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FINASTERIDE LOWERS CHOLESTEROL IN HUMANS AND DELAYS
ATHEROSCLEROSIS PROGRESSION IN *LDLR*^{-/-} MICE

BY

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THESIS

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Abstract

Plasma cholesterol and sex hormones are strong risk factors associated with atherosclerosis, the leading cause of death worldwide. The objective of this study was to determine the role of finasteride, a drug that inhibits the activation of testosterone to dihydrotestosterone, on plasma lipid profile and atherogenesis. For our clinical study, we applied regression models to examine associations between circulating lipid profile and finasteride using the National Health and Nutrition Examination Survey database. After controlling for covariates, we observed a significant reduction in total cholesterol levels in subjects taking finasteride.

In our preclinical model, we tested the effect of finasteride in atheroprone low-density lipoprotein receptor-deficient (*Ldlr*^{-/-}) mice fed increasing doses of finasteride for 12 weeks. Finasteride reduced circulating cholesterol levels and resulted in a reduction in lesion size, necrotic core area, and immune cell content. Global transcriptome analysis of liver showed an up-regulation of bile acid biosynthesis and oxidative phosphorylation, accompanied by a reduction in inflammatory markers. Our results show that finasteride is associated with a reduction in plasma cholesterol in both human and mice and highlight the potential role of this drug in the reduction of atherosclerosis.

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Chapter 1

Literature Review

Cardiovascular diseases: overview and prevalence

Cardiovascular diseases (CVDs) are the main cause of death worldwide, including the United States, where according to 2015 update by the American Heart Association, over 85.6 million people are currently living with some type of CVD. (Mozaffarian et al., 2015) CVDs are present in many forms including stroke, heart attack, deep vein thrombosis, and cardiomyopathy. (Flora et al., 2019) Shockingly, 80% of deaths caused by CVD are thought to be preventable through a combination of lifestyle changes such as increased physical activity, cessation of smoking, amelioration of stress, and others (Figure 1.1). (Buttar et al., 2005) Effective pharmacological interventions are routinely used as well. (Salvo et al., 2017) Typically, these interventions aim to reduce circulating cholesterol and blood pressure, and to lesser degree, other parameters such as circulating triglycerides and glucose levels, and obesity. Among these factors, plasma cholesterol remains one of the best clinical predictors of atherosclerosis, the main underlying cause of CVD. (Figure 1.1)

Role of hepatic lipoproteins on the initiation of atherosclerosis

Atherosclerosis is a progressive disease driven by the excessive accumulation of pro-atherogenic lipoproteins in plasma, known as very-low density lipoprotein (VLDL) and low-density lipoprotein (LDL). VLDL are produced by the liver and are characterized by the presence of a single molecule of apolipoprotein B100 (apoB100). Circulating VLDL delivers hepatic triglycerides to extrahepatic tissues slowly becoming LDL, which is smaller in size, enriched in cholesterol, and is mainly responsible for cholesterol delivery to extrahepatic tissues.

When the balance of VLDL secretion/utilization is altered, pro-atherogenic lipoproteins can accumulate in the blood, and transmigrate across the endothelial layer of the arterial wall. Once in the intima layer, these particles tend to suffer physicochemical modifications, acting as chemoattractant cues for circulating monocytes. As a result, monocytes infiltrate into the intima and differentiate to macrophages to engulf modified lipids, becoming cholesterol-laden foam cells. (Moore et al., 2013)

The liver also secretes apolipoprotein A1, which contributes to reverse cholesterol transport by collecting this lipid from plasma membranes from extrahepatic tissues, becoming high-density lipoprotein (HDL). HDL cholesterol (HDL-C) returns to the liver where it is internalized into the hepatocyte by interacting with the scavenger receptor 1B. Once in the liver, extrahepatic cholesterol can be eliminated by its conversion to bile acids. Several factors such as exercise and smoking regulate HDL levels, although recent research show that HDL function, rather than HDL-C levels are a better predictor of the incidence of CVD. (Ouimet et al., 2019)

Role of immune system in plaque formation and composition

The innate and adaptive immune system plays a key role in the development of atherosclerosis. Several cell types such as monocytes, pro-inflammatory macrophages, T lymphocytes, neutrophils, and B lymphocytes are involved in the development of the disease (Moriya, 2019). However, macrophages are still considered the main cell type involved in atherosclerosis progression. Monocytes are the precursor of macrophages, which are long-lived resident immune cells that are part of the mononuclear phagocytic family. For simplicity, macrophages are typically considered pro-inflammatory or M1 or anti-inflammatory or M2. (Murray et al., 2014) M1 cells secrete pro-inflammatory factors, showing a strong

phagocytic/cytotoxic phenotype. M2 macrophages release anti-inflammatory mediators and are involved in tissue remodeling. Developing atherosclerotic lesions, also known as plaques, are typically enriched in M1-like cells that secrete pro-inflammatory factors, contribute to monocyte recruitment/macrophage differentiation, and lesion growth.

While some studies show that plaque macrophages are predominantly generated due to local proliferation, (Robbins et al, 2013) it is widely accepted that many lesion macrophages are monocyte-derived macrophages. Human monocytes are classified according to the content of CD14 and CD16 (Ziegler-Hetbrock et al., 2010) but with new techniques such as multicolor flow cytometry, research has been able to discriminate more subtypes of these cells. Like macrophages, murine monocytes are divided into two subtypes that reflect the expression of the lymphocyte antigen six complex (Ly6C). Again, for simplicity, Ly6Chi monocytes, which express higher levels of C-C motif chemokine receptor 2 (CCR2), are proinflammatory and have been identified as precursors for M1 macrophages. On the other hand, Ly6Clo monocytes have lower expression of CCR2 and are anti-inflammatory. (Shi et al., 2011)

Besides macrophages, other cell types and extracellular components such as collagen, cholesterol crystals, and calcium deposits compose atherosclerotic lesions. Over time, the microenvironment of larger plaques can become anoxic and toxic for cells, resulting in the formation of necrotic cores. These structures are constituted by lipid-rich acellular regions that attract macrophages. (Bentzon et al., 2014) The presence and size of necrotic cores correlate with increased incidence of CVD in human, as they directly contribute to plaque vulnerability and rupture. (Ohayon et al., 2008)

Contribution of hypercholesterolemia to myelopoiesis and atherosclerosis

Several factors such as stress and diabetes contribute to monocyte proliferation. Additionally, hypercholesterolemia promotes monocytosis by promoting myelopoiesis in the bone marrow and the spleen. (Nagareddy et al., 2013) Similarly, defective reverse cholesterol transport, favors hematopoiesis and monocytosis, as revealed by studies employing experimental models in which HDL receptors in myeloid cells were absent or circulating apoA-I genetically eliminated. (Yvan-Charvet et al., 2010)

Preclinical studies recently reviewed in Current Opinion in Lipidology show that the accumulation of cholesterol in the plasma membrane of hematopoietic cells promotes cell proliferation, which can be inhibited by providing cholesterol acceptors, like HDL and apoA-I, to reverse hypercholesterolemia-mediated hematopoiesis. (Amengual et al., 2019) A recent study showed that the sterol regulatory element-binding protein 2, which is mobilized in response to intracellular cholesterol, regulates myelopoiesis expansion by activating proliferation pathways in zebrafish. (Gu et al., 2019) Overall, these data highlight the importance of cholesterol and monocyte circulating levels as crucial drivers of the pathogenesis of atherosclerosis, and therefore, CVD development.

Role of Biological Sex and Sex Hormones on the Development of Atherosclerosis

Epidemiological studies highlight the effect of biological sex on the risk of CVD. Men are statistically more likely to experience heart attacks and strokes than premenopausal women, which contributes to a significant decrease in lifespan in males (78) when compared to females (83) in the United States (Rodgers et al., 2019). The physiological differences between males and females are very profound and mediated, at least in part, by the amount of sex hormone production. Sex hormones are responsible for the formation of primary sexual organs during

early development, but they also play crucial roles during adulthood. Male sex hormones, or androgens, promote the development of the primary sexual characteristics, as well as the maintenance of the male secondary sexual characteristics such as facial and body hair growth. (Davey et al., 2016) Female sex hormones, or estrogens, mediate the development of the reproductive tract and to maintain the secondary sexual characteristics such as fat accumulation in the breast. (Cui et al., 2012)

Both androgens and estrogens are metabolic derivatives of cholesterol by a complex and tightly regulated biosynthetic pathways involving several intermediates. (Figure 1.6.) For simplification, uptake of LDL occurs upon its interaction with its receptor the LDLR. The mechanism by which cholesterol is taken-up and utilized by cells led to the discovery and characterization of the receptor-mediated endocytosis. (Brown et al., 1986) Briefly, the LDL particle passed along the cell through early and late-stage endosomes and is eventually hydrolyzed after it fuses with a lysosome. In the lysosome, lipases will hydrolyze cholesterol esters to cholesterol, which can then be metabolized by several pathways. Cholesterol can also be synthesized endogenously by some cells, including the liver. Hepatocytes can esterify free cholesterol into cholesterol esters for storage in lipid droplets or secrete it as VLDL. In the hepatocyte, free cholesterol can be converted into bile acids, which is transported to the gallbladder. Bile acids are crucial for the fat emulsification, but also for the recycling and elimination of cholesterol. Cholesterol secreted as VLDL can travel to the testes where steroid hormone synthesis occurs in Leydig cells. (Figure 1.2.) This not only highlights the importance of the liver in the processing and synthesis of cholesterol, but also further implicates the interrelatedness between cholesterol and steroid hormones in the body.

Testosterone and dihydrotestosterone

In females, a majority of testosterone is produced in the ovary although small amounts are produced in the adrenal glands. (Bolour et al., 2005) In males, testosterone is the main cholesterol-derived androgen. Testosterone is synthesized both in the adrenals and by Leydig cells in the testes and testosterone production stimulated by the hypothalamus and anterior pituitary gland. (Smith et al., 2014) There are two main pathways of testosterone synthesis. As its' rate limiting step, cholesterol can be converted to pregnenolone in the mitochondria after gonadotropin-releasing hormone is secreted by the hypothalamus and luteinizing hormone is released from the pituitary gland. (Miller et al., 2011) (Figure 1.4.) Pregnenolone is then subsequently converted to 17α -hydroxyprognelone, dehydroepiandrosterone, and 4-androstane-3, 17-dione before it is fully converted to testosterone. The alternative pathway involves conversion of cholesterol into progesterone, then to 17α -hydroxyprogesterone and 4-androstane-3, 17-dione before conversion to testosterone. (Durdiaková et al., 2011) (Figure 1.5.)

Testosterone can be converted to estradiol, which is considered an estrogen, or to a more potent androgen, DHT, further highlighting the complex metabolic fate of sex hormones. (Ishikawa et al., 2006) (Figure 1.6.) Circulating testosterone in males follows a normal bimodal distribution, with the lower end of the male spectrum being 4-5 times higher than the highest end in females. (Clark et al., 2018). Although testosterone is vital for sexual expression and health, it exerts most of its biological functions in males upon conversion to its active derivative DHT. Among the different androgens, DHT is the strongest paracrine hormone amongst them. (Horton, 1992) In comparison to its precursor testosterone, DHT binds the androgen receptor with the twice the binding affinity, and its dissociation rate is five times slower. (Marchetti et al., 2013)

Indeed, DHT regulates cell growth and survival in the prostate as well as hair follicle growth as it exerts its function at the level of peripheral tissues and does not tend to be present in large amounts in circulation. (Deslypere et al., 1992) During early development, DHT contributes to the generation of male genitalia. Later in childhood, and with the development of the adrenal gland and puberty, DHT is responsible for penal and scrotum development, and body hair growth, among others. In adulthood, DHT plays minor roles in normal physiology other than prostate enlargement and male-pattern hair loss. (Kinter et al., 2021)

The conversion of testosterone to its active metabolite DHT is catalyzed by three distinct 5- α reductases. Type I 5 α reductase (SRD5A1) is expressed primarily in hepatocytes, the sebaceous glands, dermal papillae cells, and in follicular and epidermal keratinocytes. (Azzouni et al., 2012) Type II 5 α reductase (SRD5A2) is present in root sheathes of hair follicle, epididymis, vas deferens, seminal vesicles, and prostate, while type III 5 α reductase (SRD5A3) is expressed mostly in androgen mediated tissues such as skeletal muscle and prostate tissues, although it can also be found in malignant basal epithelial tissues. SRD5A2 is the most biologically active enzyme of the three. (Marchetti et al., 2013) Besides their role in the conversion of testosterone to DHT, 5 α reductases catalyze the reduction of several-cholesterol-derived hormones to their 5 α derivatives. (Marks, 2004)

Hormone replacement therapies and CVD

Testosterone levels vary during age, being at its highest during puberty, decreasing after sexual and developmental maturity in males. Because testosterone decreases over time, testosterone replacement therapy as a common practice to combat issues such as decreased sexual libido, loss of muscle, and other related dysfunctions. (Bassil et al., 2009) There is

evidence that exogenous testosterone replacement increases lean muscle mass and decreases fat mass but the impact on physiological function is unknown. (Snyder et al., 1999) Several association studies suggest that a decrease in testosterone during aging in men is associated with adverse effects on CVD, although randomized control trials using testosterone therapy in older men failed to show a clear outcome. (Terentes-Printzios et al., 2021) The use of hormone replacement therapy in women to prevent the development of CVD has raised several concerns over the past years. Research shows that estrogens contribute to the prevention of CVD, and that decline experienced in women because of aging, can contribute to the increased risk observed in women as they age. (Rodgers et al., 2019) Currently, however, hormone therapy replacement in women is not recommended to reduce the risk of CVD. (Chester et al., 2018)

History of 5 α reductase deficiency

The path to discovering finasteride and other 5 α reductase inhibitors, such as dutasteride, is set by a fascinating historical context. In the mid 20th century, James Hamilton, who was a scholar at Yale University became increasingly aware of the fact that sexually mature males were much more predisposed to balding, or alopecia. He took the opportunity to research this topic using 104 men with testicular impotency, referred to as “eunuchs” or “eunuchoids” as a model. From the study, Hamilton was able to conclude that hormonal pathway stimulation is implicated in those with male-pattern baldness. (Gupta et al., 2016) This set the stage for further research pertaining to endocrine function and sex hormone regulation as potential factors that underlie common pathology, such as androgenic alopecia.

