosterone level may have an acute effect on disease development and progression. In the Testosterone in Older Men (TOM) trial, one of the earliest trials that investigated the effect of testosterone therapy on cardiovascular event incidence, the researchers reported cardiovascular events in the treatment group were associated with notable changes in serum testosterone levels; those that experienced events had a greater increase and substantial variations in testosterone levels compared to those

who did not. The sudden increase of testosterone levels above 17.34-34.67 nmol/L (target on-treatment range) in participants who already had a higher baseline risk of cardiovascular disease given their comorbidities may make them more susceptible to additional cardiovascular events, though the underlying mechanism remains unclear [88]. In animal studies, wild-type mice that went through castration experienced a pronounced testosterone decline within hours. Sudden testosterone declines induced macrophage infiltration and elevated inflammation biomarkers such as interleukin-6 (IL-6) and interleukin-1 beta (IL-1β), and lipid profiles such as cholesterol and triglyceride remarkably increased in the castrated mice as compared to the sham-operated mice. As a result, the castrated mice manifested exacerbated aortic aneurysm, a pathological phenotype of vascular aging, five weeks after castration [65].

Previous studies suggest that age-related testosterone decline rather than testosterone per se, plays a major role in the higher mortality rate observed in males [89]. Compared to a natural and slow decrease of testosterone levels during males' aging process, abnormally rapid declines or sudden drop-offs may result in various disease outcomes. St Sauver *et al.* found faster declines in testosterone level was associated with faster increases in prostate volume and lower urinary tract symptoms, and faster decreases in maximum flow rate [90]. Our recent study [91] investigating the dynamic patterns of testosterone on prostate cancer development found that hypogonadal men with a notable testosterone level decline during the study period had a higher risk of prostate cancer. Given the critical role that testosterone plays in maintaining the normal physiological function, it may also be important to know whether pronounced testosterone declines influence the risk of other adverse health outcomes such as cardiovascular disease, especially for those who already experienced testosterone deficiency that posed a baseline higher risk for many chronic conditions including cardiovascular disease. If notable testosterone declines are

confirmed to be associated with an increased risk of cardiovascular disease, monitoring of testosterone levels might be necessary to deliver timely interventions when pronounced drop-offs of testosterone are detected in order to maintain a normal metabolic function and prevent cardiovascular events in men.

In this study, we used a clinical dataset of 376 hypogonadal men whose testosterone levels were below 12.1 nmol/L at study entry from a registry study in Germany, and conducted survival data analyses to investigate the effect of sudden testosterone declines on the risk of cardiovascular events. Given the potential discrepancies in comorbidity conditions among patients with prior cardiovascular events and those without, all the analyses were stratified by patients' prior cardiovascular event status. Whether the effects of testosterone drop-offs on the risk of cardiovascular events were actually different among patients with prior cardiovascular events and those without was also examined.

Materials and Methods

Study Population

We used de-identified data from a registry study in Germany. Seven hundred and seventy-six hypogonadal men were recruited from one urology center in Bremerhaven, Germany from 2004 to 2016. Hypogonadism diagnosis was confirmed if they had total testosterone level≤12.1 nmol/L and symptoms such as decreased libido, erectile dysfunction, depression, and fatigue, as assessed by the Aging Males' Symptoms scale (AMS). The threshold of 12.1 nmol/L was selected based on clinical experience and confirmed by Bhasin *et al.* [83]. Ethical guidelines formulated by the German Ärztekammer (German Medical Association) for observational studies in patients receiving standard treatment were followed. After receiving an explanation about the nature and the purpose of the study, all patients provided written consent to be included

in the registry and have their data analyzed. Eligible participants without contraindications were given option of testosterone therapy at study entry. Four hundred of them decided to receive testosterone therapy, whereas three hundred and seventy-six opted against testosterone therapy for various reasons including the concern about the testosterone therapy safety issues. This study only used data from the participants who did not take treatment to avoid the influence of exogenous testosterone administration.

Testosterone Measurement and Cardiovascular Outcome Ascertainment

Participants were followed semi-annually for updates in serum testosterone levels and several other physical, laboratory, and imaging test results. Cardiovascular events (i.e., myocardial infarction and stroke) occurring during follow-up were recorded. Cardiovascular events were partly reported in the form of "physician letters" from the hospital or the cardiologist/neurologist/family physician, and partly by patients themselves or relatives. The latter usually occurred when already scheduled patient visits had to be postponed due to an event.

Statistical Analysis

In the descriptive analysis, characteristics were compared in the patients who experienced cardiovascular events during the follow-up period and those who did not.

A time-varying "testosterone drop" variable that is equal to the testosterone level measured at the last visit minus the testosterone level measured at the current visit was created. A positive value indicates the testosterone level declines since the last visit; a negative value indicates the testosterone level increases since the last visit. The larger the value of this variable, the larger change (drop/decline) this patient experienced between two adjacent visits.

Cox proportional hazards regression models were fitted to the data to investigate the association between testosterone drop since the last visit and the risk of cardiovascular events.

Time to event was the duration from study entry to the time when the cardiovascular event was recorded or the end of the study. Event is denoted 1 if a cardiovascular event was present during the follow-up period, or 0 if not (censored). Covariates that are closely related to the risk of cardiovascular risk including age at study entry, baseline smoking/drinking status, baseline comorbidities (type 2 diabetes, hypertension, dyslipidemia), and family history of coronary heart disease were included in the model. HR describing the risk of cardiovascular events and its 95% confidence interval (CI) was presented comparing (1) observations differing in 1 nmol/L testosterone drop; (2) observations of which testosterone drop was greater than or equal to the median to those of which testosterone drop was smaller than the median; (3) observations of which testosterone drop was greater than or equal to 75th percentile to those of which testosterone drop was smaller than 75th percentile; (4) observations of which testosterone drop was greater than or equal to 90th percentile to those of which testosterone drop was smaller than 90th percentile. We considered both negative and positive values of the testosterone drop variable for all the participants and all the visits when calculating the percentiles.

Analyses were stratified by participants' prior cardiovascular event status; those with prior cardiovascular events and those without prior cardiovascular events. The significance of the interaction between testosterone drop since the last visit and patients' prior cardiovascular event status was also tested to examine whether the effects of testosterone drop since the last visit were actually different in the two strata. Outcome variables were myocardial infarction, stroke, or any cardiovascular event (either myocardial infarction or stroke). Mean testosterone levels at each visit for patients who experience cardiovascular events during the study period and those who did not were presented separately. Testosterone changes over time for selected participants in each group were also depicted.

All analyses were performed with Stata/MP 14.0.

Results

As shown in **Table 6**, the baseline characteristics were in general different in the two groups.

Table 6 Characteristics of participants, by outcome status

Characteristics	Had CV events (n=79)		Did not have CV events (n=297)		*p*-value [a]
	Mean ± SD N (%)	Range	Mean ± SD N (%)	Range	
Age at study entry (years)	63.49 ± 4.75	49-73	64.06 ± 4.66	45-74	0.3456
Baseline testosterone (nmol/L)	9.49 ± 1.22	5.89-12.13	9.75 ± 1.12	5.89-11.79	0.0941
BMI (kg/m^2)	31.86 ± 4.77	23.20-46.02	29.66 ± 3.94	22.15-46.98	0.0003
Smoker	42 (53.16)	-	96 (32.32)	-	0.001
Alcohol user	50 (63.29)	-	135 (45.45)	-	0.005
Hypertension	68 (86.08)	-	216 (72.73)	-	0.014
Diabetes	46 (58.23)	-	107 (36.03)	-	<0.001
Dyslipidemia	55 (69.62)	-	179 (60.27)	-	0.128
Family history of coronary heart disease	37 (46.84)	-	62 (20.88)	-	<0.001
Prior CV event	34 (43.04)	-	73 (24.58)	-	0.001
Follow-up (years)	7.82 ± 1.51	4-10	7.35 ± 1.79	2-11	0.0181

[a] *p*-value for t-test if the variable is continuous; *p*-value for Chi-Square test if the variable is categorical
CV: cardiovascular
SD: standard deviation
BMI: body mass index

As compared to the participants who did not experience cardiovascular events, those who had cardiovascular events during the study period had higher BMI, were more likely to be a smoker and alcohol user, have hypertension, diabetes, family history of coronary heart disease, and prior cardiovascular events, and had longer follow-up (p-value<0.05). There was no

difference in baseline age, testosterone levels, and the proportions of patients with dyslipidemia in the two groups (p-value>0.05).

