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Acute Sex Steroid Withdrawal Increases Cholesterol Efflux Capacity and HDL-Associated Clusterin in Men

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Abstract

Exogenous androgens can lower HDL-cholesterol (HDL-C) concentrations, yet men with low serum testosterone have elevated rates of cardiovascular disease (CVD). HDL function may better predict CVD risk than absolute HDL-C quantity. We evaluated the acute effects of medical castration in men on HDL-C, cholesterol efflux capacity and HDL protein composition. Twenty-one healthy men, ages 18–55, received the GnRH antagonist acyline and one of the following for 28 days: Group 1: placebo, Group 2: transdermal testosterone gel and placebo, Group 3: transdermal testosterone gel and an aromatase inhibitor. Sex steroids, fasting lipids, and cholesterol efflux to apoB-depleted serum were measured in all subjects. The HDL proteome was assessed in Group 1 subjects only. In Group 1, serum testosterone concentrations were reduced by >95%, and HDL-C and cholesterol efflux capacity increased ($p=0.02$ and $p=0.04$ vs. baseline, respectively). HDL-associated clusterin increased significantly with sex steroid withdrawal ($p=0.007$ vs. baseline). Testosterone withdrawal in young, healthy men increases HDL-C and cholesterol efflux capacity. Moreover, sex steroid deprivation changes HDL protein composition. Further investigation of the effects of sex steroids on HDL composition and function may help resolve the apparently conflicting data regarding testosterone, HDL-C, and CVD risk.

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Keywords

testosterone; estradiol; HDL cholesterol; cardiovascular disease; apolipoproteins; atherosclerosis

Introduction

Male sex is associated with an increased risk for the development of cardiovascular disease (CVD). The mechanisms underlying this elevated risk remain unclear, but it has been suggested that testosterone may play a role in promoting CVD. This assumption is predicated in part on the well-recognized effects of sex steroids on plasma lipids. Androgen deprivation therapy (ADT) for the treatment of prostate cancer increases high density lipoprotein-associated cholesterol (HDL-C) concentration, whereas the administration of exogenous testosterone can lower HDL-C and induce modest decreases in low density lipoprotein-associated cholesterol (LDL-C) [1]. In apparent contrast to these effects on HDL-C, recent epidemiologic data demonstrate an elevated risk of CVD and mortality among men with low circulating androgen levels [2, 3]. Moreover, intervention studies demonstrate that marked increases in HDL-C secondary to pharmacologic treatment can be associated with increased CVD and mortality risks [4]. The effects of androgens on HDL-C concentration alone are therefore unlikely to explain fully the impact of androgens on CVD risk in men.

In an effort to better understand the mechanisms by which HDL impacts CVD risk, recent investigations have focused on alternative metrics, including HDL function and protein composition [5, 6]. The cholesterol efflux capacity of serum HDL, for example, has been proposed as a better predictor of extant coronary artery disease than fasting HDL-C concentration [7]. Importantly, HDL particles comprise a complex mixture of proteins and lipids. For example, greater than 50 proteins have been identified in HDL isolated by centrifugation, and detailed analyses have revealed significant differences in constituent proteins between subjects with and without CVD or CVD risk factors [6, 8, 9], suggesting that functional and compositional variation in HDL associates with CVD risk.

We performed a placebo-controlled intervention study to determine the metabolic effects of acute androgen deprivation in young, healthy men [10]. We used samples from this study to explore the effects of testosterone withdrawal on cholesterol efflux capacity. In addition, we investigated changes in the HDL proteome conferred by sex steroid withdrawal.

Experimental

Study protocol

Subject recruitment and the study design have been reported previously [10]. All study visits were performed at the University of Washington Medical Center where the Institutional Review Board approved all study procedures. In brief, healthy men ages 18–55 were recruited through advertisement. Study participants had no chronic medical or reproductive conditions, were taking no medications including steroids, and had normal baseline physical examinations, serum chemistries, complete blood counts, gonadotropins, and normal serum total testosterone concentrations (10.4–34.7 nmol/L). Informed consent was obtained from all study participants.