A few decades later, bewildering reports of girls turning into boys in the Caribbean raised many questions around the world. More specifically, in Dominican Republic these children, were

referred to as *Guevedoces* (meaning “testes at 12”) or *Machihembras* (meaning first woman, now man) because they presented identifiable female genitalia at birth that began developing into male genitalia at the onset of puberty. The increased rates of these pseudohermaphroditic children, which was calculated to be as high as 2% of Dominican born in the 1970’s, made researchers ponder the possible underlying pathology afflicting this population. (Imperato-McGinley et al., 1974)

At the top of this list was Dr. Julianne Imperato-McGinley, a decorated endocrinologist from Cornell University who traveled to the Dominican Republic to study these children. In 1974, she published a landmark paper that highlighted 5 α reductase deficiency, a clinical disorder that had not been previously studied, as being the underlying pathology of the afflicted children. (Imperato-McGinley et al., 1974) People with 5 α reductase deficiencies have since continued to be utilized as a unique and natural model to study the effects of testosterone and DHT on male sexual and development. (Azzouni et al., 2012)

Later, there came a preponderance of evidence showing that insufficiency of 5 α reductase was not only associated with reduced prostate sizes but is also associated with reduced alopecia. Finasteride (Proscar/Propecia) was found to successfully inhibit type II 5 α reductase and was released at 5 mg dose to fight prostate diseases in 1992. In 1998, after more clinical trials, Merck and Co. released the drug at a lower 1 mg dose to treat androgenic alopecia. Nowadays, 1 mg of finasteride is the only oral drug approved by the United States Food and Drug Administration (FDA) to treat androgenic alopecia. (fda.gov)

Finasteride; an inhibitor of testosterone conversion to DHT

Finasteride inhibits both type I and type II 5 α -reductase with a 360 and 69 nM K_i , respectively (Figure 1.7.). (Schmidt et al., 2011). Like cholesterol and testosterone, the structure of finasteride contains a central sterol nucleus made of four hydrocarbon rings (Figure 1.3.). Type 1 is primarily expressed in the hepatocytes and type 2 is expressed in the prostate and hair follicles. They typically convert testosterone to its active metabolite 5 α -dihydrotestosterone. (Zhu et al., 2005) Recently, finasteride was shown to also bind and inhibit the human steroid 5 β -reductase, also involved in the reduction of certain steroid hormones, with a 2.1 μ M K_i . (Figure 1.7.) (Drury et al., 2009). Unlike the 5 α -reduction, 5 β -reduction typically deactivates steroid hormones. This protein is highly expressed in the liver, where it deactivates testosterone by its conversion to 5 β -dihydrotestosterone and contributes to the formation of bile acids (Figure 1.8.). (Nikolaou et al., 2021) Several studies have demonstrated the safety of finasteride in humans, and consistently showed that finasteride inhibits the conversion of testosterone to DHT. For instance, supplementation with finasteride decreases prostatic DHT levels by 70% (Stephen et al., 2004) in prostate disease patients and scalp levels of DHT by 70% in subjects with clinical androgenic alopecia. (Drake et al., 1999)

Metabolism of finasteride

Finasteride is among the top 100 most prescribed medications in the US. (Xiao et al., 2020) It is a white crystalline hydrophobic compound taken daily. It is completely taken up by the liver 6-8 hours after ingestion, although peak mean blood concentrations have been measured around the second hour after consumption. (Carlin et al., 1992). Phase I catabolism of finasteride is mediated by enzymes in the cytochrome P450 family, which convert finasteride to ω -

hydroxyfinasteride and finasteride- ω -oic acid (Huskey et al., 1995) (Wrighton et al., 1992).

Phase II is mediated via glucuronidation, common in endobiotic and xenobiotic metabolism (Dutton et al., 1966) (Wells et al., 2004).

Cytochrome P450 enzymes in phase I of finasteride metabolism include CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (Nebert et al., 2002). Interestingly, the CYP3A family is crucial in the oxidation of testosterone and the manipulation of steroid metabolites. (Waxman et al., 1988) UTG1A4 and UTG1A3 are two of the phase II enzymes that contribute to the elimination of finasteride. These proteins can be found in the liver, biliary tissue, stomach, and colon, they are responsible for the conversion of ω -hydroxyfinasteride and finasteride- ω -oic acid to hydroxyfinasteride glucuronide and finasteride carboxylic acid glucuronide, respectively. (Soars et al., 2002, Tukey et al., 2000) The derivative 6 α hydroxyfinasteride is the main human metabolite of finasteride biotransformation that has been identified in bile. This metabolite has been proposed to be hydrolyzed and subsequently reabsorbed by the intestine and reenter enterohepatic circulation, possibly affecting half-life of the drug. (Lundahl et al., 2009, Liu et al., 1993). Previous literature has shown that finasteride is excreted in both the urine and in the feces, although there is contradictory evidence for the relative amounts for each. (Mysore, 2012)

Finasteride as a medication for androgenic alopecia

Androgenic alopecia, or male-pattern baldness, is a progressive disorder in which DHT accumulates in the hair follicle leading to follicular miniaturization and death. In 1998, finasteride became the first and only FDA-approved oral medication to treat androgenic alopecia at 1 mg/day and according to the Medical Expenditure Panel Survey it is estimated there are around 8,986,897 prescriptions in the United States (Stough D et al 2005) (Agency for

Healthcare and Research Quality). Finasteride has been shown many times to be effective in treating androgenic alopecia, reducing scalp DHT levels by up to 70%. (Drake et al., 1999).

Minidoxil is the second medication approved by the FDA to treat androgenic alopecia, although it can only be used directly on the scalp. In a randomized control trial of 99 male patients with androgenic alopecia, minodoxil and finasteride were shown to have similar efficacy. (Saraswat et al., 2003)

Finasteride as a medication for prostate diseases

Finasteride is used to mitigate symptoms associated with benign prostatic hyperplasia which is characterized by epithelial and stromal proliferation in the transition zone that surrounds the urethra. (Ng et al., 2021) This disorder is prevalent in most men older than 40 years, and its incidence increases over time, being approximately 80-90% in males over the age of 70. (Lim Kb et al. 2017) Histological changes associated with benign prostate hyperplasia can lead to clinical implications including bladder outflow obstruction and lower urinary tract symptoms. Early studies showed that finasteride was able to shrink the size of the prostate by a mean of 28% over 6 months. (Stoner, 1990)

The safety of finasteride has been questioned, as its effects on prostate size and reduction of prostate-specific antigen levels could mask prostate malignancies. Prostate cancer afflicts around 16.5% of men in the United States over the lifespan and is the most common male non-skin cancer in the world. (Siegal et al., 2013) Despite the rarity of its use, there is evidence in using finasteride to treat prostate cancer as well. In a 10-year prostate cancer prevention trial in 18,882 men, finasteride was shown to reduce prostate cancer risk by about one third. (Thompson et al., 2013). Other studies confirm the reduction in prostate cancer risk although there are

reports that malignant forms of the cancer may increase. (Wang et al., 2020) This evidence shows that finasteride is a potent pharmacological option whose use should be considered by doctors when treating patients at risk of suffering prostate hyperplasia or cancer.

Relationship between finasteride, lipid profile and the development of atherosclerosis

While the role of sex hormones on CVD has been extensively studied, the implication of finasteride on this disease is not well established. Clinical studies aimed to establish the safety and efficacy of finasteride revealed conflictive results regarding plasma cholesterol, triglyceride, and glucose levels. Few studies show negative impact on lipid parameters— including one looking at the effects of three and six months of 1 mg/day finasteride treatment lipid parameters in 13 patients in which they saw an increase in HDL3 and Lp(a). (Denti et al., 2000) Tenover et al. showed in 2,112 men that 12 months of 5mg/day finasteride (for benign prostatic hyperplasia efficacy study) treatment did not significantly alter plasma lipid profile. (Tenover et al., 1997). In another study that looked at 50 older men with low levels of testosterone, treated with 5 finasteride per day for 36 months, finasteride increased lean body mass and lowered total cholesterol in addition to the LDL fraction. (Page et al., 2005)

Recently, a preclinical model for atherosclerosis using apolipoprotein e deficient mice showed that finasteride exposure results in a reduction in plasma triglycerides, total cholesterol, LDL-C, and HDL-C. (Liu et al., 2020) These changes also resulted in a reduction in plaque size, suggesting that finasteride could serve as a therapeutic agent to delay the progression of atherosclerosis. However, the authors failed to pursue the molecular mechanisms by which finasteride reduces plaque size in mice.

In our study, we examined the effect of finasteride in a clinical and a preclinical model. Using the NHANES database, we observed that the consumption of finasteride is associated with reduced total cholesterol in subjects above 50 years of age. These results were validated in a preclinical model, which also showed that finasteride treatment results in a reduction in plaque size, confirming previous findings in a comparable mouse model. (Liu et al., 2020) Further, we show that finasteride reduces plaque inflammation and monocyte infiltration. Whole genome analysis of the liver suggests that finasteride mitigates liver inflammation, favors oxidative phosphorylation, and promotes bile acid biosynthesis, which could explain the positive effects of finasteride in health

Figures and tables

Figures

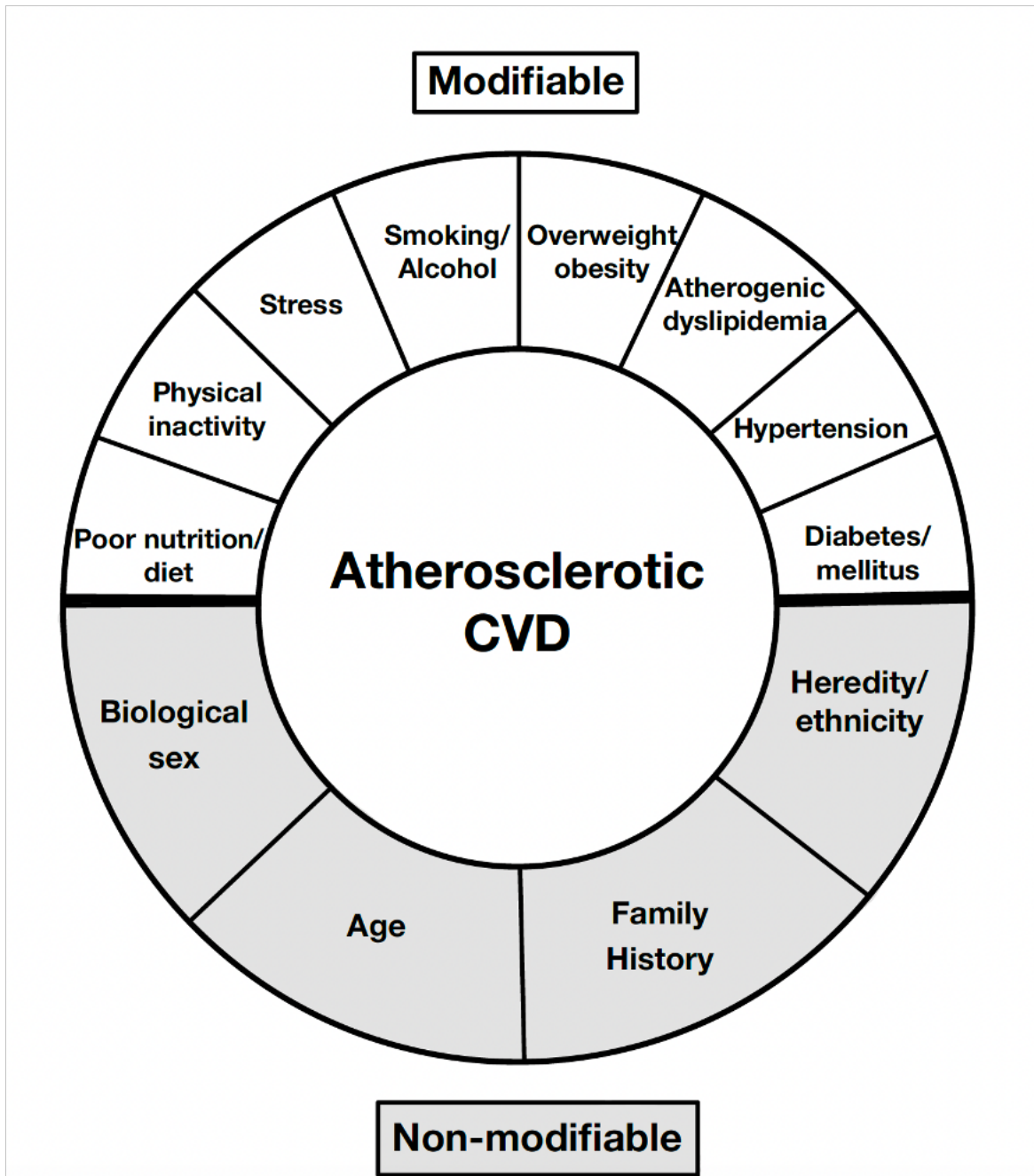


Figure 1.1 Modifiable and non-modifiable factors associated with atherosclerotic cardiovascular disease (CVD).