Table 7 Cox proportional hazards regression of cardiovascular events on testosterone drop, after adjustment, by prior cardiovascular event status

	Without prior CV events (N=269)			With prior CV events (N=107)		
	HR (95%CI)			HR (95%CI)		
	Any CV event	MI	Stroke	Any CV Event	MI	Stroke
T drop (continuous)	1.00 (0.76, 1.29)	1.11 (0.81, 1.53)	1.07 (0.74, 1.56)	1.26 (0.96, 1.67)	1.14 (0.79, 1.66)	1.28 (0.88, 1.87)
T drop≥1.39 [a]	1.33 (0.73, 2.43)	2.04 (0.99, 4.20)	1.05 (0.43, 2.53)	1.14 (0.59, 2.20)	0.95 (0.38, 2.37)	1.53 (0.66, 3.54)
T drop≥2.08 [b]	1.21 (0.66, 2.23)	1.78 (0.86, 3.67)	1.05 (0.43, 2.54)	1.30 (0.67, 2.45)	1.05 (0.42, 2.64)	1.76 (0.76, 4.04)
T drop≥3.12 [c]	1.34 (0.66, 2.72)	2.06 (0.94, 4.52)	1.00 (0.33, 2.98)	2.72 (1.37, 5.41)	1.87 (0.69, 5.08)	3.01 (1.22, 7.40)

[a] 50th percentile of testosterone drop (nmol/L)
[b] 75th percentile of testosterone drop (nmol/L)
[c] 90th percentile of testosterone drop (nmol/L)
CV: cardiovascular
HR: hazard ratio
CI: confidence interval
MI: myocardial infarction
Adjusting for age at study entry, BMI, smoking and drinking status, family history of coronary heart disease, and baseline comorbidity (hypertension, diabetes, dyslipidemia)

Table 7 presents the Cox regression analysis results, stratified by patients' prior cardiovascular event status. After adjusting for age at study entry, BMI, smoking and drinking status, family history of coronary heart disease, and baseline comorbidity (hypertension, diabetes, dyslipidemia), participants who experienced greater testosterone drop-offs in general had a higher risk of cardiovascular events, with HRs ranging from 0.95 to 3.01. For participants with prior cardiovascular events, those whose testosterone drop since the last visit was greater or equal to 3.12 nmol/L (90th percentile) had a 2.72 (95%CI: 1.37, 5.41) times higher risk of any cardiovascular event and 3.01 (95%CI: 1.22, 7.40) times higher risk of stroke. For other HRs,

although a higher risk of cardiovascular events was observed in patients with larger testosterone drop-offs, those ratios were not different from 1 (p-value>0.05).

Table 8 Cox proportional hazards regression of cardiovascular events on testosterone drop, after adjustment

	Total (N=376)		
	HR (95%CI)		
	Any CV event	**MI**	**Stroke**
T drop (continuous)	1.11 (0.93, 1.33)	1.09 (0.82, 1.47)	1.15 (0.90, 1.47)
T drop≥1.39 [a]	1.29 (0.83, 2.00)	1.58 (0.90, 2.77)	1.29 (0.71, 2.36)
T drop≥2.08 [b]	1.29 (0.83, 2.01)	1.52 (0.86, 2.67)	1.39 (0.76, 2.54)
T drop≥3.12 [c]	1.93 (1.19, 3.11)	2.09 (1.14, 3.86)	1.71 (0.87, 3.33)

[a] 50th percentile of testosterone drop (nmol/L)
[b] 75th percentile of testosterone drop (nmol/L)
[c] 90th percentile of testosterone drop (nmol/L)
CV: cardiovascular
HR: hazard ratio
CI: confidence interval
MI: myocardial infarction
Adjusting for age at study entry, BMI, smoking and drinking status, family history of coronary heart disease, and baseline comorbidity (hypertension, diabetes, dyslipidemia), and prior cardiovascular event status

Though the stratum-specific HRs were slightly different among patients with prior cardiovascular events and those without, the interactions between testosterone declines since the last visit and patients' prior cardiovascular event status were not different from 0 (p>0.05, results not shown), indicating the effects of testosterone drop on the risk of cardiovascular events were not different in the two strata, based on formal statistical tests. Thus, a collapsed table describing this association, regardless of the patients' prior cardiovascular event status was presented.

As shown in **Table 8**, patients whose testosterone decline since the last visit was greater or equal to 3.12 nmol/L (90th percentile) had a 1.93 (95%CI: 1.19, 3.11) times higher risk of any cardiovascular event and 2.09 (95%CI: 1.14, 3.86) times higher risk of myocardial infarction,

after adjustment. A potential dose-response relationship between testosterone decline since the last visit and risk of cardiovascular events was observed; patients with higher testosterone declines since the last visit had a higher risk of cardiovascular events. This result matched our *post hoc* analysis results using stratified proportional hazards models (results not shown).

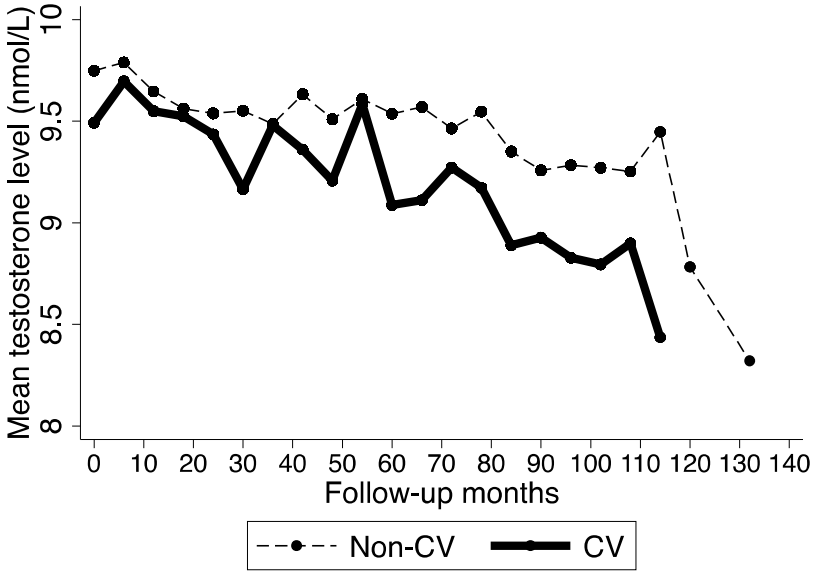

Figure 1 Mean testosterone levels over time, by outcome status

Figure 1 depicts the mean testosterone levels during the follow-up period in the patients who experienced cardiovascular events and those who did not. Both cardiovascular cases and non-cases had decreasing testosterone levels during the study period, as no exogenous testosterone was administrated to these patients. As compared to patients who did not have cardiovascular events, those who developed events had more variable changes in testosterone levels and overall "steeper" slope for the decreasing trend. There were also notable drop-offs

observed around month 30, 60, and 110 for cases, and post 110 months for non-cases. Data on later follow-up periods beyond 110 months might be less precise due to the limited number of observations (n=18).

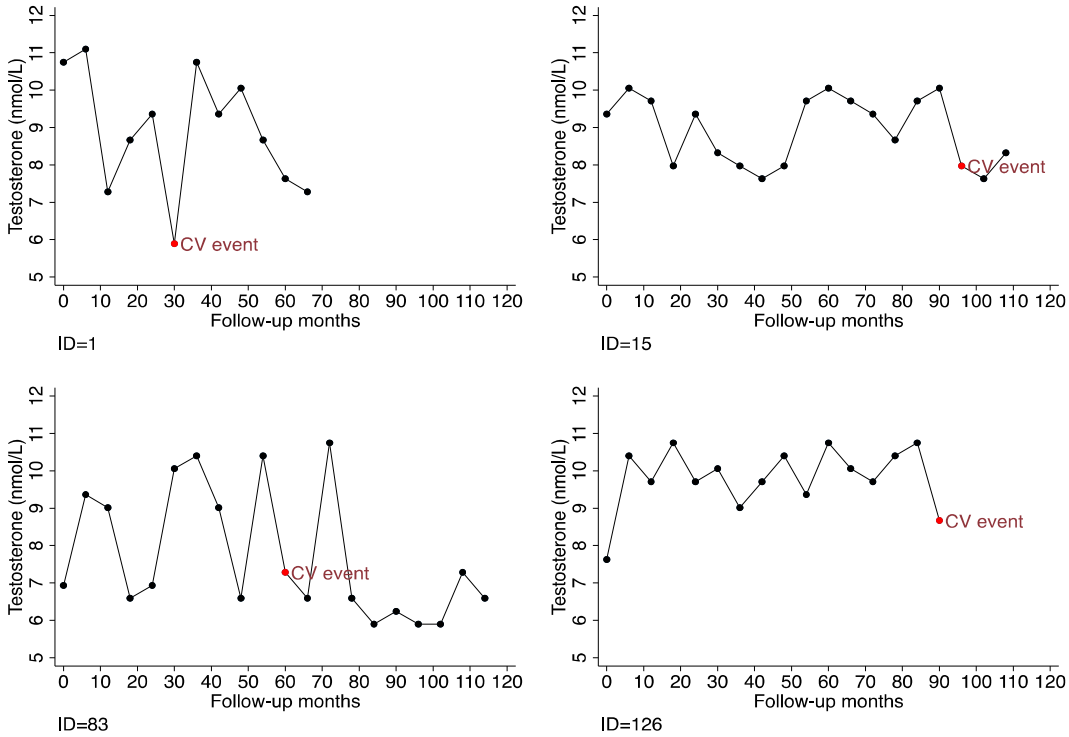

Figure 2 Testosterone changes over time for selected patients who experienced CV events during the study period

Figure 2 depicts the testosterone changes over time for selected patients who experienced cardiovascular events during the study period. They all experienced pronounced testosterone drop-offs before the event. For comparison, **Figure 3** shows the testosterone changes for selected patients who did not experience cardiovascular events during the study period. Though the overall trend was decreasing, they did not have remarkable testosterone declines.