To suppress testosterone production, all subjects received the gonadotropin releasing hormone (GnRH) antagonist acyline 300 mcg/kg by subcutaneous injection every 2 weeks for 2 cycles [11]. The first 8 enrolled subjects were assigned to Group 1 and received daily placebo transdermal gel and daily oral placebo pills for 28 days. The next 16 enrolled

subjects were randomly assigned to Groups 2 and 3. In addition to acyline, these subjects received 10 grams of transdermal testosterone gel (1% Testim, Auxilium Pharmaceuticals, New Jersey) daily. Group 2 subjects also received daily placebo pills while Group 3 subjects were administered 1 mg oral anastrozole daily (Arimidex, AstraZeneca, Wilmington, DE) to selectively suppress estradiol production. Subjects returned on Days 0, 14, 28, and 56 (follow-up) for study visits that included a physical examination, a fasting blood draw, and adverse event monitoring. Lipid data were analyzed from all subjects with normal baseline HDL-C values (0.78–1.81 mmol/L). Twenty-two subjects completed all study procedures, and 21 were included in the final analysis (7 in Group 1, 6 in Group 2 and 8 in Group 3).

Laboratory assessments

Specimen collection and processing were performed by only 2 study personnel according to rigorous protocol guidelines that were followed uniformly across study subjects. Samples in the present study were held at 4°C immediately subsequent to collection, and serum was stored at –80 °C until completion of the study. Assays were run in a single batch for all study participants. Serum estradiol, total testosterone, and sex hormone binding globulin (SHBG) were measured by radioimmunoassay [10]. Fasting serum lipids (LDL-C, total cholesterol, HDL-C, triglycerides) were measured by the Northwest Lipid Research Center using standard assays (Seattle, WA)[10].

Cholesterol efflux capacity of apoB-depleted serum

The cholesterol efflux capacity of apolipoprotein B (apoB)-depleted serum was evaluated on Days 0, 28, and 56 in a single assay for all groups. Serum cholesterol efflux capacity was determined using a recently described method characterized by intra- and interassay coefficients of variation of 5% and 9%, respectively [7]. Briefly, J774 cells were cultured in DMEM with 10% fetal bovine serum. Cellular pools of free and total cholesterol were radiolabeled by incubation with [³H] cholesterol (1 µCi/ml, Perkin Elmer) in DMEM containing 1 mg/ml fatty acid-free bovine serum albumin (FAFA) and the ACAT inhibitor Sandoz 58–035 (5 µg/ml, Sigma) overnight. After two washes, cells were treated overnight with 0.5 mM 8-Br-cAMP to induce ABCA1 expression. To assay cholesterol efflux, the cells then were incubated with DMEM/FAFA with or without 2.8% apoB-depleted serum (equivalent to 2% serum) for 4 hours at 37°C. ApoB-containing particles were depleted from serum by polyethylene glycol precipitation [12]. At the end of the incubation, the cells were chilled on ice, medium was collected and filtered to remove detached cells, and the [³H] cholesterol content of medium and cells was measured. Cholesterol efflux assay results are expressed as fraction of total [³H] cholesterol released into the medium after subtraction of values obtained in the absence of serum. Samples were run in duplicate, and mean values were used for statistical analysis.

HDL protein composition

The HDL proteome was analyzed by mass spectrometry on Days 0, 28, and 56 for Group 1 subjects only. Analysis was performed by a method previously described [6]. Briefly, HDL (d=1.063–1.210 g/mL) was purified from EDTA-anti-coagulated human plasma using sequential density gradient ultracentrifugation. After dialysis to remove KBr, 10 µg HDL was denatured with Rapigest (Waters, Milford, MA), reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (trypsin:protein, 1:50). Peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an LTQ linear ion trap mass spectrometer (Thermo, Waltham, MA) equipped with a Magic C18AQ column (0.10 × 150 mm, Micron) and a 180 min gradient of acetonitrile/0.1% formic acid. Tandem mass spectra were searched against the human proteome database (human IPI v. 3.76) and interpreted using Sequest, Peptide Prophet, and Protein Prophet algorithms [13–

15]. Only HDL-associated proteins with mean spectral counts >5 were included in the final analysis.

Serum clusterin

Serum concentrations of clusterin (apolipoprotein J) were determined using a commercially available ELISA according to the manufacturer's instructions (Biovendor, Brno, Czech Republic).

Statistical analysis

As Group 1 was recruited independently from Groups 2 and 3, statistical analyses were limited to changes from baseline within a given group, and between-group comparisons were not performed. Comparison of results from the end of treatment and recovery with baseline were made using a Wilcoxon sign-rank test. Correlations were performed using Spearman's technique. Statistical analyses were performed using STATA version 10 (College Park, TX, USA). Proteomic data were analyzed with paired, 2-tailed Student's *t* test using spectral count totals for each identified protein. To correct for multiple comparisons, *p* value thresholds for statistical significance were determined using a modified Bonferroni correction [16] with classes of variables determined by Pearson correlations and a threshold of 0.8. For cholesterol efflux capacity, a *p* value <0.05 was considered statistically significant. For fasting lipids, a *p* value of <0.036 was significant, and for HDL-associated proteins, a *p* value <0.015 was considered significant.