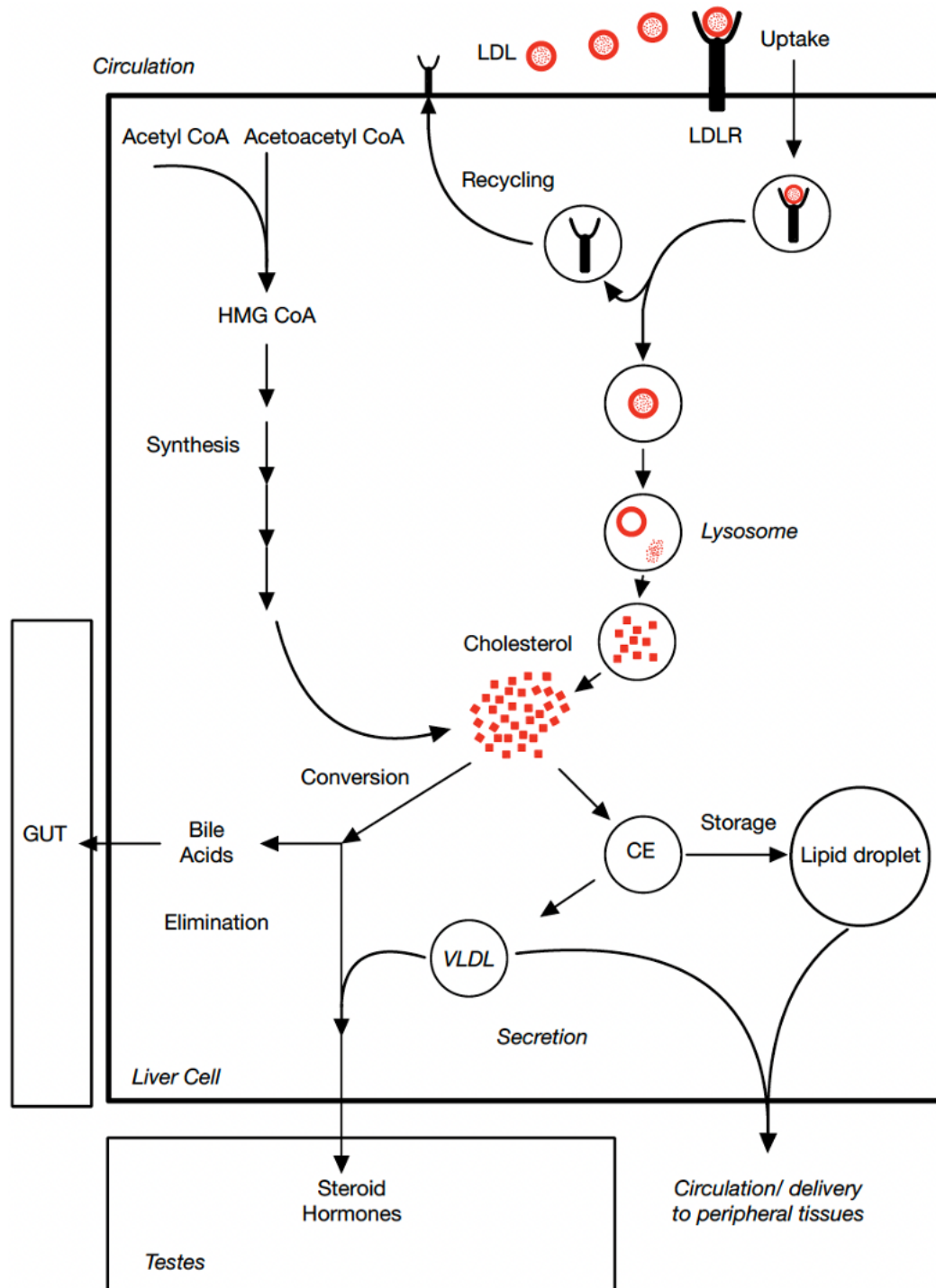


Figure 1.2 Cholesterol uptake, synthesis, and excretory pathways in the hepatocyte. LDL, low-density lipoprotein; LDLR, LDL receptor; HMG CoA, β -Hydroxy β -methylglutaryl-CoA; CE, cholesteryl esters; VLDL; very low-density lipoprotein.

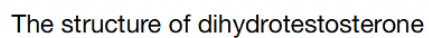
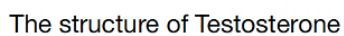
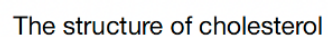
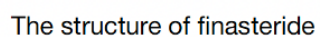


Figure 1.3. The structures of finasteride, cholesterol, testosterone, and dihydrotestosterone.

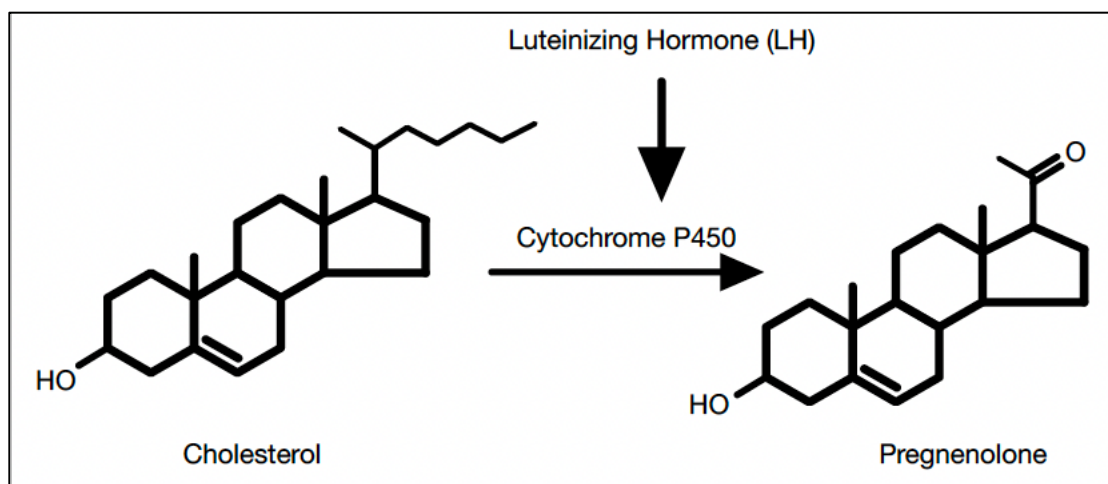


Figure 1.4. The rate limiting step in the synthesis of testosterone. Cholesterol is converted to pregnenolone after stimulation from luteinizing hormone.

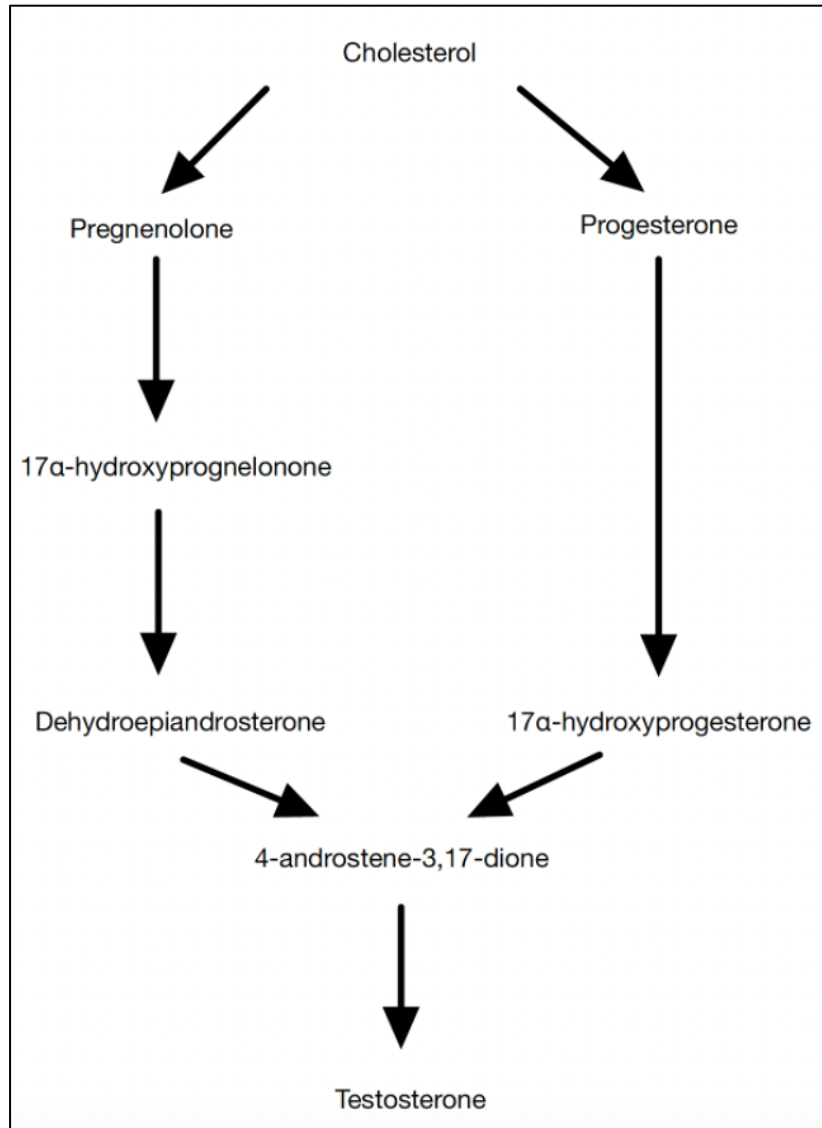


Figure 1.5. Two pathway syntheses of testosterone from cholesterol.

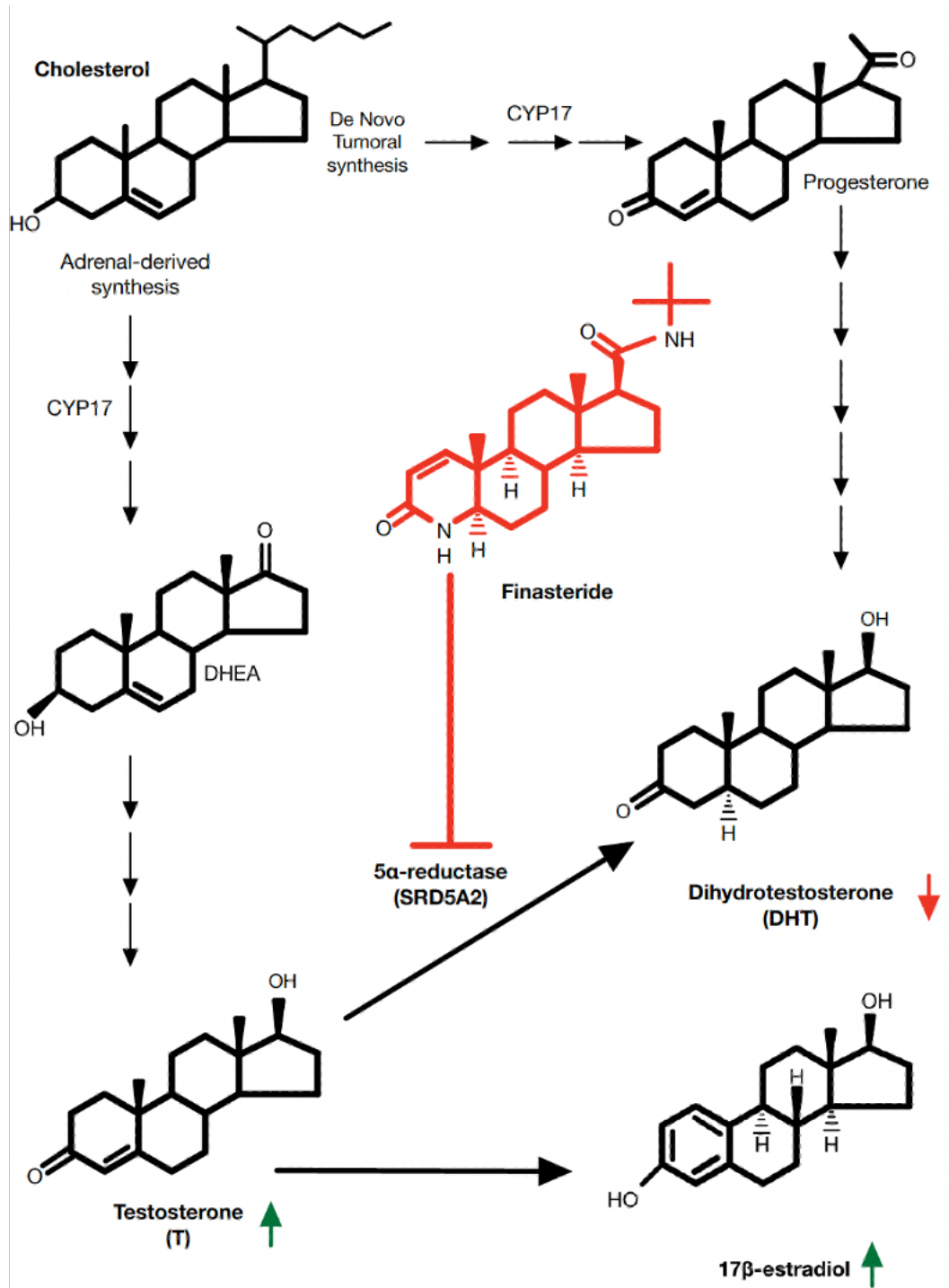


Figure 1.6. Pathways connecting cholesterol, progesterone, DHEA: dehydroepiandrosterone, T: testosterone, DHT: dihydrotestosterone, 17β-estradiol, and finasteride. Finasteride inhibits SRD5A2: Type II 5α reductase, decreasing DHT, increasing testosterone and 17β-estradiol

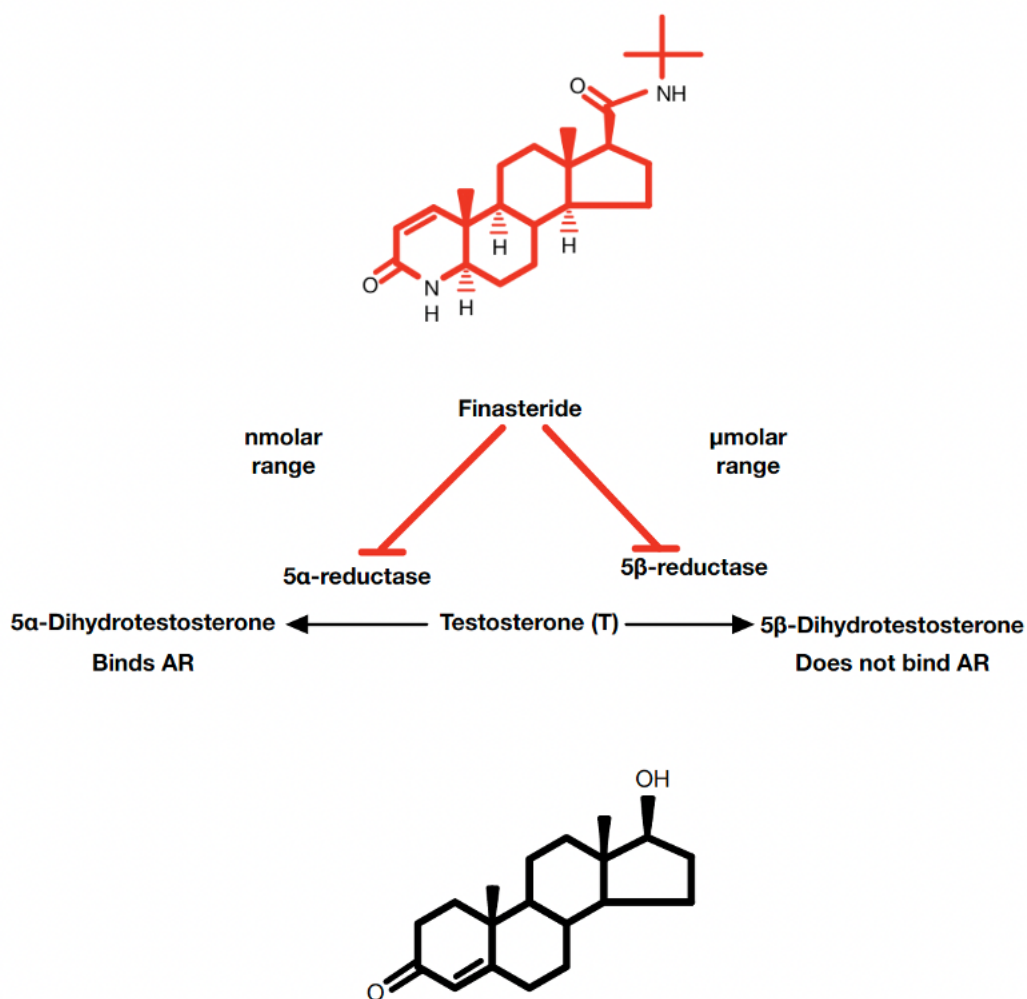


Figure 1.7. Finasteride inhibits 5 α - and 5 β -reductases. Finasteride inhibits both type 1 and type 2 5 α -reductase with a 360 and 69 nM Ki, respectively. (Schmidt et al., 2011) Type 1 is primarily expressed in the hepatocytes and type 2 is expressed in the prostate and hair follicles. They typically convert testosterone to its active metabolite 5 α -dihydrotestosterone. A recent study shows that finasteride also inhibits 5 β - reductase with a 2.1 μ M Ki. (Drury et al., 2009) 5 β - reductase is primarily expressed in hepatocytes, where it can deactivate testosterone by its conversion to 5 β -dihydrotestosterone. Unlike type 1 and 2 5 α -reductases, 5 β -reductase can act on several substrates, being typically involved in bile acid synthesis.