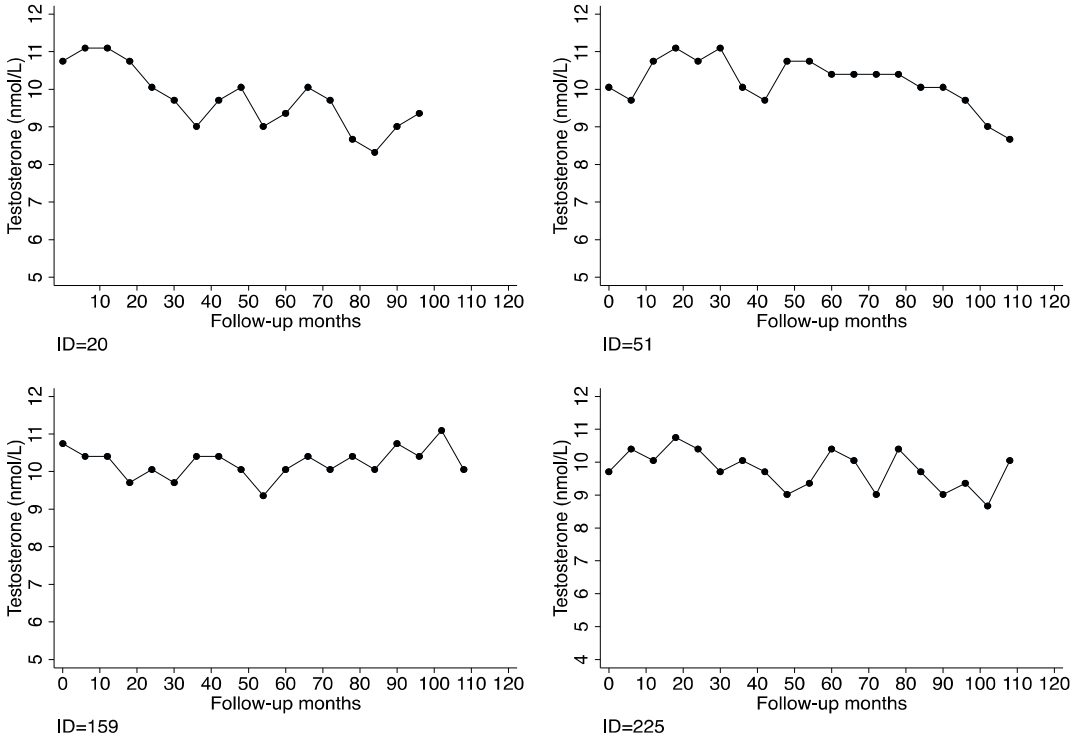

Figure 3 Testosterone changes over time for selected patients who did not experience CV events during the study period

Discussion

In this cohort study, we explored the association between recent testosterone drop-offs and the risk of cardiovascular events, stratified by patients' prior cardiovascular event status. We found the effects of testosterone declines since the last visit on the risk of cardiovascular events were not different among patients with prior cardiovascular events and those without, based on formal statistical tests. Regardless of patients' prior cardiovascular event status, patients with larger testosterone declines (≥3.12 nmol/L, 90th percentile) since the last visit were more likely to experience myocardial infarction, but not stroke.

In contrast to women, who have a sudden cessation of gonadal function and pronounced sex hormone changes around menopause [59], men typically experience a slow reduction in male hypothalamic-pituitary-gonadal axis function during the aging process, which results in a mild and gradual decline in testosterone levels through both central (hypothalamic-pituitary level, alterations in gonadotropin secretion) and peripheral (gonadal level, failure of Leydig cell function in the testicle) mechanisms [92]. There is a natural decline of testosterone levels in men with advancing age, typically 1% - 3% per year [58]. On average, this change is small, and the levels remain within the normal range in most men; approximately 25% of men experience a progressive decline and are diagnosed of testosterone deficiency, or hypogonadism during their 60s or 70s [57], a condition of suppressed circulating testosterone levels and symptoms such as erection dysfunction and decreased libido. In addition to poor physical performance and increased frailty, low testosterone level has been associated with obesity, greater waist circumference, diabetes, hypertension, and lower level of high-density lipoprotein (HDL) cholesterol, which are strong predictors of future cardiovascular events [57,61]. Hypogonadism is common in men with metabolic syndrome and associated with poor glucose and lipid control, which are well-established risk factors for cardiovascular disease [93]. Researchers also found testosterone deficiency was associated with premature coronary artery disease in men ≤age 45 and higher carotid intimal thickness in middle-aged diabetic men [94,95]. Atherosclerotic plaques and endothelial dysfunction were more likely to be found in patients with low testosterone [57]. Additionally, testosterone deficiency has been associated with hepatic dysfunction and elevated liver enzyme levels that are associated with vascular inflammation reflecting rupture-prone vulnerable atherosclerotic plaques [96] and impaired coronary flow reserve [97].

Among patients who already have lower than normal testosterone levels, remarkable drop-offs may exacerbate the preexisting comorbidities and contribute to additional risk for subsequent cardiovascular events. Studies have shown that androgen-deprivation therapy, which aims to lower testosterone levels to castration levels using chemical (e.g., gonadotropin-releasing hormone (GnRH) antagonists) or surgical methods (bilateral orchiectomy) among advanced prostate cancer patients, causes a rapid decrease in testosterone and leads to detrimental cardiovascular adverse effects [98]. The sudden testosterone declines resulting from androgen-deprivation therapy have been associated with an increase in fat mass, lipid panel, glycosylated hemoglobin levels, and insulin resistance within the initial months of treatment [99,100]. The metabolic changes that create an atherogenic risk following the androgen-deprivation therapy might be due to sudden loss of androgen-mediated inhabitation of stem cell differentiation into adipocytes and an increase of pro-inflammatory cytokines [98,101]. Additionally, androgen deprivation therapy-associated QT prolongation and changes in cardiomyocyte contractility may also contribute to an increased cardiovascular risk [102,103]. Many of these changes and alterations were observed greater in older individuals, who have already experienced age-related testosterone declines and shown decreased compliance to changes during the aging process. This is consistent with our study findings that among hypogonadal men with low testosterone levels, sudden testosterone drop-offs were associated with an additional risk of cardiovascular events.

As compared to men free of prior cardiovascular events, those with prior cardiovascular history might be more vulnerable to cardiovascular events if exposed to risk factors, given the pathological changes of their blood vessels and overall worse health conditions. However, in our study, we did not find any statistical difference in the effect of testosterone drop-offs and the risk of cardiovascular events among patients with prior cardiovascular events and those without.

Previous studies that examined the association between the absolute testosterone level and the risk of cardiovascular events reported that there was no such association if their study participants were restricted to patients without prior cardiovascular events, and a positive association if their study participants were those with prior cardiovascular events [81,82,84]. However, the authors used different source populations, and therefore alternative explanations might account for the difference in their study findings other than patients' prior cardiovascular event status. In our study, we initially intended to investigate whether patients with prior cardiovascular history had a higher risk of cardiovascular events when experiencing notable testosterone declines as compared to those without, in order to provide evidence for future targeted intervention in the more susceptible population. However, due to the limited sample size in the stratified analyses, we were underpowered to detect the true difference if any. Future studies are needed to verify our study findings.

Notable drop-offs or large variations of testosterone levels not only increase the risk of cardiovascular events, but also relate to the development of other diseases. Researchers reported Parkinson's Disease-related nigrostriatal pathologies in young male mice induced by castration, a surgical or chemical procedure that removes testicles that leads to sudden testosterone drops. These pathologies were normalized and the locomotor activities got improved after testosterone supplementation [104]. In our recent study that investigated the dynamic patterns of testosterone level and risk of prostate cancer using the same data source, we found that among hypogonadal men, further declines or large variations of testosterone level over the study period were associated with an increased risk of prostate cancer, and those that had a younger age at hypogonadism diagnosis were at a higher risk of developing prostate cancer [91]. In the current study, we also performed a secondary analysis to determine whether testosterone drop-offs since

the last visit has an impact on the risk of prostate cancer, and the findings show that the HR for prostate cancer associated with each 1 nmol/L testosterone drop since the last visit was 1.72 (95%CI: 1.35, 2.19). In another study that examined the association between family history of prostate cancer and age-related declines of testosterone level using nationally representative National Health and Nutrition Examination Survey (NHANES) data, we found men with a family history of prostate cancer experienced a more pronounced drop-off in testosterone levels between early to middle adulthood, which may explain, at least in part, the excess prostate cancer risk associated with family history of prostate cancer [105]. Further investigation is needed before any firm conclusion can be made. If that is the case, regular monitoring of testosterone levels in men might be useful to detect abnormal drop-offs or variations and deliver timely interventions (e.g., testosterone therapy) to prevent adverse health outcomes such as prostate cancer or cardiovascular events.