Results

Study participants

Twenty-one subjects were included in the presented analysis. There were no serious adverse events and no clinically significant changes in liver function tests, blood chemistries, or blood counts in any of the subjects [10].

At baseline, subjects in all study groups were healthy, normotensive, eugonadal men. The average age among the participants was 30.0±10.5 years, and the average body mass index (BMI) was 25.5±3.0 kg/m². No significant changes in BMI or body weight were evident in any of the treatment groups during the study [10].

Sex steroid levels

Detailed hormonal analyses have been presented previously [10]. Subcutaneous injection of acyline suppresses gonadotropins within 4 hours of administration and results in medical castration (testosterone <1.7 nmol/L) within 24 hours [11]. Accordingly, Group 1 subjects uniformly exhibited marked decreases in testosterone levels for the duration of the study period (D0: 14.8±3.3 nmol/L, D28: 0.9±0.9 nmol/L, *p*<0.0001). Substantial reduction also was observed in serum estradiol concentrations in this group (D0: 94±19 pmol/L, D28: 34±10 pmol/L, *p*<0.0001). In Group 2 subjects, transdermal testosterone administration resulted in serum concentrations of testosterone (D0: 16.3±3.3 nmol/L, D28: 17.8±5.5 nmol/L, *p*=0.65) and estradiol (D0: 118±29 pmol/L, D28: 109±29 pmol/L, *p*=0.46) that remained in the physiologic range. In Group 3 the addition of anastrozole resulted in significantly decreased concentrations of serum estradiol (D0: 96±22 pmol/L, D28: 37±14 pmol/L, *p*<0.001) without alteration in serum testosterone concentrations (D0: 16.5±2.6 nmol/L, D28: 19.0±7.3 nmol/L, *p*=0.34). No changes were observed in serum SHBG in any of the groups [10].

Fasting lipid profiles

In Group 1, sex steroid deprivation did not confer changes in total cholesterol, LDL-C, or triglycerides. As expected, a significant increase in HDL-C was evident and occurred uniformly across Group 1 subjects (Table 1, Figure 1, D0: 1.19 ± 0.2 mmol/L v. D28: 1.37 ± 0.2 mmol/L, $p=0.02$). No changes in lipid profiles were observed in Groups 2 and 3 (Table 1), indicating that the increase in HDL-C evident in Group 1 was specifically attributable to suppression of serum testosterone.

Cholesterol efflux capacity of apoB-depleted serum

Cholesterol efflux was evaluated by assessing the fraction of radiolabeled cholesterol removed from lipid-loaded macrophages after incubation with HDL-containing serum from an individual subject. Cholesterol efflux quantified by this method recently has been shown to be lower in patients with known coronary artery disease [7]. In Group 1 subjects, a significant increase in cholesterol efflux was evident with sex steroid deprivation (D0: $6.7 \pm 1\%$ v. D28: $7.4 \pm 0.7\%$, $p=0.03$). The increase in efflux was consistent across subjects, as efflux was higher on Day 28 than Day 0 in 6 of 7 subjects (Figure 2).

Moreover, cholesterol efflux returned to baseline on Day 56, after restoration of endogenous testosterone and estradiol production (Table 2). No significant changes in cholesterol efflux capacity were observed in either Group 2 or Group 3, suggesting that the changes evident in Group 1 resulted specifically from suppression of serum testosterone.

The magnitude of change in testosterone concentration did not correlate with either the change in HDL-C (data not shown) or the change in efflux capacity (Supplementary Figure S1b). Nor was any correlation observed between the magnitude of change in HDL-C and that in cholesterol efflux capacity among Group 1 subjects (Supplementary Figure S1a). No correlation was evident between serum cholesterol efflux capacity and HDL-C at baseline or after 4 weeks of acyline treatment (Supplementary Figures S2a and S2b, respectively).