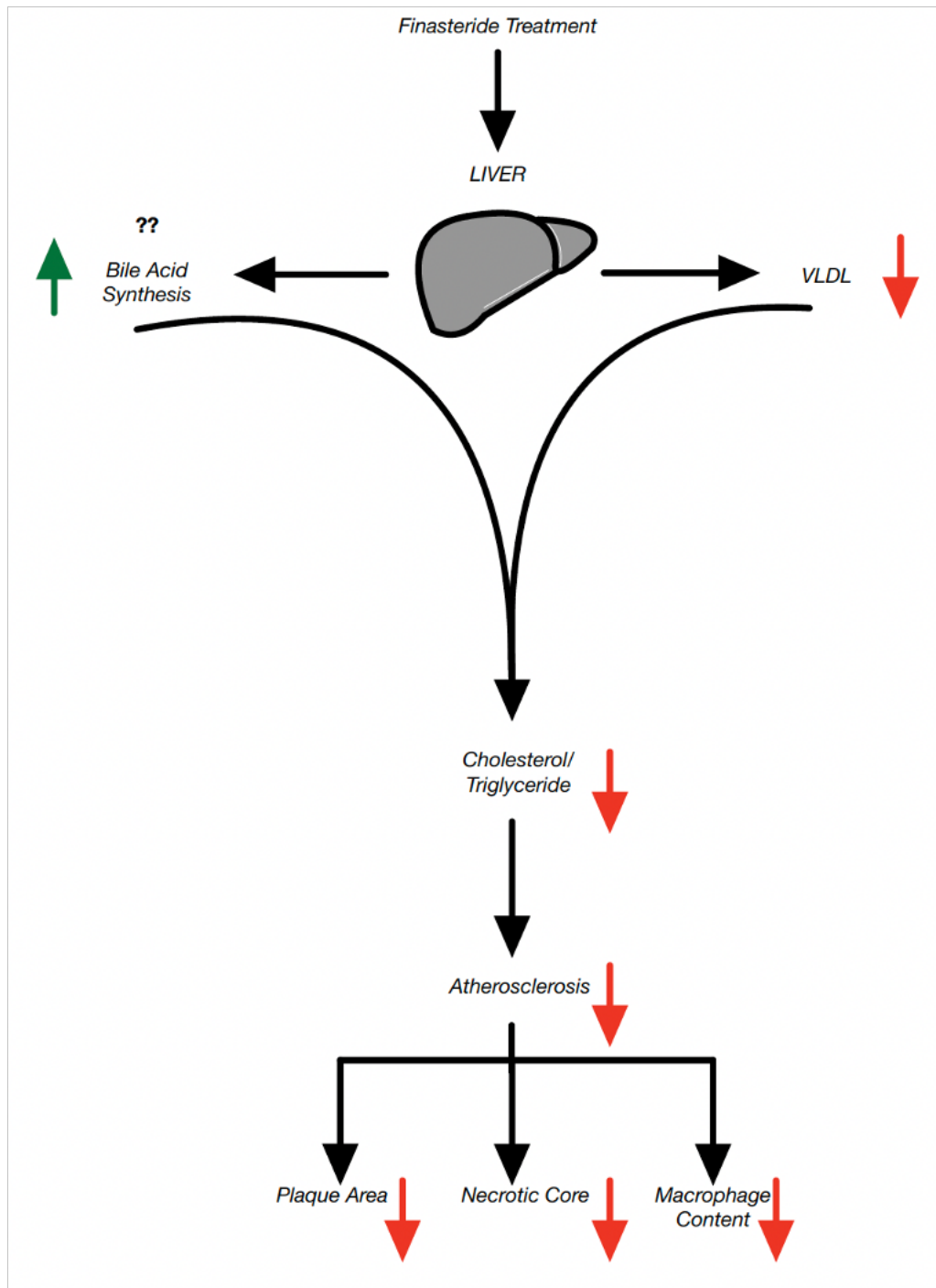


Figure 1.8. Summarized outcomes of our study. Finasteride treatment decreases cholesterol/triglyceride, and parameters associated with atherosclerosis such as plaque area, necrotic core, and macrophage content (red arrows). Whole genome analysis of finasteride treated animals showed increased bile acid synthesis (green arrows).?? Indicates ongoing experiments

Chapter 2

Finasteride treatment is associated with a reduction in plasma cholesterol in humans and a delay in atherosclerosis progression in mice

Abstract

Plasma cholesterol levels are among the best predictors of the development of atherosclerosis, but the mechanisms that regulate cholesterol production are not completely understood. The objective of this study was to determine whether finasteride, a drug used to treat benign prostate hyperplasia and male pattern hair loss, affects plasma cholesterol levels and the development of atherosclerosis. We used regression models to examine associations between circulating lipid profile and the use of finasteride using the National Health and Nutrition Examination Survey database. After controlling for covariates, we observed a significant reduction in total cholesterol levels in subjects taking finasteride. In our preclinical model, we tested the effect of oral finasteride in the atheroprone low-density lipoprotein receptor-deficient (*Ldlr*^{-/-}) mouse model. Finasteride reduced circulating cholesterol levels, plaque size, and inflammation without altering biometric parameters or causing hepatic toxicity. Global transcriptome analysis of the liver showed an increase in bile acid biosynthesis and a reduction in inflammatory markers. Our findings suggest that finasteride reduces plasma cholesterol levels by promoting bile acid formation and cholesterol intestinal elimination.

Introduction

Cardiovascular diseases (CVDs) are responsible of approximately one in four deaths worldwide. (Cardiovascular diseases) Atherosclerosis is the main underlying cause of CVDs, and it is characterized by the accumulation of cholesterol-rich lipoproteins in the intima layer of the arterial wall. As a result, circulating monocytes transmigrate into the endothelial layer of the artery to differentiate into macrophages to become cholesterol-laden foam cells. (Moore et al., 2013) The progressive accumulation of lipoproteins and foam cells in the arterial wall can cause the rupture of these lesions, resulting in thrombus formation and a cardiac event. (Moore et al., 2018)

Common non-modifiable and modifiable risk factors contribute to most of the burden of CVD. A recent study estimated that between 60% to 80% of the prognostic performance of CVD can be attributed to age, ethnicity, and sex (Pencina et al., 2019) For instance, men are twice as likely as women to die from a cardiac event. (Möller-Leimkühler et al., 2007) Part of these sex differences are attributed to differences in plasma cholesterol levels, since men are prone to hypercholesterolemia at earlier ages than women, a condition that it is reversed upon the onset of menopause (Maas et al., 2010). While the exact mechanisms underlying sex differences are not completely understood, sex hormone signaling is among the most plausible causes of these differences. (Herring et al., 2013) More specifically, some studies shows that testosterone conversion to estradiol results in decreased atherosclerosis development. (Nathan et al., 2001) Another study showed that DHT-upregulates genes related to increased atherosclerosis in cultured macrophages. (Ng et al., 2003) Several pharmacological interventions targeting these pathways are currently employed for a variety of purposes such as hormone replacement

treatments for men or women, steroids to enhance muscle mass, *in vitro* fertilization, and sex transition therapies (Bassil et al., 2009).

Among these drugs, finasteride shunts the conversion of testosterone to DHT and it is commonly prescribed to treat benign prostate hyperplasia in men, a disorder that affects many men after the age of 40. (Stoner et al., 1990) Additionally, finasteride is the only FDA approved oral medication to treat androgenic alopecia (fda.gov). Finasteride is a type II 5 α reductase inhibitor that blocks the conversion of testosterone to its active metabolite 5 α -dihydrotestosterone (DHT). Finasteride is among the top 100 prescribed medications in the United States (Xiao et al., 2020). Multiple studies show that finasteride is safe, but whether this drug affects plasma cholesterol and atherogenesis has not been studied in humans.

In these studies, we investigated whether finasteride affects plasma cholesterol and other parameters related to CVD. For this purpose, we examined finasteride use in men from the National Health and Examination Survey (NHANES) and performed a preclinical study in low-density lipoprotein receptor (LDLR)-deficient mice, a common model to study atherosclerosis. (Ishibashi et al., 1993) Our clinical data suggest that finasteride treatment is associated with lower levels of total plasma cholesterol. In agreement with these findings, *Ldlr*^{-/-} mice fed finasteride showed a reduction in plasma cholesterol and atherosclerosis, accompanied by an upregulation of hepatic bile acid synthesis. Overall, these results unveil the positive effects of finasteride on the regulation of plasma cholesterol and shed light on possible causes related to sex differences in the development of CVD.

Materials and Methods

Retrospective human study

Study design

NHANES is a continuous, cross-sectional resource database that surveilles the health and nutrition status of the non-institutionalized United States population. NHANES is administered through the National Center for Health Statistics, a part of the Centers for Disease Control and Prevention. A comprehensive report on the methods of NHANES data collection can be found in previous publications (Ahluwalia et al., 2016). All participants of the NHANES must provide written consent for the public availability and use of these data. This process is completed under the approval of the National Center for Health Statistics Institutional Review Board.

Study sample and inclusion criteria

We utilized the multi-stage data from the U.S. NHANES 2009-2010, 2011-2012, 2013-2014, and 2015-2016 datasets. Because finasteride is typically taken to treat male baldness and benign prostatic hyperplasia, we excluded women from the analysis. Only those men that completed the 24-hour health and dietary recall interviews and participated in the Mobile Examination Center sample collection were included in the study. We excluded subjects with prostate inflammation and infection, and those diagnosed with prostate cancer or with self-reported prostate cancer or that were younger than 50 years of age. (Figure 2.1.).

Variables and measurements

The dependent variables for the study were total cholesterol, cholesterol found in the LDL fraction (LDL-C), cholesterol found in the high-density lipoprotein fraction (HDL-C),

triglyceride, glucose, and glycohemoglobin in plasma. Subjects were categorized as finasteride users or not. We considered the following variables as covariates; alcohol intake (derived from ALQ150 and ALQ151), diagnosis with coronary heart disease (derived from MCQ160C), any kind of liver condition (derived from MCQ160L), cancer or malignancy of any kind (derived from MCQ220), diagnosis with diabetes or prediabetes and insulin or other diabetic medication users (derived from DIQ050 and DIQ070; and DIQ010 and DIQ160), diagnosis with high blood cholesterol level and cholesterol medication users (derived from BPQ080 and BPQ090D), smoking status (derived from SMQ040), drugs affecting lipid profile, drugs affecting glucose profile, age, body mass index (categories derived from BMXBMI), and race (RIDRETH1).

Statistical analyses

Statistical analyses were performed using SAS v9.4 software (SAS Institute, Cary, NC). Descriptive statistics were calculated using PROC MEANS and PROC FREQ procedures (Table 1) to examine if finasteride intake effects the outcomes variables (glucose, glycohemoglobin, total cholesterol, LDL-C, HDL-C, and triglyceride). Bivariate analyses for each outcome were conducted separately with each of the covariates (listed above) to see whether these variables truly were confounding the relationship between finasteride intake and outcome variables (change of >10% in beta coefficient), and if these variables qualified as interaction terms with finasteride use in the model for each of the six outcomes (at $p < 0.05$). Once subsets of candidate confounders and interacting variables were identified for each outcome model, these were added in the final linear regression model for each of the six outcomes. PROC SURVEYREG procedure was used in these analyses and least square means estimates were assessed for each significant interaction in the final model. (The SURVEYREG procedure, n.d.) All analyses were

conducted according to the NHANES analytical guidelines. Masked variance was accounted for, and appropriate sample weights were applied to represent unequal probabilities of selection, nonresponse bias, and oversampling. Eight-year weights were calculated using the method provided by National Center for Health Statistics.

Animal Studies

Study design, diets, and tissue harvesting

Studies were executed in compliance with the guidelines published in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The animal protocols were approved by the Animal Care and Use Committees of the University of Illinois at Urbana-Champaign. We used male *Ldlr*^{-/-} (#002207, Jackson Labs, Bar Harbor, ME) mice, unless otherwise stated. All mice were housed and nurtured at 24°C on a 12-hour light/dark cycle and fed *ad libitum* with free access to water. The dams and pups were fed a non-purified standard diet (Teklad global 18% protein diet: Envigo, Indianapolis, IN). Pups were weaned three weeks after birth onto a standard diet.