To our knowledge, this study is the first to investigate the effect of recent testosterone drop-offs on the risk of cardiovascular events. It provides insights into the etiologic role of testosterone in the development of cardiovascular events. In addition to the absolute testosterone level, the abnormal decline of testosterone levels among patients who already had testosterone deficiency may pose an additional cardiovascular risk for them. However, limitations should be noted. First, the study population consisted of European men who consulted urologic problems in a private urology center in Germany, and therefore our results may not be generalized to men of other nationalities or ethnicities. In addition, because the study population already had low testosterone levels, our conclusion about the effect of testosterone drop-offs on the risk of cardiovascular events may only apply to hypogonadal men. Future rigorously designed prospective cohort studies in general men population with a diverse nationality/ethnicity makeup

and a wide range of testosterone levels are needed. Second, there are several types of strokes, among which ischemic stroke is more closely related to atherosclerosis and excess lipid accumulation that sudden testosterone declines may contribute to. However, in this dataset, we did not have information on the specific type of stroke that the patient had, and it could be the reason we did not find any association between testosterone drop-offs and stroke in this study. Third, the cut-off points to categorize testosterone drop-offs in the analyses were arbitrary, given the exploratory nature of this analysis. Future longitudinal studies are needed to verify the study findings.

In conclusion, recent pronounced testosterone drop-offs may affect the risk of cardiovascular events among hypogonadal men. The effects of testosterone drop since the last visit were the same among patients with prior cardiovascular events and those without. Future longitudinal studies in male populations with different characteristics from ours (e.g., testosterone range, race/ethnicity, nationality) are needed to confirm our exploratory study findings.

CHAPTER IV

TESTOSTERONE DEFICIENCY, LONG-TERM TESTOSTERONE THERAPY, AND INFLAMMATION

Introduction

Testosterone is the principal male sex hormone responsible for the maturation of sexual organs and secondary sexual characteristics. It is also an important metabolic hormone for maintaining the overall physiological function, including carbohydrate, protein, and lipid metabolism in men [55]. A deficiency of testosterone secretion from the Leydig cells is called male hypogonadism, which can be classified as primary hypogonadism (testicular failure due to radiation therapy, trauma, torsion-induced ischemia, or infection) and secondary hypogonadism (central suppression of the hypothalamus and pituitary) [57].

Previous studies have suggested that testosterone deficiency in hypogonadal men may have an influence on atherosclerosis, a chronic inflammatory process and a major pathway of cardiovascular disease through which many risk factors affect cardiovascular health [71]. Animal studies have demonstrated that testosterone deficiency was associated with elevated blood-brain-barrier permeability accompanied by up-regulation of inflammatory molecules [69], which may trigger arterial inflammation that causes arterial hyperplasia even when the traditional risk factors were absent [70]. In an experiment investigating the mechanism through which testosterone deficiency-induced endothelial dysfunction in rats, the authors found a higher level of interleukin-6 (IL-6), nuclear factor kappa B (NF-κB), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-1, and NADPH oxidase-4 in castrated rats compared to castrated rats supplemented with testosterone and sham rats (p-value<0.01), indicating elevated oxidative

stress and inflammation in the rats undergoing testosterone deficiency and potential beneficial effects of testosterone therapy in attenuating this result [72]. However, to our knowledge, studies using large nationally representative samples of human subjects to evaluate the association between testosterone deficiency and inflammation are scarce.

Although low testosterone level has been associated with atherosclerosis and various cardiovascular risk factors [57,61], controversies exist regarding the role of exogenous testosterone therapy, a treatment to improve serum testosterone levels, in preventing cardiovascular risk. A recent study reviewed seven systematic reviews that included a total of 94 randomized controlled trials and investigated the association between exogenous testosterone and the risk of cardiovascular events [68]. Six of the reviewed studies showed no association and one showed an increased risk of cardiovascular disease associated with exogenous testosterone. The authors concluded that because of limited sample sizes and short follow-up periods, these trials might have been underpowered to detect a true difference in cardiovascular risk between treatment and control groups. Large observational studies with long follow-up periods are needed to provide real-world evidence in determining the potential beneficial effect of testosterone therapy on cardiovascular disease. Besides, whether testosterone therapy affects the cardiovascular risk by alleviating inflammation, the major pathway of cardiovascular disease, is also worth exploring.

Recent evidence has confirmed that multiple inflammatory biomarkers were elevated years before the first cardiovascular event and were highly predictive of future cardiovascular risk in otherwise health populations [71]. Of these biomarkers, C-reaction protein (C-RP), known as a systemic inflammatory biomarker, has become a standard predictor for cardiovascular risk due to its ease of measurement and abundance of clinical data. C-RP is one of the best measures of the acute-phase response to inflammation, allowing for the assessment of the effect of

testosterone deficiency and testosterone therapy on inflammation. Additionally, liver enzymes have recently been demonstrated as emerging risk factors for cardiovascular diseases. By using F-fluorodeoxyglucose positron emission tomography, researchers have suggested that abnormally elevated liver enzyme levels were associated with hepatic and vascular inflammation that reflects rupture-prone vulnerable atherosclerotic plaques [96] and impaired coronary flow reserve [97], making liver enzymes, which are low-cost, sensitive, and routinely measured for patients in clinical practice, another potential marker to help assess the effect of testosterone depletion and testosterone therapy on inflammation.

In this proposed study, we conducted a cross-sectional study using the recently released 2015-2016 National Health and Nutrition Examination Survey (NHANES) data to examine the association between testosterone deficiency and inflammation biomarkers including high sensitivity C-RP, liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the US general population. We also conducted a longitudinal study to investigate the effect of long-term testosterone therapy on inflammation biomarkers and the risk of cardiovascular events using data based on a registry study in Germany. We hypothesized (1) testosterone deficiency was associated with elevated inflammation biomarkers; (2) long-term testosterone therapy decreased levels of inflammation biomarkers and the risk of cardiovascular events.

Materials and Methods

A Cross-Sectional Study Using NHANES Data

Study Population

To examine the cross-sectional association between testosterone deficiency and inflammation biomarkers, we used data from the NHANES 2015-2016 cycle. NHANES is a

national survey that is conducted every two years to assess the population's health and nutrition status in the US through interviews, laboratory tests, and physical examinations. The sampling method for this nationwide survey is based on a stratified multistage probability design. The samples are weighted to represent the total US civilian, non-institutionalized population [106]. The NHANES 2015-2016 was approved by the Centers for Disease Control and Prevention (CDC) Institutional Review Board. Written informed consent was obtained from participants. This study was limited to male adults only because the relationship between testosterone and inflammation biomarkers may be different during puberty when hormone levels are not stable [107]. Participants who reported hepatitis (n=103) or liver cancer (n=2) in their medical history, or had extreme levels of testosterone, inflammation biomarkers, and total cholesterol levels (testosterone>50 nmol/L, n=12; C-RP>100 mg/L, n=292; ALT>200 U/L, n=6; AST>200 U/L, n=2; total cholesterol>400 mg/dL, n=3) were excluded for the concern about the underlying disease condition or treatment status that may modify the associations of interest. A total of 2,466 male adults were included in the study.

Serum Testosterone, High-Sensitivity C-RP, ALT, and AST Levels

Data on serum testosterone, high-sensitivity C-RP, ALT and AST levels were obtained from the NHANES Laboratory File. Details on the laboratory data collection and analysis were described elsewhere [108]. In brief, serum specimens were collected, stored, and shipped to the Collaborative Laboratory Services for analysis. Testosterone was performed via Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry (ID-GC/MS) method. The lower detection limit was 0.026 nmol/L [109]. High-Sensitivity C-RP was measured on the Beckman Coulter UniCel DxC 600 Synchron and the Beckman Coulter UniCel 660i Synchron Access chemistry analyzers. The lower detection limit for High-Sensitivity C-RP was 0.11 mg/L [110]. The Beckman

Coulter UniCel DxC 800 Synchron was used to measure ALT and AST. The DxC800 used a kinetic rate method to measure ALT activity and an enzymatic rate method to measure the AST activity in serum. The lower detection limit for both ALT and AST was 5 U/L [111,112]. In the original laboratory data files, entries below the lower detection limit were replaced by the value of the lower detection limit divided by the squared root of 2. In our study population, the testosterone, ALT and AST levels were all above the lower detection limit, and 150 participants had high-sensitivity C-RP below the lower detection limit.