HDL proteome

To determine whether changes in cholesterol efflux capacity were associated with changes in HDL protein composition, mass spectrometry was employed to evaluate the proteome of HDL isolated by centrifugation from serum of Group 1 subjects. As expected [17], apolipoprotein A-1 was the most abundant protein in HDL and did not vary with sex steroid manipulation. On Day 28, significant increases were observed in HDL-associated clusterin and apolipoprotein A-IV (apoA-IV) (Table 3; $p=0.007$ and $p=0.04$, respectively, for D0 v. D28). After correction for multiple comparisons, however, only the change in clusterin remained significant. Notably, clusterin levels in HDL increased in 6 of the 7 Group 1 subjects (Figure 3). Whereas apoA-IV returned to baseline levels on day 56, there was a trend toward a sustained increase in HDL-associated clusterin (D56: 12.3 ± 2.4 , $p=0.056$ v. D0) despite restoration of endogenous sex steroids. No change in individual HDL-associated proteins correlated with the observed changes in HDL-C or cholesterol efflux capacity. Changes in HDL-associated clusterin or apoA-IV did not correlate with changes in serum testosterone or estradiol concentrations.

Serum Clusterin

To determine whether the observed changes in clusterin were specific to HDL or simply represented a consequence of changes in circulating clusterin levels, we assessed the concentration of clusterin in serum in Group 1 subjects. In contrast to clusterin associated with HDL, serum clusterin levels did not change with acyline treatment (D0: 54 ± 18 $\mu\text{g/mL}$, D28: 58 ± 14 $\mu\text{g/mL}$, $p=0.34$). Furthermore, no correlation was evident between serum and

HDL-associated clusterin levels (D0: $R=-0.26$, D28: $R=-0.11$, $p=NS$). Thus, the effect of sex steroid deprivation appeared specific to clusterin associated with HDL.

Discussion

Recent investigations have focused upon understanding HDL as a heterogeneous particle with pleiotropic functions and suggest that the effects of HDL on CVD risk are not captured by HDL cholesterol content alone [7, 9, 18]. HDL particles appear to exert anti-inflammatory effects [5, 19] and play a critical role in cholesterol efflux from macrophages that reside within the artery wall [20]. In the present study, we demonstrate that acute sex steroid withdrawal increases the serum cholesterol efflux capacity in young, healthy men. Further, we found that the enhanced efflux capacity is specifically attributable to androgen rather than estradiol deprivation. Sex steroid deprivation also confers changes in the protein composition of HDL, changes that could directly impact cholesterol efflux or other HDL effector functions. These findings underscore the complexity of the relationship between HDL and sex steroid exposure.

The modulatory effects of androgens on CVD risk remain poorly understood. Historically, testosterone has been presumed to augment risk on the basis of its HDL-C lowering effect and the earlier onset of CVD in men relative to women. Yet recent prospective epidemiologic data now suggest the converse, as low endogenous circulating androgens predict CVD and mortality risk in men [2, 3]. Similarly, men who have undergone ADT for the treatment of prostate cancer exhibit a disproportionately increased incidence of CVD compared to age-matched controls [21, 22]. These inconsistent findings in part may derive from comparison of interventional and observational data, as the latter particularly may be confounded by the presence of co-morbid conditions, such as obesity and chronic illness, that independently can alter testosterone and HDL-C concentrations and overall CVD risk [1]. Alternatively, the apparent discrepancy may result from the heterogeneous biological processes that underlie atherosclerosis, many of which may be modulated by sex steroids. Further, despite the consistent, negative correlation between endogenous HDL-C concentrations and CVD risk, it remains highly uncertain whether changes in HDL-C due to clinical interventions translate into modified risk [4, 23].

Many lines of evidence support the proposal that a primary atheroprotective role of HDL is the mediation of reverse cholesterol transport, the process by which HDL unloads cholesterol from peripheral tissues and transports it to the liver [24, 25]. Animal studies provide compelling evidence that one key mechanism involves the removal of cholesterol from lipid-laden macrophages, which are of central importance in the pathogenesis of atherosclerosis [26]. Rader, Rothblat, and colleagues have recently developed an assay to determine the capacity of serum to accept cholesterol from macrophages [(7, 27)]. As this assay measures the efflux capacity of apoB-depleted serum, it has been used as a surrogate for HDL-mediated function. These investigators demonstrated that low cholesterol efflux capacity as measured by this assay predicted the presence of coronary artery disease in a clinical cohort [7, 27]. Moreover, the relationship between cholesterol efflux and coronary artery disease persisted after correction for HDL-C, suggesting that this metric of HDL function might prove a key indicator of CVD risk. Indeed, efflux capacity was a better predictor of CVD status than was HDL-C [7]. These observations raise the question of whether HDL-C alone is an adequate assessment of HDL-associated CVD risk. These findings also underscore the potential disconnect between the cholesterol content and functional significance of HDL.