To avoid litter-specific effects, we synchronized the pregnancies of the mice. Male mice born from the same litter were ear-tagged and weaned into separate cages in company of mice born from other litters with a maximum of three days apart. All cages contained comparable amounts of mice and were monitored daily during the first week to detect signs of stress or aggression. After a week of adaptation to the breeder diet, four-week-old mice were fed 12 weeks Western diet containing 0.3% of cholesterol and 41% of fat as a source of energy without finasteride (control) (n=14) or the same diet supplemented with either 10 mg/kg (n=10), 100 mg/kg (n=10), or 1000 mg/kg (n=15) of finasteride. Finasteride was purchased from Cayman

Chemical (Abb Arbor, MI), and shipped to Research Diets (New Brunswick, NJ) for diet preparation using cold extrusion to preserve finasteride's integrity. Finasteride dosing was based on previous animal studies using similar dosage (Froese et al., 2016) (Xu et al., 2006). Upon arrival, diets were kept frozen at -20° until use.

Body weights and food intake were measured once a week during the entire experiment. After 12 weeks on the corresponding Western diets, mice were fasted overnight and euthanized by injecting intraperitoneally a lethal dose of 80 mg ketamine/kg and 8 mg/kg of xylazine. Blood was drawn from the right ventricle of the heart using EDTA-coated syringes and kept on ice for plasma preparation. Mice were perfused systemically with a 10% sucrose in saline solution (0.9% NaCl in water) for approximately two minutes. Tissues were harvested and immediately placed in liquid nitrogen and stored at -80°C. Aortic roots were cleaned of surrounding fat and tissue under microscope and imbedded in optimal cutting temperature (OCT) compound (Sakura, Torrance, CA), placed on dry ice, and stored at -80°C. Plasma was collected by centrifugation of the blood for five minutes at 1000 x g and stored immediately at -80°C.

Analysis of hepatic liver toxicity

Alanine transaminase (ALT) and aspartate transaminase (AST) activities were analyzed in the plasma of wild-type mice using commercially available kits (Abcam, Cambridge, MA), as per manufacturer instructions. Plasma microRNA-122 (miR-122) was analyzed as previously described (Vogt et al., 2019). Briefly, total plasma RNA was isolated and retro-transcribed to cDNA using TRIzol LS reagent (Thermo Fisher Scientific, Waltham, MA) and TaqMan MicroRNA Reverse Transcription Kit (Applied BioSystems, Carlsbad, CA), respectively. Each sample was spiked with synthetic *C. elegans* miR-39 (cel-miR-39) (Qiagen, Hilden, Germany),

which was used as an external control. Quantitative real-time PCRs were performed using TaqMan Fast Advanced Master Mix (Applied Biosystems) Relative miR-122 to cel-miR-39 expression levels were determined using the Pfaffl method considering the reaction efficiencies, as done previously (Pfaffl, 2001).

Glucose tolerance test assay

A week before tissue harvesting, mice were fasted overnight in clean cages without access to food, but with free access to water. In the morning, mice were gavaged with glucose dissolved in saline (1.5 g/kg of body weight). Changes in glucose levels were measured with a glucometer (OneTouch) from tail-snips, following established protocols. After the last time point, mice were allowed to recover for a week with free access to the corresponding experimental diets until the day of the sacrifice.

Lipid and lipoprotein analysis

Plasma total cholesterol, HDL-C, and triglyceride concentrations were measured using colorimetric assays (FUJIFILM Wako Diagnostics, Mountain View, CA) and performed in accordance with manufacturer's instructions.

Fast performance liquid chromatography (FPLC) analyses

Plasma lipoproteins were fractionated using FPLC with two Superose 6 10/300 GL columns (GE Healthcare, Boston, MA) on a Shimadzu HPLC system (Columbia, MD) as done in the past (Zhou et al., 2020). Pooled samples from five mice per group were pooled for analysis.

Lipoprotein fractions were identified by measuring lipid content (cholesterol and triglyceride) and western blot analysis (see below).

Western blot analyses of FPLC fractions

Lipoprotein species in FPLC fractions were identified by a combination of lipid measurements and confirmed by western blot targeting for apolipoprotein B and apolipoprotein A1, which are present in the VLDL/LDL and HDL fractions, respectively. Briefly, 5 μ l of the indicated fractions were run in SDS-PAGE gels, transferred to nitrocellulose membrane, and incubated with the corresponding antibodies. For apolipoprotein B, we utilized a rabbit monoclonal antibody (Millipore) at 1:1,000 dilution. For apolipoprotein A1, we utilized rabbit polyclonal antibody (Santa Cruz).

Quantification of plaque area in the ascending aorta

Plaque area in the ascending aorta was estimated by quantifying the size of lesions present in the brachycephalic artery until its bifurcation to the right subclavian artery, and the total amount of lesions found in the ascending aorta, the left carotid artery, and the left subclavian artery.

Aortic Root Analysis

Aortic roots were sectioned using a cryostat at a thickness of 6 μ m. The sections were stained with hematoxylin/eosin to visualize the structure of the lesion, and with anti-CD68 (AbD Serotec, Oxford, UK) to quantify macrophage content in the plaque, as done previously (Zhou et al., 2020). Images were taken using a Zeiss light microscope (Axioskop 40, Carl Zeiss) 10x

magnification and plaque area and CD68⁺ content in the atherosclerotic lesions were then quantified using ImageJ software.

Flow Cytometry and Monocyte Recruitment

To examine monocyte recruitment into atherosclerotic plaques, circulating monocytes were labeled with 5-ethynyl-2'-deoxyuridine (EdU) as described (Rahman et al., 2017). Mice were injected intraperitoneally with 250 μ l of 1 μ M EdU (Life Technologies, CA) two days before the sacrifice. After 48 hours, we examined EdU-labeling efficiency in circulating monocytes by flow cytometry to measure monocyte labeling efficiency. Blood was collected via tail snip and incubated with red blood cell lysis buffer (BD Biosciences, CA). Samples were incubated with antibodies anti-mouse CD45-FITC (Clone 30-F11; BioLegend, CA), CD115-APC (Clone AFS98; BioLegend, CA), and Ly6c-PerCP-Cy5.5 (Clone RB6-8C5; BD Biosciences, CA) to identify circulating monocytes. Thereafter, cells were permeabilized and EdU detected using Click-IT EdU Pacific Blue Flow Cytometry Assay Kit (Life Technologies, CA). Flow cytometry was performed in LSR II analyzer (BD Biosciences, CA) and data analyzed using FCS Express 5.01.0092 (De Novo Software, CA).

Quantification of Newly recruited Monocytes and Macrophage Proliferation in Plaques

To visualize EdU⁺ cells in the lesions, aortic root sections were fixed in 10% formalin, permeabilized with 1% Triton X-100 in PBS, and stained for EdU using Click-IT EdU Imaging Kit and Alexa Fluor 647 azide (Life Technologies, CA). Macrophage proliferation was determined by staining slides with the proliferative marker, Ki67 (Mohammed et al., 2012). Newly recruited

monocytes in lesions were identified as EdU/CD68 double positive, Ki67 negative cells. Recruited monocyte number was corrected by the number of EdU+ circulating monocytes.

RNA isolation and sequencing (Ivan)

Liver RNA was isolated using a Zymo Research mini prep tissue kit (Orange County, CA), including DNase treatment, following the manufacturer's instructions. Samples were sequenced using the HiSeq 4000 Paired-End (Illumina, San Diego, CA) and the results were analyzed using Gene Set Enrichment Analysis (GSEA, Broad Institute Inc., MIT, Cambridge, MA). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through the GEO Series accession number (TBD).

Sequencing reads were mapped to the reference genome (mm10) using the STAR aligner (v2.5.0c) (23). Alignments were guided by a gene transfer format file. The mean read insert sizes and their standard deviations were calculated using Picard tools (v.1.126) (<http://broadinstitute.github.io/picard>). The read count tables were generated using HTSeq (v0.6.0) (24), normalized based on their library size factors using DEseq2 (25), and differential expression analysis was performed. The read per million normalized BigWig files were generated using BEDTools (v2.17.0) (26) and bedGraphToBigWig tool (v4). To compare the level of similarity among the samples and their replicates, we used two methods: principal-component analysis and Euclidean distance-based sample clustering. All the downstream statistical analyses and generating plots were performed in the R environment (v3.1.1) (<https://www.r-project.org/>).

Statistical Analyses

Data are expressed as means +/- the standard error of the mean. All statistical differences were analyzed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). The normal distribution of the control and finasteride supplemented groups was analyzed using Shapiro-Wilk normality tests. In the preclinical model, data comparing all four experimental groups were analyzed either using two-way ANOVA (GraphPad Prism) with Dunnett's multiple comparison testing or repeated-measures two-way ANOVA with Sidak's multiple comparisons test. Data comparing Western diet control group to high dose finasteride were analyzed using t test. Statistical significance was set at $P < 0.05$. For the glucose tolerance test measurements, areas under the curve were measured using Graphpad Prism software (GraphPad Software Inc., San Diego, CA).

Results

Human Study - Demographics of the Participants

A total of 4,791 participants met our exclusion criteria; 4,636 participants that did not take finasteride and were compared to 155 participants that reported taking finasteride in the survey (Figure 2.1.). The distribution by race, body mass index, and age of participants is summarized in Tables 1, 2 respectively.

Human Study - Finasteride Treatment Is Associated with a Reduction in Total Plasma Cholesterol

Table 2.1. shows the changes in total plasma cholesterol concentrations. Finasteride treatment was not associated with alterations in total cholesterol when alcohol was considered as

an interaction variable. However, plasma cholesterol was reduced in subjects reporting the use of finasteride and previously diagnosed with CVD ($p = 0.0018$). Similarly, finasteride treatment was associated with a reduction in total cholesterol in those subjects also taking drugs that affect glucose metabolism ($p = 0.016$).

Table 2.2. and 2.3. shows the results obtained for the associations between finasteride treatment LDL-C and HDL-C, respectively. Finasteride was not associated with alterations in LDL-C or HDL-C levels when compared using common interaction variables. In all cases, however, LDL-C and HDL-C levels showed a tendency towards a decrease in those subjects that reported the use of finasteride (Table 2.2. and 2.3.).

Table 2.4. shows the results obtained for triglyceride concentration of the NHANES cohort. We did not observe any significant differences in triglyceride levels in subjects taking finasteride for any of the interaction variables evaluated.

Human Study - Finasteride Treatment in Plasma Glucose and Glycohemoglobin levels

Table 2.5 and 2.6. show the glucose and glycohemoglobin concentrations in the NHANES cohort, respectively. Finasteride treatment was associated with a reduction of circulating glucose in subjects diagnosed with diabetes currently taking antidiabetic medications of any kind ($p=0.045$). Glycohemoglobin concentration is used as indicator of glycemic levels of the subject over the past two to three months, and its use in clinical practice is standardized (Little et al., 2018). Our data show that finasteride intake was associated with a reduction in glycohemoglobin in smokers ($p=0.0022$). When we fractionated our cohort using body mass index as interaction variable, finasteride was associated with an increase in glycohemoglobin only in underweight subjects ($p=0.0046$). For the remaining interaction variables considered for

glucose and glycohemoglobin levels, our model did not show significant differences between subjects treated with and without finasteride use or not, although our data showed a trend towards a decrease in these parameters in subjects taking the drug (Tables 2.5. and 2.6.).

Mouse Study - Finasteride Decreases Plasma Cholesterol in *Ldlr*^{-/-} Mice

Our clinical study suggests that finasteride treatment ameliorates lipid and glucose profiles in humans, suggesting that finasteride reduce the risk for the development of atherosclerosis. To test this, we fed male *Ldlr*^{-/-} mice a Western diet containing increasing doses of finasteride (Figure 2.2.A). Finasteride did not alter food intake, liver size, or circulated parameters commonly used as indicators of liver toxicity (Table 2.8). We did not observe differences in total body weight or body adiposity between experimental groups (Figure 2.2.B, 2.2.C) As previously reported (Canene-Adams et al., 2007), finasteride caused a reduction in prostate size in mice, in agreement with previous reports using comparable experimental conditions (Figure 2.2.D).

Finasteride treatment decreased circulating total plasma cholesterol when administered at 1000 mg/kg, failing to alter HDL-C at any of the doses (Figure 2.2.E, 2.2.F). In comparison to control-fed mice, mice fed 1000 mg/kg also had a significant reduction in fasting plasma triglyceride (Figure 2.2.G). Next, we compared plasma cholesterol and triglyceride partitioning between control-fed and finasteride fed mice at 1000 mg/kg by FPLC using pooled samples (5 mice/group). The cholesterol and triglyceride content in the VLDL and LDL fractions were reduced in finasteride-treated mice, while a non-significant increase in HDL-C was observed in finasteride-fed mice (Figure 2.2.H, 2.2.I). Finasteride treatment did not affect fasting glucose levels or glucose tolerance test under our experimental conditions (Figure 2.5.)

Mouse Study – Finasteride reduces lesion size and inflammatory parameters in atherosclerotic lesions of $Ldlr^{-/-}$ Mice

To determine the effects of finasteride on atherogenesis, we visualized lesion size in the ascending aorta (Figure 2.3.A). Lesion size quantification showed a reduction in total plaque in mice exposed to 1000 mg/kg of finasteride (Figure 2.3.B). These data were confirmed by characterizing lesion size at the level of the aortic root (Figure 2.3.C). Our morphometric analyses revealed that mice fed 1000 mg/kg finasteride presented with a reduction in plaque size and macrophage content in comparison to control-fed mice (Figure 2.3.D, 2.3.E). We next quantified the necrotic core area in the lesions, a parameter negatively associated with plaque stability in humans. (Ohayon et al., 2008) Mice fed 1000 mg/kg of finasteride showed a reduction in the presence of necrotic cores in comparison to control-fed mice (Figure 2.3.F).

Histological changes were accompanied by a reduced monocytoxis in mice exposed to 1000 mg/kg of finasteride in comparison to control-fed mice, although the ratio between $Ly6c^{hi/low}$ -expressing monocytes, which represents the pro-inflammatory phenotype of circulating monocytes, was comparable between among all the experimental conditions (Figure 2.3.G, 2.3.H). Our ongoing experiments will examine the monocyte recruitment rate to the plaques in mice exposed to 1000 mg/kg in comparison to control-fed mice as well as the number of proliferative macrophages at the moment of the sacrifice (Figure 2.3.I).