Covariates

Testosterone deficiency as the main variable of interest was defined as serum testosterone level≤12.1 nmol/L. The threshold of 12.1 nmol/L was selected based on clinical experience and confirmed by Bhasin *et al.* [83]. Age, race, comorbidity condition, and smoking and drinking status that are related to the inflammation biomarkers were included in the model as confounders. Age (years), race/ethnicity (Non-Hispanic White, Non-Hispanic Black, Hispanic, Other), ever diagnosed with coronary heart disease (yes/no), diabetes (yes/no), and hypertension (yes/no) were self-reported during the NHANES interview and extracted from the NHANES Questionnaire File. Body mass index (BMI) was categorized into four categories (Underweight: <18.5 kg/m^2; Normal: 18.5-<25 kg/m^2; Overweight: 25-<30 kg/m^2: Obese: ≥30 kg/m^2), using the BMI data from the NHANES Examination File. Alcohol consumption was categorized into two categories (≥5 drinks every day or not), using the alcohol use data from the NHANES Questionnaire File. Smoking status was categorized into two categories (active smoker (serum cotinine>10ng/mL [113]) or not), using the serum cotinine level data from the NHANES Laboratory File. Total cholesterol was categorized into three categories (Normal: <200 mg/dL;

Borderline High: 200-239 mg/dL; High: ≥240 mg/dL) [114], using the cholesterol data from the NHANES Laboratory File.

Statistical Analysis

Descriptive analyses comparing the characteristics in the testosterone deficiency group and non-testosterone deficiency group using t-tests and Chi-Square tests were conducted. For continuous variables, means and standard deviations were presented. For categorical variables, counts and proportions were presented.

Multiple linear regression models were fitted to the data to assess the association between testosterone deficiency and inflammation biomarker levels. Changes of inflammation biomarkers associated with testosterone deficiency and 95% confidence intervals (CIs) were presented. Multiple logistic regression models were also fitted to the data to examine the association between testosterone deficiency and the odds of having elevated inflammation biomarkers. Dichotomization cutoff points for C-RP (High: C-RP≥3 mg/L; Normal: C-RP<3 mg/L), ALT (High: ALT>40 U/L; Normal: ALT≤40 U/L), and AST (High: AST>40 U/L; Normal: AST≤40 U/L) were based on prior literature and clinical experience [106,115]. Odds ratios (ORs) for elevated inflammation biomarkers associated with testosterone deficiency and 95%CIs were presented.

All analyses were performed with Stata/MP 14.0.

A Longitudinal Study Using Data Based on a Registry Study in Germany

Study Population

To investigate the longitudinal effect of testosterone therapy on inflammation, we used de-identified data from a registry study in Germany that contain participants' treatment status and repeated measurement of testosterone and inflammation biomarkers. Seven hundred and seventy-six hypogonadal men were recruited from one urology center in Bremerhaven, Germany

from 2004 to 2016. Hypogonadism diagnosis was confirmed if they had total testosterone level≤12.1 nmol/L and symptoms such as decreased libido and erectile dysfunction, as assessed by the Aging Males' Symptoms scale (AMS). The threshold of 12.1 nmol/L was selected based on clinical experience and confirmed by Bhasin *et al.* [83]. Ethical guidelines formulated by the German Ärztekammer (German Medical Association) for observational studies in patients receiving standard treatment were followed. After receiving an explanation about the nature and the purpose of the study, all patients provided written consent to be included in the registry and have their data analyzed. Participants were followed semi-annually for updates in serum testosterone level (nmol/L), C-RP (mg/L), ALT (U/L), AST (U/L), and several other physical, laboratory, and imaging test results. We did not dichotomize the outcome variables, as in that case we were not able to capture the potential treatment effect on alleviating inflammation if inflammation biomarker levels had not yet been reduced to normal range.

Cardiovascular Outcome Ascertainment

Cardiovascular events (i.e., myocardial infarction and stroke) occurring during follow-up were recorded. Cardiovascular events were partly reported in the form of "physician letters" from the hospital or the cardiologist/neurologist/family physician, and partly by patients themselves or relatives. The latter usually occurred when already scheduled patient visits had to be postponed due to an event.

Treatment Assignment

Patients with prostate-specific antigen (PSA) levels less than 4 ng/mL were given the option of testosterone therapy at the beginning of the study. However, the final decision on whether or not to receive testosterone therapy was based on the patients' own choice. Patients who decided to take testosterone therapy were classified as the treatment group (n=400), and

those who opted against testosterone therapy were classified as the control group (n=376). As described previously [55,116], patients on testosterone therapy received injections of 1,000 mg of testosterone undecanoate with the second injection 6 weeks after the first injection, followed by injections at 12-week intervals throughout the observation time. Since every injection was administered and documented in the urology office, the adherence to testosterone therapy was 100%. No participants changed their treatment status during the study period.

Statistical Analysis

In the descriptive analysis, baseline testosterone, C-RP, AST, ALT levels, and other characteristics were compared in the treatment and control groups using t-tests or Chi-square tests. Mean testosterone, C-RP, ALT, AST levels were plotted to visually compare the changes in the treatment and control groups.

Cardiovascular event (myocardial infarction and stroke) incidence rates in the treatment and control groups were compared using an incidence rate difference and its 95%CI.

Linear mixed-effect models with a random intercept, a random slope for time (month), and fixed effects of treatment, time (month), and an interaction between treatment and time (month) were fitted to the data to investigate the longitudinal effect of testosterone therapy on C-RP, ALT, and AST. Covariates that are closely related to C-RP, ALT, and AST including age, BMI, smoking/drinking status, comorbidities (type 2 diabetes, hypertension, dyslipidemia), and coronary heart disease were included in the model.

All analyses were performed with Stata/MP 14.0.

Results

Results for the Cross-Sectional Study Using NHANES Data

As showed in **Table 9**, characteristics were different between the testosterone deficiency group and the non-testosterone deficiency group.

Table 9 Characteristics of participants by testosterone deficiency status, NHANES 2015-2016

	TD (N=982)	Non-TD (N=1484)	
	N (% [a])	N (% [a])	*p*-value [b]
Age (Mean ± SD; years)	48.93±16.80	44.92±17.52	0.0109
Race/Ethnicity			
Non-Hispanic White	350 (64.6)	500 (64.4)	0.2868
Non-Hispanic Black	172 (8.9)	321 (10.9)	
Hispanic	299 (16.3)	423 (15.8)	
Other	161 (10.2)	240 (8.9)	
BMI (kg/m^2)			
<18.5 (Underweight)	3 (0.1)	30 (2.1)	<0.0001
18.5-<24 (Normal)	133 (11.3)	519 (32.8)	
24-<30 (Overweight)	324 (31.3)	560 (38.2)	
≥30 (Obese)	506 (56.0)	362 (25.6)	
Active smoker	237 (24.5)	477 (31.4)	0.0029
≥5 drinks per day	204 (20.7)	269 (18.7)	0.3360
Hypertension	346 (31.3)	316 (19.2)	0.0001
Diabetes	225 (16.4)	164 (9.2)	0.0003
Cholesterol (mg/dL)			
<200	658 (64.6)	997 (66.7)	0.1928
200-239	218 (22.6)	337 (24.0)	
≥240	106 (12.8)	150 (9.3)	
Coronary Heart Disease	73 (5.4)	57 (3.4)	0.0522

[a] Numbers may not add up to total because of missing values.
[b] *p*-value for t-test if the variable is continuous; *p*-value for Chi-Square test if the variable is categorical
NHANES: National Health and Nutrition Examination Survey
TD: testosterone deficiency
SD: standard deviation
BMI: body mass index
Active smoker: serum cotinine level>10 ng/mL
Age, race/ethnicity, alcohol consumption, hypertension, diabetes, and coronary heart disease were self-reported by the participants during the survey. BMI was calculated based on height and weight measured in the mobile examination center during the survey. Total cholesterol levels and serum cotinine levels were measured during the survey.

As compared to the participants without testosterone deficiency, those with testosterone deficiency were older, more likely to be obese, none activate smokers, have diabetes and

hypertension (p-value<0.05). There was no difference in race/ethnicity, drinking status, cholesterol levels, and coronary heart disease status between the two groups (p-value>0.05).