While our findings suggest that androgen withdrawal increases cholesterol efflux from macrophages, prior work *in vitro* has suggested that androgen supplementation with

physiologic and supraphysiologic doses of testosterone might augment HDL₃-mediated cholesterol efflux from macrophages [28]. Although it is possible that both sex steroid withdrawal and replacement produce similar changes in efflux, these apparently discrepant results instead may be attributable to the different experimental methods employed. In our study, we manipulated sex steroids in men for 4 weeks, allowing time for *in vivo* modifications of HDL to occur, and then examined functional capacity of total HDL. In contrast, previous analyses applied sex steroids *in vitro* and only examined efflux mediated by the small, dense HDL₃ subfraction. Notably, these investigators found that testosterone treatment increased hepatocyte and macrophage expression of scavenger receptor B1 (SRB1), a receptor implicated in HDL uptake in the liver [28]; accordingly, the reduction in HDL-C often observed with testosterone treatment potentially could result from accelerated hepatic uptake of HDL-derived cholesterol and paradoxically predict reduced atherosclerotic risk. Of note, the assay employed in our study quantifies cholesterol efflux predominantly mediated by the ABCA1 transporter; future studies are warranted to determine whether sex steroids exert differential effects on pathways mediated by other cholesterol transport proteins *in vivo*.

Previous models also have demonstrated the *in vivo* effects of sex steroids on HDL-C, HDL-associated proteins, and cholesterol efflux capacity. The synthetic steroid tibolone decreased whereas conjugated equine estrogens increased serum efflux capacity in a primate model [29]; the effect of tibolone may be dose-dependent, as decreases in cholesterol efflux have been observed only at higher doses in association with substantial reductions in HDL-C [29, 30]. Acute sex steroid suppression in men further has been shown to exert effects on serum concentrations of HDL-C and HDL-associated proteins [31–33]. A novel aspect of our study is inclusion of a treatment group rendered selectively estrogen-deficient to discriminate between androgen- and estrogen-mediated effects on HDL-C and cholesterol efflux. In addition, we examined cholesterol efflux using a macrophage-based assay that strongly associates with coronary artery disease [7], but larger trials with purely HDL-specific assays are needed to fully define the impact of androgens on HDL-mediated cholesterol efflux. Importantly, too, no data have yet demonstrated that modifying HDL efflux capacity alters CVD risk. Thus, intervention trials with long-term follow-up are required to understand the clinical implications of our findings.

In contrast to anticipated findings, selective estradiol deprivation (Group 3) did not confer changes in HDL-C. In a previous study with a similar design, estradiol deprivation was associated with a decrease in HDL-C [34], and supraphysiologic, exogenous estradiol administration to men undergoing ADT increased HDL-C concentrations [35]. The fact that we did not observe changes in HDL-C with selective estradiol withdrawal may be a function of our small sample size or may reflect the lesser degree of estradiol suppression achieved in our study compared to previous studies using aromatase inhibitors [34].

The functional capacity of HDL appears to be determined in part by its protein composition, and increasing evidence suggests that the protein constituents are modifiable and associate with CVD risk [6, 36]. We therefore investigated whether sex steroid withdrawal modulates the HDL proteome. After only 4 weeks of sex steroid deprivation, we found significant increases in HDL-associated clusterin and apoA-IV, though only the change in clusterin remained significant after correction for multiple comparisons. Consistent with these findings, clusterin is an androgen-responsive target gene in the prostate also known as testosterone-repressed prostate message-2 (TRPM-2), and elevations in prostatic clusterin have been observed in men after ADT [37]. Interestingly, low levels of HDL-associated clusterin have been correlated with CVD risk factors in patients with metabolic syndrome [8]. We also observed a partially sustained increase in HDL-clusterin after restoration of endogenous sex steroids, suggesting that even transient changes in sex steroid exposure

might confer more enduring effects on HDL protein composition. Finally, the observed increase was evident in HDL-associated but not serum clusterin and therefore implicates sex steroids in the specific modulation of HDL protein composition.