Mouse Study – Finasteride reduces hepatic inflammation, stimulates bile acid biosynthesis and oxidative phosphorylation in $Ldlr^{-/-}$ mice

Our data show that finasteride treatment results in a reduction in plasma cholesterol and the burden of atherosclerosis in mice (Figure 2.2., 2.3.). Indeed, plaque size correlates with

plasma cholesterol levels, suggesting that finasteride does not act at the local level in the plaque (Figure 2.6.).

Because plasma cholesterol levels are largely regulated by the rate of VLDL secretion, we next wondered the effect of finasteride in the liver. For this, we performed hepatic whole-genome transcriptome analysis in mice fed Western diet (control) and the same diet supplemented with 1000 mg/kg of finasteride.

Principal component analyses showed a clear distribution between both experimental groups (Figure 2.4.A). A total of 2102 genes were significantly regulated with an adjusted p value < 0.05 (Figure 2.4.B, 2.4.C). Pathway analysis revealed an upregulation of genes involved in xenobiotic catabolism, bile acid metabolism, and oxidative phosphorylation in finasteride fed mice. These changes were accompanied by a downregulation in inflammatory markers (Figure 2.4.D).

Discussion

Over the past decades, several observational studies showed that sex is among the strongest predictors of atherosclerotic CVD. Men are more susceptible to experiencing complications related to CVD earlier in life than women due to, at least in part, increased total plasma cholesterol levels and reduced HDL-C levels in comparison to women. While these differences are not completely understood, sex hormones have been pinpointed among the driving causes in these changes. There are several sex hormones, among which testosterone is the primary androgen in men. The association between testosterone levels and the risk of CVD is controversial, but the current scientific consensus suggests that low testosterone levels in men is associated with increased CVD and other comorbidities such as obesity and diabetes.

Additionally, testosterone-replacement therapies are not associated with CVD in men suffering hypogonadism, overall suggesting that elevated levels of testosterone are not the responsible of the increase in CVD risk in men (Elagizi et al., 2018). As with the rest of sex hormones, testosterone acts at the level of gene expression. Testosterone binds and activates the androgen receptor, which is expressed in a variety of tissues including the liver.

In this study, we determined the effect of finasteride on glucose and lipid parameters commonly associated with atherosclerotic CVD risk. Finasteride inhibits the conversion of testosterone to its active metabolite DHT. Finasteride is among the top 100 prescribed medications in the US, being typically prescribed to treat prostate hyperplasia as well as male pattern hair loss. Overall, our clinical data suggested that the consumption of finasteride was associated with positive health effects, especially regarding total cholesterol and glycohemoglobin levels (Table 2.1. and 2.6.). Consumption of alcohol associated with a greater decrease in total cholesterol could be due to the synergistic impact of finasteride and alcohol on hormones. (Table 2.1.) Several associations were not statistically significant but exhibited trend towards positive health outcomes in parameters such as LDL-C, triglyceride, and glucose levels (Tables 2.9., 2.4., 2.5.). While these data were properly controlled, only a relatively small number of participants (n=155) received finasteride in the NHANES survey. Controlled clinical studies using finasteride showed controversial outcomes regarding lipid and glucose parameters, which might be explained by several confounding factors or the duration of the trials, which typically did not examine the effects of finasteride after 1 year. (Denti et al., 2000) (Tenover et al., 1997) (Page et al., 2005).

Our clinical data suggest that finasteride could have positive effects in metabolic parameters linked to atherosclerotic CVD. However, the study of atherosclerosis in humans is

challenging as it typically involves complicated imaging techniques or post-mortem analyses of atherosclerotic lesions. This prompted us to perform a dose-response study with finasteride to *Ldlr*^{-/-} male mice, a commonly used mouse model to study atherosclerosis. Finasteride did not cause any signs of hepatic toxicity and resulted in a clear reduction in prostate size at the end of the experiment, consistently with results observed in human and rodents (Edwards et al., 2002).

The highest dose of finasteride, which is comparable to doses reported previously in the literature (Froese et al., 2016), showed a significant reduction in total cholesterol and triglyceride levels in the VLDL/LDL fractions, as evidenced by our lipoprotein fractionation. In contrast, our preclinical data failed to reproduce alterations in glucose metabolism, as highlighted by fasting glucose and glucose tolerance tests measurements.

Finasteride treatment also resulted in a reduction in plaque size, in agreement with a recent report showing that finasteride supplementation in the water reduces plaque size in apolipoprotein E-deficient mice (Liu et al., 2020). In our study, however, we examined the composition of the atherosclerotic lesions, which showed that plaque macrophage content, and necrotic areas were reduced in finasteride-treated mice in comparison to control-fed mice. Since fasting circulating lipid levels are largely driven by liver-derived lipoproteins, we turned our attention towards understanding the effect of finasteride in the liver. We performed an RNA-seq transcriptome profiling in livers of mice fed control or the highest dose of finasteride. Our data show that mice fed finasteride presented with an overall upregulation of pathways related to drug catabolism, indicative of an enhanced activity related to finasteride degradation. Interestingly, finasteride-fed mice also showed an increased oxidative phosphorylation and bile acid synthesis, suggesting that lipid metabolism was upregulated in these mice.

Bile acid synthesis is among the best characterized pathways by which the liver drives the elimination of endogenous cholesterol. (Boyer et al., 2013) Taking a closer look at the genes regulated by finasteride, the 5 β - reductase caught our attention. This enzyme is responsible for the reduction of a large variety of lipids, most of which are precursors of bile acids. It also reduces sex hormones including testosterone to 5 β - inactive derivatives, unlike 5 α - reductases on testosterone, which results in an enhanced binding activity to the androgen receptor. Recently, finasteride was identified as an inhibitor of 5 β - reductase, and our RNA seq data show that finasteride treatment results in the upregulation of 5 β - reductase (Figure 2.7). While finasteride did not alter the expression of type 1 and 2 5 α - reductases, which are also inhibited by finasteride, our results indicate that the alteration of 5 β - reductase levels could result in an enhanced cholesterol conversion to bile acids. Indeed, loss of function mutations in the 5 β - reductase in humans results a reduction of bile acid synthesis, causing developmental bile acid syndrome. (Drury et al., 2010) This disease was first identified in 1988 in neonatal siblings suffering intrahepatic cholestasis. (Setchell et al., 1988) Whether alterations in this enzyme are the main driver of our clinical and preclinical data, will need further investigation.

Our RNA-seq data also revealed an overall downregulation of hepatic inflammatory markers commonly regarded as macrophage markers (Figure 2.7). These results agree with our recent findings in which reduced circulating cholesterol levels cause a reduction in hepatic macrophages (data not shown). Whether hepatic inflammation directly can affect atherosclerosis development is not clearly elucidated, but our data would point out that this reduction can be explained by alterations in our lipid profile.

Acknowledgments

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Figures and Tables

Figures

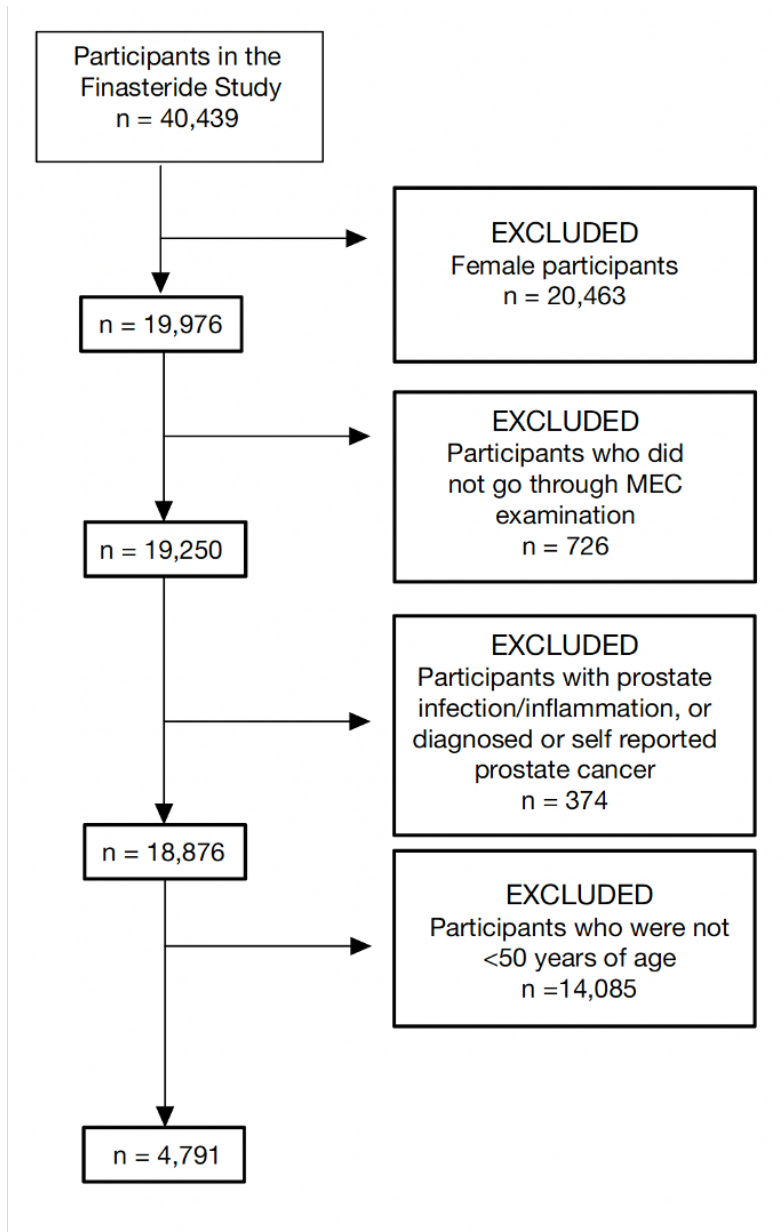


Figure 2.1. Exclusion flow chart of the study protocol. MEC; mobile examination center

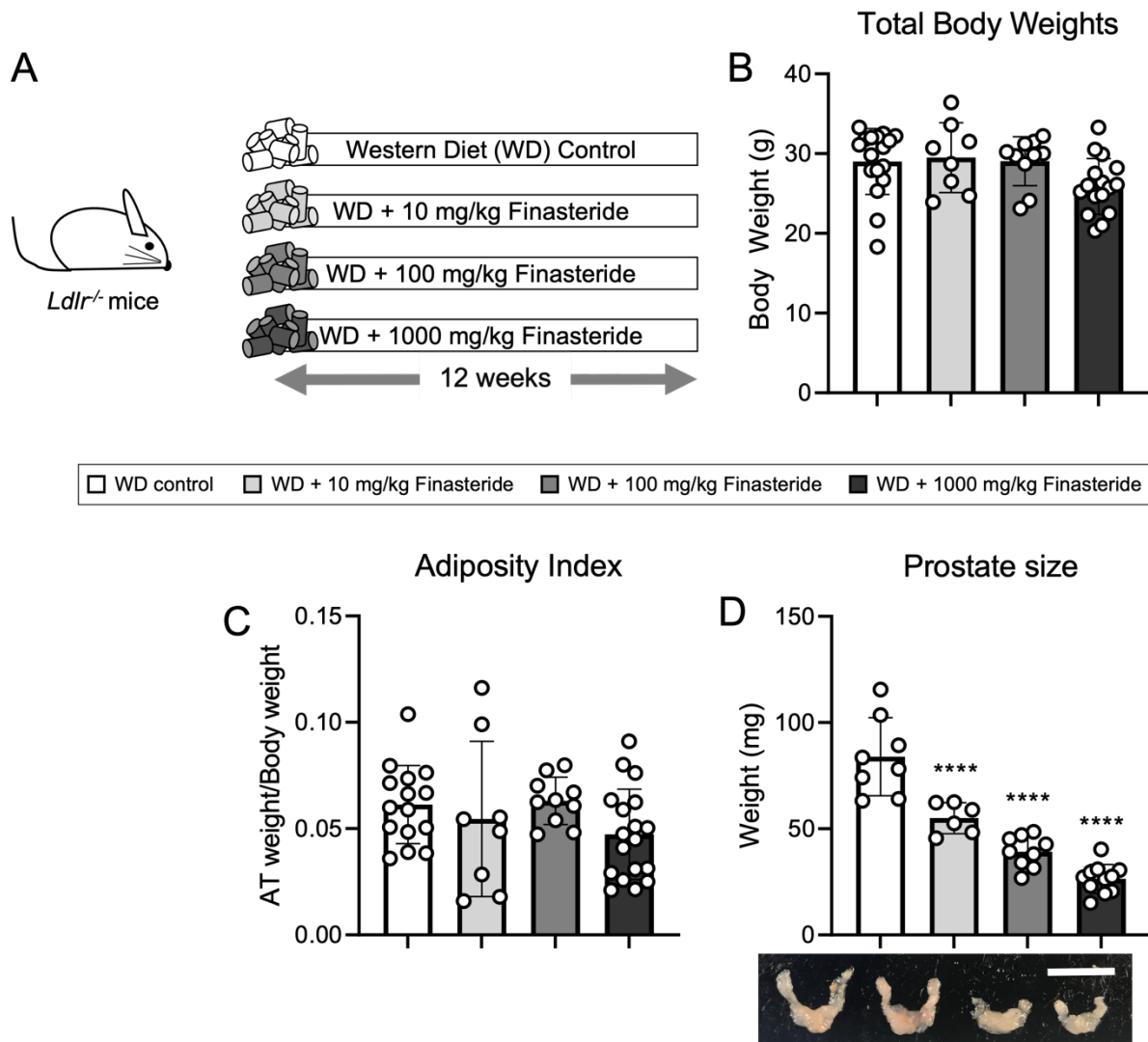


Figure 2.2. Finasteride supplementation reduces total cholesterol and triglyceride plasma levels in *Ldlr*^{-/-} mice. (A) Experimental design. Four-week-old male *Ldlr*^{-/-} mice were fed Western diet with increasing doses of finasteride for 12 weeks before tissue and blood harvesting. (B) body weight (n=55), (C) adiposity index, and (D) prostate size at the moment of the sacrifice. Statistical differences were evaluated using one-way ANOVA ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$ considering *Ldlr*^{-/-} mice fed Western diet Control as reference group.