Table 10 Multiple linear regression results by outcome, NHANES 2015-2016

	C-RP (mg/L)	ALT (U/L)	AST (U/L)
	β (95%CI)	β (95%CI)	β (95%CI)
Testosterone deficiency	1.53 (0.93, 2.13)	2.17 (0.68, 3.66)	-0.05 (-1.18, 1.07)
Age (years)	0.02 (0.002, 0.04)	-0.19 (-0.23, -0.15)	-0.06 (-0.09, -0.02)
Race/Ethnicity			
Non-Hispanic White	Reference	Reference	Reference
Non-Hispanic Black	0.55 (-0.22, 1.32)	-3.38 (-5.30, -1.46)	-0.45 (-1.90, 1.00)
Hispanic	0.49 (-0.20, 1.18)	2.38 (0.67, 4.10)	0.24 (-1.06, 1.54)
Other	-0.47 (-1.36, 0.42)	-0.11 (-2.32, 2.11)	-0.66 (-2.33, 1.02)
BMI (kg/m^2)			
<24 (Underweight+Normal)	Reference	Reference	Reference
24-<30 (Overweight)	-0.43 (-1.16, 0.30)	3.93 (2.12, 5.73)	0.77 (-0.60, 2.13)
≥30 (Obese)	0.59 (-0.18, 1.36)	9.09 (7.18, 11.01)	1.72 (0.27, 3.16)
Active smoker	0.69 (0.07, 1.32)	-2.41 (-3.96, -0.85)	-1.74 (-2.91, -0.57)
≥5 drinks per day	1.13 (0.46, 1.80)	0.18 (-1.49, 1.84)	1.11 (-0.14, 2.37)
Hypertension	0.42 (-0.26, 1.11)	1.98 (0.27, 3.68)	2.08 (0.79, 3.37)
Diabetes	1.01 (0.19, 1.84)	-0.53 (-2.59, 1.52)	-0.51 (-2.06, 1.04)
Cholesterol (mg/dL)			
<200	Reference	Reference	Reference
200-239	-0.05 (-0.72, 0.62)	4.55 (2.88, 6.22)	2.01 (0.75, 3.27)
≥240	-0.30 (-1.21, 0.61)	7.93 (5.66, 10.20)	3.70 (1.99, 5.41)
Coronary Heart Disease	0.81(-0.45, 2.07)	-0.63 (-3.78, 2.51)	-0.23 (-2.61, 2.14)

NHANES: National Health and Nutrition Examination Survey
C-RP: C-reaction protein
ALT: alanine aminotransferase
AST: aspartate aminotransferase
CI: confidence interval
BMI: body mass index
Active smoker: serum cotinine level>10 ng/mL
Age, race/ethnicity, alcohol consumption, hypertension, diabetes, and coronary heart disease were self-reported by the participants during the survey. BMI was calculated based on height and weight measured in the mobile examination center during the survey. Total cholesterol levels and serum cotinine levels were measured during the survey.

The multiple linear regression results were presented in **Table 10**. Because of very few observations in the underweight BMI category (n=33), the underweight BMI category and

normal BMI category were collapsed in the analyses. As compared to men without testosterone deficiency, those with testosterone deficiency had 1.53 mg/L higher C-RP (p-value<0.001), 2.17 U/L higher ALT (p-value=0.004), and 0.05 U/L lower AST (p-value=0.926), after adjustment.

Table 11 Multiple logistic regression results by outcome, NHANES 2015-2016

	C-RP≥3mg/L	ALT>40U/L	AST>40U/L
	OR (95%CI)	OR (95%CI)	OR (95%CI)
Testosterone deficiency	1.81 (1.46, 2.24)	1.46 (1.10, 1.95)	0.99 (0.69, 1.42)
Age (years)	1.01 (1.00, 1.02)	0.96 (0.95, 0.98)	0.98 (0.97, 0.99)
Race/Ethnicity			
Non-Hispanic White	Reference	Reference	Reference
Non-Hispanic Black	1.29 (0.97, 1.72)	0.58 (0.38, 0.91)	0.85 (0.52, 1.39)
Hispanic	1.17 (0.91, 1.51)	1.37 (0.99, 1.89)	1.02 (0.68, 1.54)
Other	0.96 (0.67, 1.37)	1.12 (0.72, 1.74)	0.87 (0.49, 1.54)
BMI (kg/m^2)			
<24 (Underweight+Normal)	Reference	Reference	Reference
24-<30 (Overweight)	1.79 (1.31, 2.43)	2.24 (1.40, 3.58)	1.39 (0.83, 2.32)
≥30 (Obese)	4.14 (3.04, 5.64)	4.47 (2.82, 7.07)	1.92 (1.15, 3.19)
Active smoker	1.68 (1.33, 2.12)	0.70 (0.50, 0.96)	0.67 (0.45, 1.01)
≥5 drinks per day	1.40 (1.10, 1.77)	1.30 (0.93, 1.84)	1.53 (1.04, 2.24)
Hypertension	1.21 (0.95, 1.54)	1.55 (0.87, 2.77)	1.36 (0.90, 2.05)
Diabetes	1.42 (1.07, 1.89)	1.00 (0.65, 1.53)	1.33 (0.82, 2.15)
Cholesterol (mg/dL)			
<200	Reference	Reference	Reference
200-239	1.35 (1.05, 1.72)	1.93 (1.40, 2.65)	1.38 (0.92, 2.06)
≥240	1.12 (0.80, 1.57)	2.75 (1.88, 4.04)	1.89 (1.17, 3.07)
Coronary Heart Disease	0.99 (0.64, 1.53)	1.12 (0.54, 2.30)	1.12 (0.51, 2.48)

NHANES: National Health and Nutrition Examination Survey
C-RP: C-reaction protein
ALT: alanine aminotransferase
AST: aspartate aminotransferase
OR: odds ratio
CI: confidence interval
BMI: body mass index
Active smoker: serum cotinine level>10 ng/mL
Age, race/ethnicity, alcohol consumption, hypertension, diabetes, and coronary heart disease were self-reported by the participants during the survey. BMI was calculated based on height and weight measured in the mobile examination center during the survey. Total cholesterol levels and serum cotinine levels were measured during the survey.

Table 12 Characteristics of participants, by treatment status, German registry study

Characteristics	Control (n=376)		Treatment (n=400)		*p*-value [a]
	Mean ± SD N (%)	Range	Mean ± SD N (%)	Range	
Baseline					
Age at study entry (years)	63.94 ± 4.68	45-74	57.70 ± 7.40	33-71	<0.001
Testosterone (nmol/L)	9.70 ± 1.15	5.89-12.13	9.82 ± 1.24	5.89-12.13	0.147
C-RP (mg/L)	1.31 ± 1.24	0.1-7.8	5.26 ± 6.85	0.1-55.7	<0.001
ALT (U/L)	30.28 ± 8.01	19-69	40.60 ± 15.32	20-118	<0.001
AST (U/L)	26.18 ± 7.92	16-60	38.41 ± 15.51	15-98	<0.001
BMI (kg/m^2)	30.12 ± 4.22	22.15-46.98	33.12 ± 5.42	21.91-46.51	<0.001
Smoker	138 (36.70)	-	154 (38.50)	-	0.657
Alcohol user	186 (49.47)	-	135 (33.75)	-	<0.001
Hypertension	284 (75.53)	-	365 (91.25)	-	<0.001
Diabetes	153 (40.69)	-	133 (33.25)	-	<0.001
Dyslipidemia	234 (62.23)		400 (100.00)		<0.001
Prior CV event	107 (28.46)		73 (18.25)	-	0.001
Endpoint					
Myocardial infarction	50 (13.30)	-	0 (0)	-	<0.001
Stroke	46 (12.23)		0 (0)		<0.001
Follow-up (years)	7.25 ± 1.69	2-11	7.01 ± 2.75	1.25-10.75	0.067

[a] *p*-value for t-test if the variable is continuous; *p*-value for Chi-Square test if the variable is categorical.
SD: standard deviation
BMI: body mass index
C-RP: C-reaction protein
ALT: alanine aminotransferase
AST: aspartate aminotransferase
CV: cardiovascular

The multiple logistic regression results were presented in **Table 11**. After adjustment, the odds of having high-sensitivity C-RP≥3mg/L in participants with testosterone deficiency was 1.81 times that in those without testosterone deficiency (p-value<0.001); the odds of having ALT>40U/L in participants with testosterone deficiency was 1.46 times that in those without testosterone deficiency (p-value=0.009); the odds of having AST>40U/L in participants with

testosterone deficiency was 0.99 times that in those without testosterone deficiency (p-value=0.971).

Results for the Longitudinal Study Using Data Based on a Registry Study in Germany

As shown in **Table 12**, the total follow-up duration for the control group was between 2 and 11 years, and 1.25 to 10.75 years for the treatment group. There was no difference in the mean follow-up duration between the treatment and control groups (7.25 vs 7.01, p-value=0.067). The baseline characteristics were in general different in the two groups. As compared to the control group, men in the treatment group had higher BMI, C-RP, ALT, and AST, were more likely to have hypertension, and dyslipidemia, but on average were younger, had a lower proportion of alcohol users, diabetic patients, and patients with prior cardiovascular events (p-value<0.001). There was no difference in baseline testosterone levels and the proportions of smokers in the two groups.