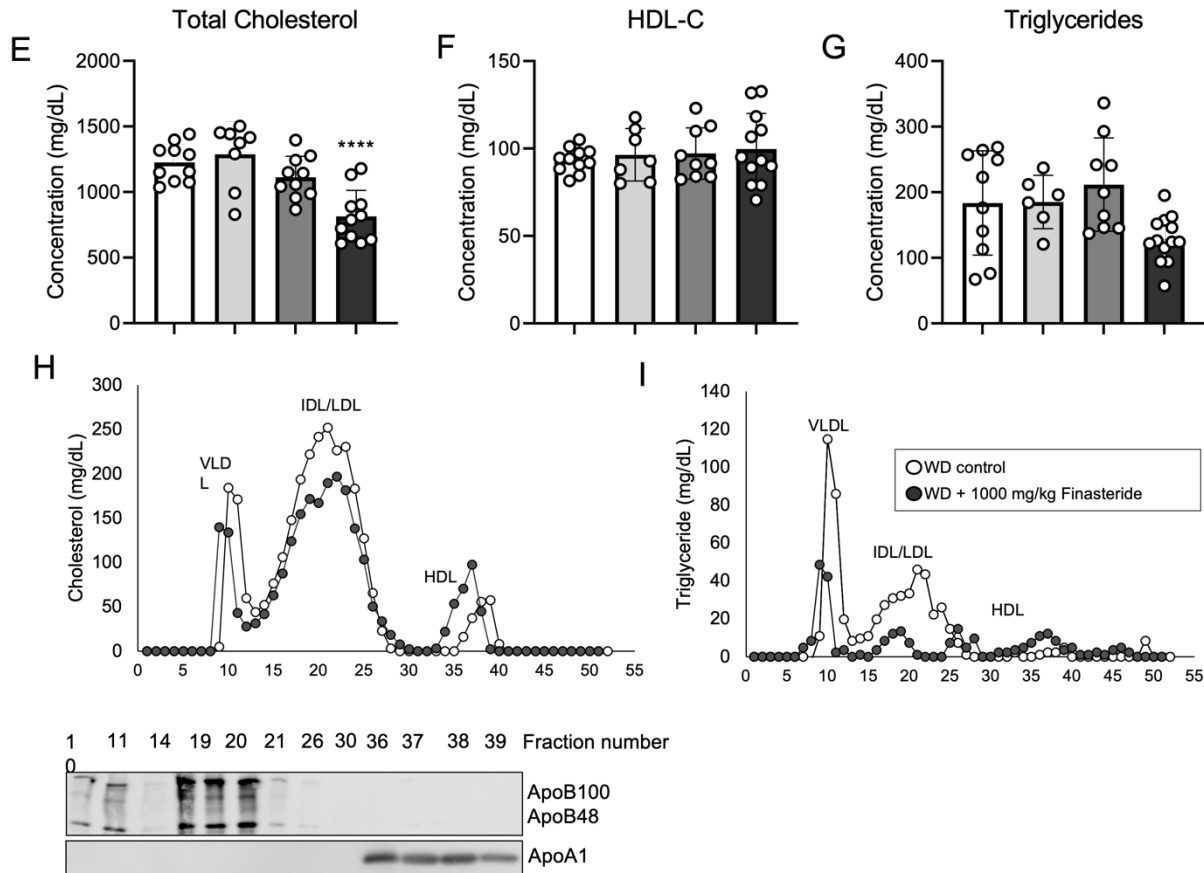


Figure 2.2. (cont.) Finasteride supplementation reduces total cholesterol and triglyceride plasma levels in *Ldlr*^{-/-} mice. (E) Total plasma cholesterol, (F) cholesterol levels in the high-density lipoprotein fraction (HDL-C), and (G) total triglyceride levels were measured in plasma after overnight fasting. Lipoprotein profiles for (H) plasma cholesterol and (I) plasma triglyceride from *Ldlr*^{-/-} mice fed Western diet control or Western diet + 1000 mg/kg of finasteride. The identity of the different fractions was confirmed by western blot. Statistical differences were evaluated using one-way ANOVA ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$ considering *Ldlr*^{-/-} mice fed Western diet Control as reference group.

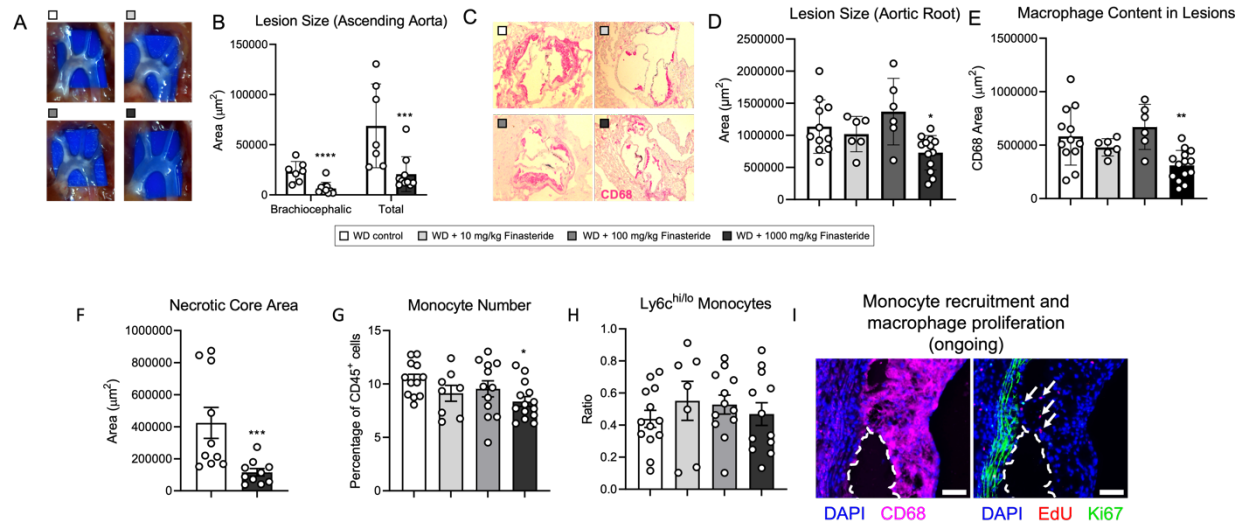


Figure 2.3. Finasteride decreases plaque size and macrophage content in *Ldlr*^{-/-} mice. Four-week-old male *Ldlr*^{-/-} mice were fed Western diet with increasing doses of finasteride for 12 weeks before tissue and blood harvesting. **(A, B)** Atherosclerotic lesions in the aortic arch, and lesion size quantification. **(C)** Atherosclerotic lesion size stained for CD68 and hematoxylin/eosin. **(D)** Quantification of the lesion size in the aortic root, and **(E)** CD68+ cell area, representative of plaque macrophages. **(F)** Necrotic core area **(G)** Percentage of monocytes in the blood, and **(H)** ratio of pro-inflammatory monocytes. **(I)** Representative image of newly recruited monocytes (red) and proliferating macrophages (green) identified by co-staining with CD68 (pink), white arrows (see methods for more detail). White dotted line represents a necrotic core area in the lesion. (Quantification – ongoing). Statistical differences were evaluated using one-way ANOVA or two-tailed student's t test ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$ considering *Ldlr*^{-/-} mice fed Western diet Control as reference group.

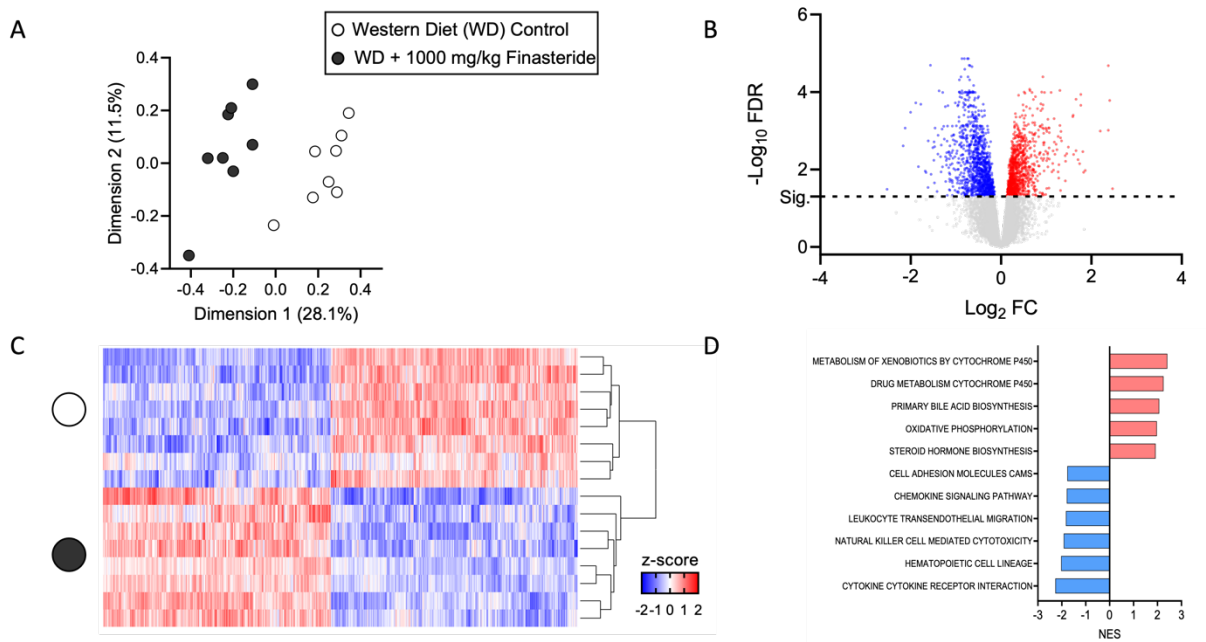


Figure 2.4. Finasteride treatment upregulates bile acid synthesis and downregulates inflammatory pathways in the liver of *Ldlr*^{-/-} mice. Four-week-old male *Ldlr*^{-/-} mice were fed Western diet Control (n=6) or with the same diet supplemented with 1000 mg/kg of finasteride (n=6) for 12 weeks. Total liver mRNA was extracted and sequenced. (A) Principal component analysis, and (B, C) volcano plot and heat map representing genes significantly up-regulated (red) and down-regulated (blue) in response to finasteride supplementation. (D) Significantly upregulated (red) and down-regulated (blue) enriched pathways in the liver of mice.



Figure 2.5. Finasteride does not alter fasting glucose levels or glucose tolerance test. Four-week-old male *Ldlr*^{-/-} mice were fed Western diet with increasing doses of finasteride for 11 weeks before performing a glucose tolerance test (see methods for more details). **(A)** Fasting glucose levels, and **(B)** Glucose tolerance tests were performed under fasting conditions. **(C)** Area under the curve (AUC) calculated for each animal. Statistical differences were evaluated using one-way ANOVA ($p < 0.05$).

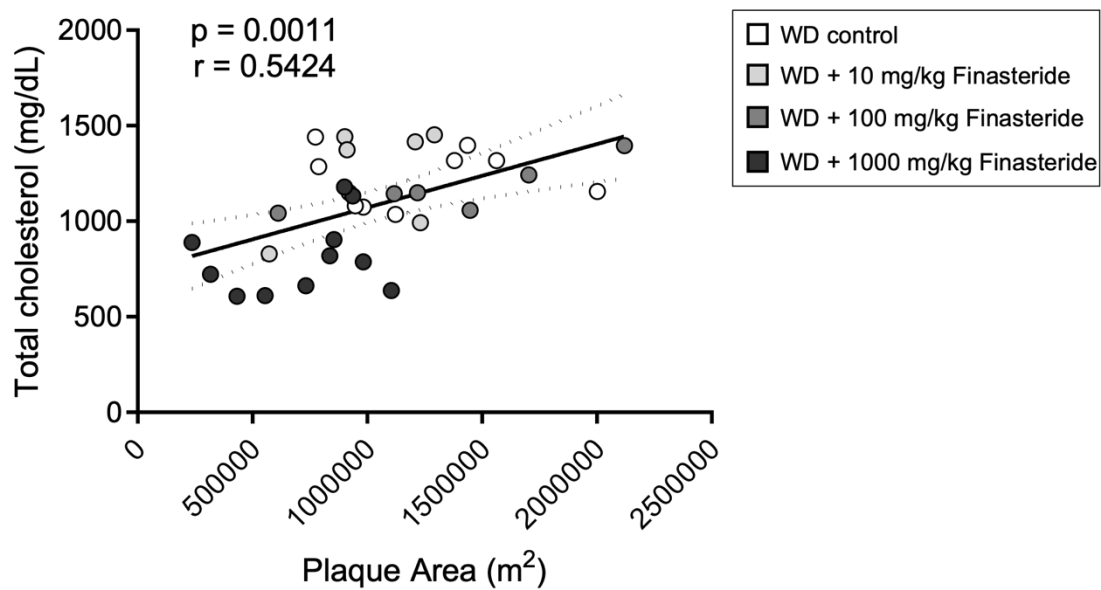
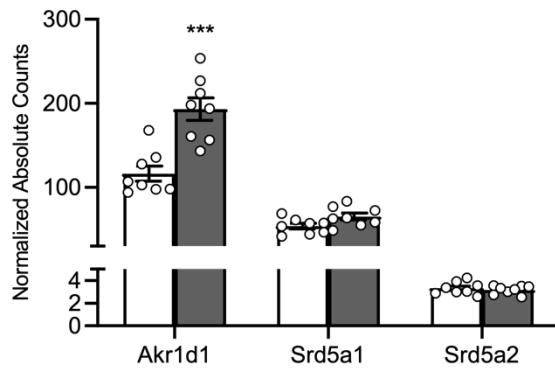


Figure 2.6. Correlation between plaque size and total cholesterol in *Ldlr*^{-/-} mice. Four-week-old male *Ldlr*^{-/-} mice were fed Western diet with increasing doses of finasteride for 12 weeks before tissue and blood harvesting. Lesion area at the level of the aortic root and total cholesterol levels at the moment of the sacrifice.

A



B

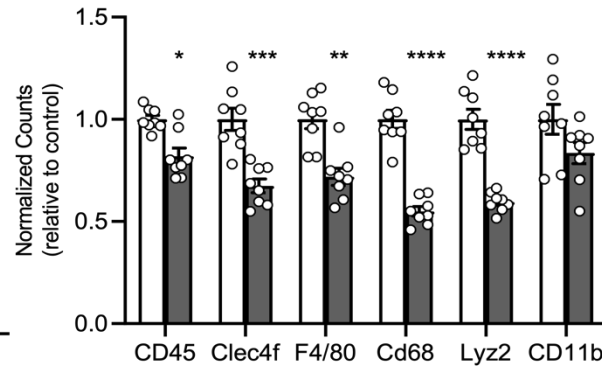
Control 930
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Figure 2.7. Four-week-old male *Ldlr*^{-/-} mice were fed Western diet Control or with the same diet supplemented with 1000 mg/kg of finasteride for 12 weeks. Total liver mRNA was extracted and sequenced. (A) mRNA levels of three finasteride targets. Finasteride is a competitive inhibitor of the 5 β -reductase (*Akr1d1*), and 5 α -reductases *Srd5a1* and *Srd5a2*. (B) Gene expression changes in myeloid cell marker (*CD45*), Kupffer cell marker (*Clec4f*), and macrophage markers (*F4/80*, *Cd68*, *Lyz2*, *CD11b*). Statistical differences were evaluated using adjusted p value (q) obtained from our RNA seq analysis. * q < 0.05, ** q < 0.01, *** q < 0.005, **** q < 0.001 considering *Ldlr*^{-/-} mice fed Western diet Control as reference group.

Tables

Total Cholesterol levels in subjects taking finasteride: NHANES 2009-2016						
Interaction Variable		No Finasteride	Finasteride	p	Test for interaction	
		Mean ± SE	Mean ± SE		F	p
Alcohol > 4/5 drinks per day	Yes	174.17 ± 3.16	137.88 ± 7.96	0.10	2.78	0.1006
	No	174.24 ± 3.04	151.86 ± 4.99			
Diagnosed with cardiovascular disease	Yes	170.41 ± 4.00	123.52 ± 9.12	0.0018	10.6	0.0018
	No	178.00 ± 2.79	166.22 ± 4.64			
Taking drugs that affect glucose metabolism	Yes	170.66 ± 2.84	154.75 ± 5.19	0.016	6.12	0.0161
	No	177.74 ± 3.80	134.99 ± 9.42			

Table 2.1. Total Cholesterol analysis of male patients treated with and without Finasteride: NHANES 2009-2016¹

¹Footnote: Statistical differences were considered significant when $p < 0.05$ and $P < 0.15$ for differences in means between No finasteride and Finasteride groups and the Test for interaction, respectively.

LDL cholesterol levels in subjects taking finasteride: NHANES 2009-2016						
Interaction Variable		No Finasteride	Finasteride	p	Test for interaction	
		Mean ± SE	Mean ± SE		F	p
Diagnosed high blood cholesterol (Diagnosed) and/or taking medications for blood cholesterol (meds)	Diagnosed + meds	103.00 ± 5.52	82.9966 ± 8.79	0.33	2.3	0.086
	Diagnosed + No med	129.12 ± 8.05	102.13 ± 10.21	0.10		
	No diagnosis + meds	90.34 ± 6.57	60.7686 ± 10.59	0.14		
	No diagnosis + No med	102.48 ± 4.69	91.47 ± 6.70			
Taking drugs that affect glucose metabolism	Yes	101.79 ± 4.69	86.8691 ± 5.16	0.063	3.58	0.063
	No	110.68 ± 6.45	81.8128 ± 7.82			

Table 2.2. LDL analysis of male patients treated with and without Finasteride: NHANES 2009-2016

¹Footnote: Statistical differences were considered significant when $p < 0.05$ and $P < 0.15$ for differences in means between No finasteride and Finasteride groups and the Test for interaction, respectively.

HDL cholesterol levels in subjects taking finasteride: NHANES 2009-2016						
		<u>No Finasteride</u>	<u>Finasteride</u>	<u>Test for interaction</u>		
Interaction Variable		Mean ± SE	Mean ± SE	p	F	p
Diagnosed with liver condition	Yes	50.82 ± 3.95	39.06 ± 5.19	0.12	2.48	0.12
	No	49.68 ± 1.78	47.26 ± 2.91			

Table 2.3. HDL analysis of male patients treated with and without Finasteride: NHANES 2009-2016

¹Footnote: Statistical differences were considered significant when $p < 0.05$ and $P < 0.15$ for differences in means between No finasteride and Finasteride groups and the Test for interaction, respectively.

Triglyceride levels in subjects taking finasteride: NHANES 2009-2016						
		<u>No Finasteride</u>	<u>Finasteride</u>	<u>Test for interaction</u>		
Interaction Variable		Mean ± SE	Mean ± SE	p	F	p
Diagnosed with cardiovascular disease	Yes	111.80 ± 17.95	71.46 ± 23.64	0.14	2.15	0.15
	No	137.01 ± 16.92	124.64 ± 14.03			
Diagnosed high blood cholesterol (Diagnosed) and/or taking medications for blood cholesterol (meds)	Diagnosed +Meds	131.96 ± 18.09	72.5278 ± 16.25	0.09	3.41	0.023
	Diagnosed + No meds	135.06 ± 21.11	137.71 ± 26.60	0.059		
	No diagnosis + Med	125.56 ± 21.86	110.16 ± 28.28	0.58		
	No diagnosis + No med	105.05 ± 15.40	71.80 ± 17.76			
Taking drugs that affect glucose metabolism	Yes	129.37 ± 15.40	118.65 ± 12.11	0.096	2.86	0.096
	No	119.44 ± 18.10	77.45 ± 25.24			

Table 2.4. Triglyceride analysis of male patients treated with and without Finasteride: NHANES 2009-2016

¹Footnote: Statistical differences were considered significant when $p < 0.05$ and $P < 0.15$ for differences in means between No finasteride and Finasteride groups and the Test for interaction, respectively.

Glucose levels in subjects taking finasteride: NHANES 2009-2016						
Interaction Variable		No Finasteride	Finasteride	p	Test for interaction	
		Mean ± SE	Mean ± SE		F	p
Diagnosed with liver condition	Yes	130.0 ± 6.8	97.7 ± 12.0	0.12	2.5	0.1
	No	125.8 ± 3.2	116.7 ± 6.9			
Diagnosed with diabetes (Diagnosed), taking anti-diabetic medications (medications)	Diagnosed + medications	153.4 ± 6.9	115.5 ± 9.6	0.045	2.1	0.1
	Diagnosed + no medications	119.8 ± 3.9	104.3 ± 13.8	0.55		
	No diabetes nor prediabetes	110.5 ± 3.5	101.8 ± 7.8			
Diagnosed high blood cholesterol (Diagnosed) and/or taking medications for blood cholesterol (meds)	Diagnosed + meds	129.7 ± 4.8	115.76 ± 9.7	0.91	2.4	0.07
	Diagnosed + no meds	125.1 ± 5.1	129.1 ± 13.1	0.086		
	No diagnosis + meds	132.6 ± 6.5	74.7 ± 21.2	0.095		
	No diagnosis + no meds	124.1 ± 4.5	109.2 ± 9.3			

Table 2.5. Association between finasteride treatment and glucose concentration: NHANES 2009-2016

¹Footnote: Statistical differences were considered significant when $p < 0.05$ and $P < 0.15$ for differences in means between No finasteride and Finasteride groups and the Test for interaction, respectively.

Glycohemoglobin levels in subjects taking finasteride: NHANES 2009-2016						
		No Finasteride	Finasteride	p	Test for interaction	
		Mean ± SE	Mean ± SE		F	p
Smoker	Yes	6.4 ± 0.1	5.7 ± 0.2	0.0022	10.2	0.002
	No	6.2 ± 0.08	6.3 ± 0.1			
Body mass index	Underweight (below 18.5)	6.2 ± 0.1	6.6 ± 0.3	0.0046	4.7	0.004
	Normal (18.5 – 24.9)	6.3 ± 0.06	5.7 ± 0.2	0.52		
	Overweight (25 - 29.9)	6.3 ± 0.07	5.9 ± 0.1	0.84		
	Obese (30 and above)	6.5 ± 0.09	5.9 ± 0.2			

Table 2.6. Glycohemoglobin analysis of male patients treated with and without Finasteride: NHANES 2009-2016

¹Footnote: Statistical differences were considered significant when $p < 0.05$ and $P < 0.15$ for differences in means between No finasteride and Finasteride groups and the Test for interaction, respectively.

Parameter	Control	10 mg/kg	100 mg/kg	1000 mg/kg	P-Value
White blood cells (10^9 L)	2.82 ± 0.3	2.93 ± 0.26	3.096 ± 0.22	$3.25 \pm .69$	0.69
Neutrophils (10^9 L)	0.67 ± 0.08	0.75 ± 0.11	0.91 ± 0.15	0.98 ± 0.16	0.98
Lymphocytes (10^9 L)	1.95 ± 0.22	1.92 ± 0.16	1.90 ± 0.07	1.87 ± 0.36	0.36
Monocytes (10^9 L)	0.063 ± 0.01	0.77 ± 0.02	0.084 ± 0.001	0.194 ± 0.07	0.17
Eosinophils (10^9 L)	0.11 ± 0.02	0.13 ± 0.04	0.162 ± 0.03	0.098 ± 0.05	0.098
Basophils (10^9 L)	0.044 ± 0.01	0.05 ± 0.03	0.04 ± 0.007	0.038 ± 0.03	0.0375
Red blood cells (10^9 L)	8.38 ± 0.27	7.66 ± 0.65	8.05 ± 0.084	8.90 ± 0.14	0.14
Hemoglobin (10^9 L)	12.8 ± 0.4	13.6 ± 1.05	12.9 ± 0.16	14.2 ± 0.5	0.50
Platelets (10^9 L)	904 ± 141.9	1005 ± 95.5	750 ± 24.5	795 ± 53.7	0.53

Table 2.7. Effects of finasteride supplementation on circulating immune cells.

Parameter	Control	10 mg/kg	100 mg/kg	1000 mg/kg	P-value
Average food Intake, (g/mouse/day)	2.7 ± .15	2.6 ± .14	2.6 ± .20	2.4 ± .12	0.5741
Liver weight (g)	60.7 ± 5.1	44.8 ± 6.8	63.1 ± 3.5	47.4 ± 5.0	.060
Plasma ALT activity, (mU/mL)	8.2 ± 1.16	13 ± 0.89	15.23 ± 0.94	13 ± 1.3	0.87
Plasma AST activity, (mU/mL)	19.17 ± 1.58	16.17 ± 1.68	19.67 ± 2.52	16.83 ± 2.07	0.70
<i>miRNA-122</i> expression (relative to cel-miR-39)	1.0 ± .29	0.59 ± .19	0.66 ± 0.14	0.49 ± .25	0.58

Table 2.8. Average food intake and liver toxicity in *Ldlr*^{-/-} mice fed finasteride. ALT, alanine transaminase; AST, aspartate aminotransferase

Chapter 3

Future Studies

The reasons for the known sex differences in CVD requires further insight. In our preclinical and clinical models, we specifically focused on the relationship between alterations in sex hormones through finasteride treatment and the progression of atherosclerosis. Finasteride, a specific inhibitor of the conversion of testosterone to DHT, is used by a diverse population throughout the lifetime: Younger populations tend to take finasteride at a lower dose (1mg/kg day) at the onset of androgenic alopecia and will take for years if not decades. Likewise, older men tend to take finasteride at a higher dose (5mg/kg day) to combat prostate diseases. Current literature on atherosclerotic CVD focuses on the pathology of progression but new models allow us to study the potential of atherosclerotic regression. For this purpose, the future aims of this study are twofold:

Atherosclerosis Progression

Progression of atherosclerosis begins early in life and is exacerbated by years of modifiable and non-modifiable risk factors (Figure 1.1.). Many finasteride users are exposed to treatment long term and could be model to further investigate this aspect of the disease. Our findings suggest finasteride has a beneficial impact on pro-atherogenic parameters in humans and in a preclinical, progression model (*Ldlr*^{-/-} mice). Liver RNA sequencing revealed an approximately 2x increase in expression of 5 β reductase (AKR1D1), a highly abundant protein in the liver (top 5% more expressed genes in the liver in our RNA seq dataset). The activity of AKR1D1 is minimally inhibited by finasteride. However, its dramatic upregulation in the liver of

finasteride-treated mice could be the causal link in increased cholesterol elimination in *Ldlr*^{-/-} mice.

Others in our lab have used liver-specific adeno-associated viral vectors (AAV) to overexpress enzymes in mice, a strategy that could be a useful tool to overexpress AKR1D1 in mice. With this strategy, we could investigate if this enzyme is responsible of the positive effects of finasteride reported in our study. Comparing these findings with the patients treated with finasteride for androgenic alopecia is important in the search for combination therapeutics to slow atherosclerosis before any cardiovascular event takes place.

Atherosclerosis Regression

Many older populations do not take measures to combat the progression of atherosclerotic CVD until it presents in some sort of cardiovascular event such as stroke or myocardial infarction. For this population, we need therapeutics that induce regression of atherosclerosis, since the disease has developed (=progressed) throughout their entire lifespan. Atherosclerosis regression can be studied using several strategies including anti-sense oligonucleotides targeting the LDLR. (Basu et al., 2018) We have utilized this approach in our lab, and we have observed that wild-type mice fed a Western diet and injected weekly with ASO-LDLR develop atherosclerosis. Following this approach, we could utilize wild-type mice to induce atherosclerosis for 16 weeks. After this time, we would kill a baseline group, and induce atherosclerosis regression by discontinuing the use of ASO-LDLR. After a few days, mice will express the LDLR, resulting in the normalization of plasma cholesterol levels and starting atherosclerosis regression. During this time, we would feed a cohort of mice a control diet and the same diet containing 1000 mg/kg of finasteride for four more weeks. This approach would allow us to determine whether finasteride stimulates atherosclerosis regression.

Since our data suggest that finasteride favors hepatic cholesterol elimination, it is possible that a combined treatment using this drug, together with cholesterol-lowering agents such as statins, could have an additive effect on atherosclerosis regression.

Comparing potential patients that require combination therapeutics, there is an interesting connection to the population of older individuals who are treated with finasteride for prostate diseases. Further investigation into this population compared to the regression animal model could give a more complete picture of the potential for using testosterone altering drugs on the development of CVDs and more specifically, the rationale for using finasteride as a CVD therapy.

Conclusion

Further research into combination therapy for atherosclerotic CVD are necessary. From this study, we observed that finasteride treatment lowered total cholesterol in humans and reduced pro-atherogenic parameters in an atheroprone mouse model. The diverse patient population taking finasteride offers a pertinent way to examine the relationship between androgen alterations and CVD.

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