Table 13 Incidence rate and incidence rate difference for myocardial infarction and stroke, German registry study

	Myocardial infarction			
	# of events	**Total person-month**	**Incidence rate**	**Incidence rate difference (95%CI)**
Treatment	0	33645	0	-0.0016
Control	50	30822	0.0016	(-0.0021, -0.0012)
	Stroke			
	# of events	**Total person-month**	**Incidence rate**	**Incidence rate difference (95%CI)**
Treatment	0	33645	0	-0.0015
Control	46	31278	0.0015	(-0.0019, -0.0010)

CI: confidence interval

Table 14 Linear mixed effect model results, by outcome, German registry study

	CRP (mg/L)	ALT (U/L)	AST (U/L)
Predictors	β (95%CI)	β (95%CI)	β (95%CI)
Treatment	2.93 (2.35, 3.52)	-5.59 (-7.06, -4.11)	-3.12 (-4.50, -1.75)
Month	0.003 (-0.001, 0.007)	0.157 (0.145, 0.170)	0.147 (0.136, 0.159)
Treatment*Month	-0.054 (-0.060, -0.047)	-0.299 (-0.317, -0.282)	-0.295 (-0.310, -0.279)
Age at study entry	0.018 (-0.021, 0.058)	0.08 (-0.02, 0.18)	0.09 (-0.002, 0.18)
BMI (kg/m^2)	-0.14 (-0.19, -0.08)	-0.07 (-0.21, 0.07)	-0.04 (-0.17, 0.09)
Smoker	-0.11 (-0.52, 0.30)	-0.20 (-1.36, 0.96)	-0.35 (-1.42, 0.73)
Alcohol user	-1.31 (-1.57, -1.04)	-0.67 (-1.60, 0.27)	-0.69 (-1.52, 0.15)
Type 2 diabetes	-0.15 (-0.68, 0.39)	-0.62 (-1.92, 0.68)	-0.52 (-1.74, 0.70)
Hypertension	0.33 (-0.22, 0.89)	0.08 (-1.49, 1.65)	0.09 (-1.36, 1.54)
Dyslipidemia	-0.11 (-0.39, 0.18)	4.34 (3.12, 5.55)	4.56 (3.50, 5.62)
Prior CV event	-0.06 (-0.64, 0.53)	-1.00 (-2.41, 0.41)	-0.59 (-1.91, 0.73)

C-RP: C-reaction protein
ALT: alanine aminotransferase
AST: aspartate aminotransferase
CI: confidence interval
BMI: body mass index
CV: cardiovascular

Table 13 presents the incidence rates and incidence rate difference in the treatment and control groups. All 50 myocardial infarctions and 46 strokes occurred in the control group, resulting in a lower incidence rate in the treatment group as compared to the control group (p-value<0.001).

Table 14 presents the longitudinal data analysis results using mixed-effect models. After adjustment, C-RP levels in the control group increased by 0.003 (95%CI: -0.001, 0.007) mg/L per month, while C-RP levels in the treatment group decreased by 0.05 (95%CI: -0.055, -0.046) mg/L per month; the variance of residual was 1.53, the variance of the random intercept was 11.77, the variance of month (slope) was 0.0015, and the covariance between the random intercept and month (slope) was -0.13; ALT levels in the control group increased by 0.157 U/L (95%CI: 0.145, 0.170) per month, while the ALT levels in the treatment group

decreased by 0.142 U/L (95%CI: -0.154, -0.130) per month; the variance of residual was 43.18, the variance of the random intercept was 71.57, the variance of month (slope) was 0.01, and the covariance between the random intercept and month (slope) was -0.72; AST levels in the control group increased by 0.147 (95%CI: 0.136, 0.159) U/L per month, while the AST levels in the treatment group decreased by 0.148 (95%CI: -0.158, -0.137) U/L per month; the variance of residual was 30.13, the variance of the random intercept was 62.23, the variance of month (slope) was 0.009, and the covariance between the random intercept and month (slope) was -0.62.

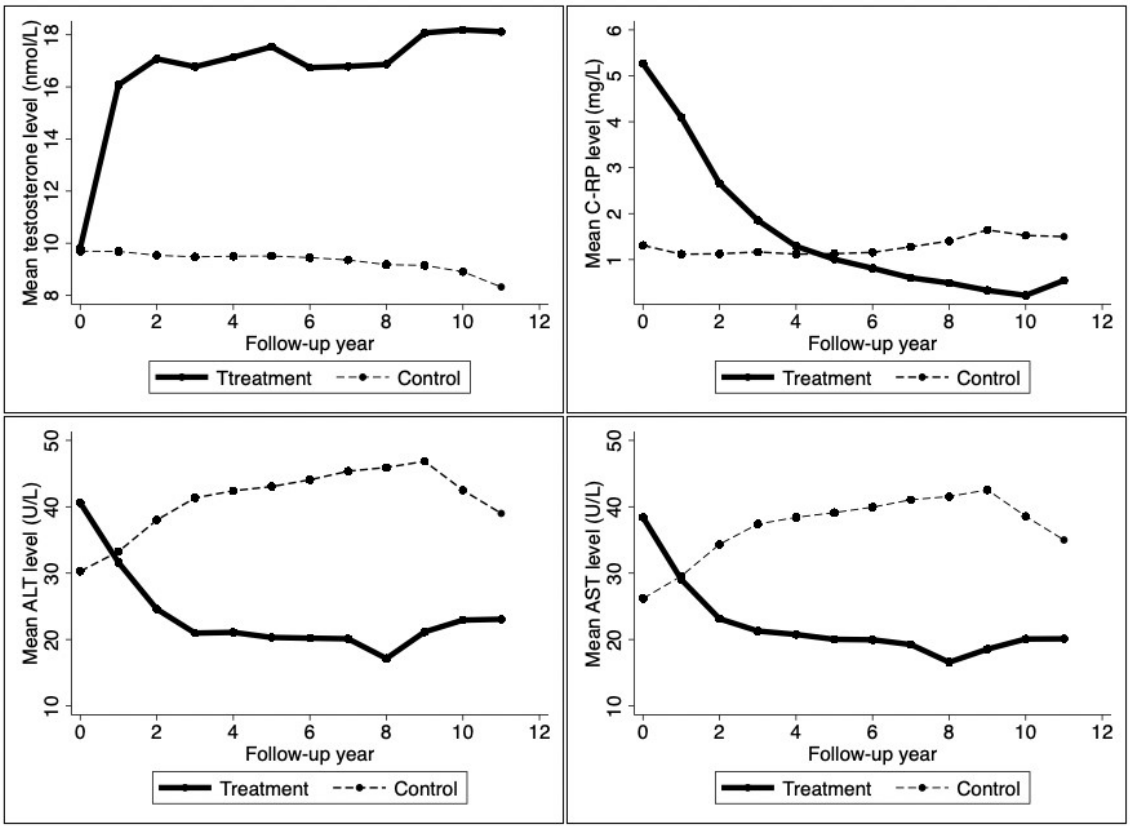

Figure 4 Mean testosterone, C-RP, ALT, and AST levels over time, by treatment status

Figures 4 depicts the changes of mean testosterone, C-RP, ALT, and AST levels in the two groups during the follow-up period with more fluctuations in the later years due to fewer number of observations. Over time, in the treatment group, testosterone level increased, and three inflammation biomarkers decreased, whereas in the control group, testosterone level decreased, and three inflammation biomarkers increased.

Discussion

In our cross-sectional study using the recently released NHANES 2015-2016 cycle data, we found participants with testosterone deficiency had higher levels of inflammation biomarkers including C-RP and ALT as compared to the participants without testosterone deficiency. In this longitudinal study using data from 776 hypogonadal men in a registry study in Germany, we found long-term testosterone therapy was associated with decreased C-RP, ALT, AST, and cardiovascular event incidence during the study period among hypogonadal men.

Very few studies have investigated the association between testosterone and C-RP levels using nationally representative samples. A cross-sectional study (n=809) using earlier survey cycles of NHANES data (1999-2004) examined the association of quintiles of testosterone levels with C-RP among males aged 20 and over. The authors found adult men who had total testosterone levels lower than 10.49 nmol/L (lowest quintile) had a 1.61 (95%CI: 1.00, 2.61) times higher odds of having C-RP≥3mg/L, compared to those in the higher quintiles[106]. Another cross-sectional study (n=1,490) using NHANES III (1988-1991) data to investigate the linear association between tertiles of testosterone level and C-RP, in men aged 18 years and over. The authors reported that compared to adult men who had testosterone level≤13.80 nmol/L (lowest tertile), the C-RP in those that had testosterone level>19.21nmol/L (highest tertile) was

0.16mg/L lower (95%CI: -0.29, -0.02) [107]. Though findings of these studies were consistent with ours, they did not investigate the effect of testosterone deficiency on C-RP.

By using the nationally representative samples, we also examined the association between testosterone deficiency and liver enzymes in the US general population, which has not been investigated before. We only identified a positive association of testosterone deficiency with elevated ALT, but not with AST. Studies have shown that testicular feminized mice with very low testosterone levels had increased lipid deposition in the liver [86], and low testosterone level was independently associated with non-alcoholic fatty liver disease [117], the hepatic component of the metabolic syndrome that is a strong predictor for cardiovascular disease [97,118]. Non-alcoholic fatty liver disease, which affects cardiovascular health in many ways including increased liver and chronic low-grade inflammation and reduced coronary flow reserve due to liver fibrosis, is characterized by hepatic steatosis and injured hepatocyte that may result in increased permeability of hepatic cell membrane and release of liver enzymes [97,119]. Liver enzymes such as aminotransferases ALT and AST are produced in the liver and used to synthesize glycogen, a stored form of glucose that provides energy for the body. Most glycogens are stored in the liver, and a few are distributed to other organs. Unlike ALT that is mainly found in the liver, AST is “scattered” in various organs including the liver, cardiac muscle, and skeletal muscles [120]. Damage of any of these tissues may lead to increased AST levels. Thus, AST may not be as specific to the liver as ALT; this could be one potential explanation that we did not observe a direct association between AST and testosterone deficiency that may lead to the pathological changes on the liver.

Inflammation is a pathophysiological process as well as a pathway to the development of many chronic inflammatory diseases such as diabetes and coronary artery disease [121]. C-RP and

liver enzymes are well-recognized inflammation biomarkers and emerging risk factors for cardiovascular disease [96,122]. In the Physician's Health Study, the risks of myocardial infarction and ischemic stroke were 2.9 (95%CI: 1.8, 4.6) and 1.9 (95%CI: 1.1, 3.3) times higher in the highest quartile as compared to those in the lowest quartile of the baseline C-RP levels [123]. Lee *et al.* reported a positive association between elevated ALT or AST and cardiovascular mortality in the Korean population, with adjusted hazard ratios (HRs) of 1.95 (95%CI: 1.07, 3.56) and 2.29 (95%CI: 1.27, 4.12) [124]. In a review study, Kunutsor *et al.* performed a meta-analysis of 29 cohort studies and reported pooled adjusted relative risks (RRs) of 1.23 (95%CI: 1.16, 1.29) and 1.08 (95%CI: 1.03, 1.14) for cardiovascular disease, associated with one-standard-deviation increase in log baseline gamma glutamyltransferase (GGT) and alkaline phosphatase (ALP) levels, two other liver enzymes commonly used for assessing liver dysfunction and inflammation. It was implied that interventions that suppress the inflammation responses and therefore decrease the levels of these inflammation biomarkers might be considered as a strategy to prevent cardiovascular events [125].

Testosterone has been reported to elicit anti-inflammatory effects in tissues and organs by regulating the function of the cellular components of the immune system (e.g., macrophages, neutrophils, mast cells) and reducing the release of pro-inflammatory cytokines (e.g., tumor necrosis factor-alpha (TNF-α), IL-6, IL-1β) that are linked to endothelial injury in vessels [121,126]. In an animal study investigating the effect of testosterone depletion and administration on abdominal aortic aneurysm formation (a pathological phenotype of vascular aging) in male mice, the authors found that for male mice under depletion of testosterone (castrated), exacerbated aortic aneurysm characterized with enhanced aortic diameter was formed as compared to sham-operated mice. They also conducted histological analyses that showed macrophages infiltrating

in the destroyed aorta and excessive expression of IL-6 and IL-1β, indicating that the formation of aortic aneurysm in the castrated male mice was probably a result of pronounced inflammation. They found after 9-week testosterone administration, the aortic expression of IL-6 and IL-1β was decreased, and the dilated aortic diameter was also ameliorated, indicating the anti-inflammatory actions of testosterone administration on vascular pathologies [65]. In another animal study, the authors reported testosterone therapy improved lipid accumulation in rabbits that were fed fat-rich diets. A decrease in liver and plasma TNF-α was also observed in these rabbits [127]. This implies testosterone therapy may ameliorate the liver inflammation resulting from abnormal lipid profiles due to fat-rich diet and eventually reduces cardiovascular events.

Few previous trials reported a protective effect of testosterone therapy on inflammation biomarkers in human subjects. In a crossover trial among 20 hypogonadal men who were diagnosed with type 2 diabetes, an inverse correlation was observed between C-RP and testosterone levels at baseline (r=-0.59, p-value=0.01), but no testosterone treatment effect was found on C-RP (0.55±0.57mg/L, p-value=0.35). Each patient had two treatment phases (testosterone therapy and placebo) of three months, and a washout period of one month [128]. Another double-blind randomized controlled trial assessed the effect of an exercise program (12 weeks), with and without testosterone therapy, on various outcomes such as high-sensitivity C-RP, in 41 male patients with testosterone deficiency and chronic heart failure. They found C-RP levels were mostly unchanged [129]. In a one-year follow-up study in Germany (n=88), the authors assessed the safety of testosterone therapy, and no elevation or reduction in liver enzymes was found. The treatment duration in these studies might be too short for testosterone therapy to have an effect on inflammation biomarkers. Besides, because of the fairly small sample sizes, it is also likely that these studies were underpowered to detect the changes in the outcomes. Unlike these

trials, the longer duration of treatment in our study, repeated measurement of inflammation biomarkers, and a larger sample size increase the study power to detect the potential longitudinal effect of testosterone therapy on inflammation and cardiovascular risk.

To our knowledge, this is the first study investigating the long-term treatment effect of testosterone therapy on liver enzymes and inflammation biomarkers using longitudinal data with up to eleven year's follow-up. The finding of this study provides insights into the potential pathways through which long-term testosterone therapy plays a role in preventing the risk of cardiovascular events. By using mixed-effect models, we were able to evaluate the testosterone treatment effect on predictors of cardiovascular disease with taking into account individual's variabilities. Besides, the cross-sectional study investigating the association between testosterone deficiency and inflammation biomarkers was conducted using large nationally representative samples. This not only increases the external validity of the study findings and also increases the study power to detect this potential association. However, limitations should be noted. First, the study results are specific to the population studied and may not be generalizable. Second, the treatment assignment was not randomized and may therefore be subject to the placement of generally healthier participants in the treatment group. Because there were zero cases in the treatment group, we were not able to get adjusted HR associated with treatment therapy using Cox proportional hazards models. Third, due to the nature of observational study design, residual confounding may still exist. However, after adjusting for major comorbidities and lifestyle characteristics, residual confounding alone is unlikely to explain the study findings.

In conclusion, by using nationally representative samples, we found a positive association between testosterone deficiency and inflammation biomarkers C-RP and ALT; by using clinical data based on a registry study in Germany, we found long-term testosterone therapy alleviated

inflammation, the major pathway of cardiovascular disease, which may explain in part how testosterone therapy reduced the risk of cardiovascular disease in our study population.

CHAPTER V

CONCLUSIONS

Summary

In this cohort study with up to eleven years of follow-up among 776 hypogonadal men aged from 31 to 74 years, we mainly investigated the association of time-varying testosterone level and testosterone level change since the last visit with the risk of cardiovascular events, stratified by patients' prior cardiovascular event status. To investigate the underlying mechanism through which testosterone deficiency affects the risk of cardiovascular events, we used the National Health and Nutrition Examination Survey (NHANES) data to examine the association between testosterone deficiency and inflammation biomarkers, a major pathway of cardiovascular disease. Additionally, we assessed the long-term effect of testosterone therapy, a treatment to improve serum testosterone level, on the risk of cardiovascular events and inflammation biomarkers over time.

We found lower time-varying testosterone level and greater testosterone declines since the last visit were associated with a higher risk of myocardial infarction. The association between testosterone and risk of cardiovascular events was not different among patients with prior cardiovascular events and those without. Testosterone deficiency was associated with higher levels of inflammation biomarkers including C-Reaction Protein (C-RP) and alanine aminotransferase (ALT); lower risk of cardiovascular events and a decrease in inflammation biomarkers were found in the testosterone therapy group.

Clinical Implications

Testosterone deficiency or recent testosterone declines should be considered when assessing cardiovascular risk in aging men. Patients should be informed of the potential adverse outcomes including cardiovascular events if low testosterone is detected during well visits. One single measurement of testosterone level may not be sufficient to evaluate one's cardiovascular risk, given the dynamic changes of testosterone levels over men's life course. Regular monitoring of testosterone levels in aging men might be useful to detect abnormal drop-offs or variations and deliver timely interventions to prevent adverse health outcomes such as cardiovascular events. Testosterone therapy may suppress the inflammation responses, one of the major cardiovascular disease pathways, and therefore decrease the levels of inflammation biomarkers, indicating its additional beneficial effect in addition to the alleviation of hypogonadal symptoms among hypogonadal men.

Future Direction

There are a number of gaps in our knowledge around testosterone, testosterone therapy, and cardiovascular disease that follow from our findings. It may be helpful to examine the effect of age at hypogonadism diagnosis on cardiovascular risk and whether a younger age at hypogonadism predicts an early onset of cardiovascular disease. Additionally, future large cohort studies with diverse race/ethnicity makeup will enable the researchers to examine whether testosterone deficiency is one of the contributing factors that account for the disproportionally high cardiovascular morbidity and mortality in certain race/ethnicity groups. Last but not least, it may be worth exploring whether restoring testosterone to different levels (above/in/below normal range) via testosterone therapy would make any difference in affecting cardiovascular event occurrence or recurrence, especially whether there is any adverse effect when it goes

beyond the normal range. Treatment is not always beneficial unless it is properly used (i.e., clear indication, timing). Off-label testosterone descriptions should be avoided. Safety issues should always be considered.

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