Although the *in vivo* significance of apoA-IV remains uncertain, *in vitro* data suggest it may play a role as a regulator of cholesterol ester transport protein (CETP), phospholipid transfer protein (PLTP), and lecithin-cholesterol acyltransferase (LCAT) [38, 39], enzymes that modify the lipid, phospholipid, and protein composition of HDL. HDL isolated from patients with CVD exhibited significant enrichment in apoA-IV compared to HDL from healthy controls [9], though overexpression of apoA-IV significantly attenuated atherosclerosis in a transgenic mouse model [40]. Rodent models have demonstrated sex steroid regulation of apoA-IV, but these findings pertained to mRNA expression rather than HDL-associated protein [41]. Although the increase in apoA-IV did not remain statistically significant after correction for multiple comparisons, the relationship between sex steroid exposure and HDL-associated apoA-IV merits further study in larger trials.

As the HDL proteome was evaluated only in Group 1 subjects, additional investigation is needed to determine whether androgens or estrogens mediate the observed effects on HDL protein composition and the impact of longer-term changes in sex steroids on the HDL proteome. Larger-scale studies eventually might be able to identify composite protein signatures, analogous to a genetic haplotype, that correspond to changes in HDL function.

Conclusion

The current study represents the first intervention trial to examine the effects of differential sex steroid exposure on HDL function and composition men. We found that androgen deprivation increased cholesterol efflux from macrophages and demonstrate that sex steroid manipulation modifies the HDL proteome. Additional research is needed to determine whether sex steroids similarly confer changes in other metrics of HDL function and to clarify the relative contributions of androgens and estrogens to the observed changes in the HDL proteome. Further studies also are warranted to determine whether changes in HDL are evident in the setting of testosterone replacement in hypogonadal men or are specific to sex steroid withdrawal. Most importantly, larger, prospective intervention studies are necessary to determine whether these alterations in HDL function and composition translate into modulation of overall CVD risk in men.

Highlights

- The effects of androgens on cardiovascular disease risk in men remain unclear.
- Androgen deprivation increases cholesterol efflux to apoB-depleted serum in healthy men.
- Sex steroid withdrawal in men increases HDL-associated clusterin.
- Sex steroids appear to impact HDL composition and function in men.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ABCA1 ATP-binding cassette transporter 1

ADT	androgen deprivation therapy
apoA-IV	apolipoprotein A-IV
BMI	body mass index
CETP	cholesterol ester transport protein
CVD	cardiovascular disease
FAFA	fatty acid-free bovine serum albumin
HDL-C	high density lipoprotein-associated cholesterol
LCAT	lecithin-cholesterol acyltransferase
LDL-C	low density lipoprotein-associated cholesterol
PLTP	phospholipid transfer protein
SHBG	sex hormone binding globulin
TRPM-2	testosterone-repressed prostate message-2

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References

1. Wu FC, von Eckardstein A. Androgens and coronary artery disease. *Endocr Rev.* 2003; 24:183–217. [PubMed: 12700179]
2. Laughlin GA, Barrett-Connor E, Bergstrom J. Low serum testosterone and mortality in older men. *J Clin Endocrinol Metab.* 2008; 93:68–75. [PubMed: 17911176]
3. Vikan T, Schirmer H, Njolstad I, Svartberg J. Endogenous sex hormones and the prospective association with cardiovascular disease and mortality in men: the Tromso Study. *Eur J Endocrinol.* 2009; 161:435–442. [PubMed: 19542243]
4. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, et al. Effects of torcetrapib in patients at high risk for coronary events. *N Engl J Med.* 2007; 357:2109–2122. [PubMed: 17984165]
5. Gordon SM, Hofmann S, Askew DS, Davidson WS. High density lipoprotein: it's not just about lipid transport anymore. *Trends Endocrinol Metab.* 2010; 22:9–15. [PubMed: 21067941]
6. Vaisar T, Pennathur S, Green PS, Gharib SA, Hoofnagle AN, Cheung MC, et al. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *J Clin Invest.* 2007; 117:746–756. [PubMed: 17332893]
7. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med.* 2011; 364:127–135. [PubMed: 21226578]
8. Hoofnagle AN, Wu M, Gosmanova AK, Becker JO, Wijsman EM, Brunzell JD, et al. Low clusterin levels in high-density lipoprotein associate with insulin resistance, obesity, and dyslipoproteinemia. *Arterioscler Thromb Vasc Biol.* 2010; 30:2528–2534. [PubMed: 20847305]

9. Vaisar T, Mayer P, Nilsson E, Zhao XQ, Knopp R, Prazen BJ. HDL in humans with cardiovascular disease exhibits a proteomic signature. *Clin Chim Acta*. 2010; 411:972–979. [PubMed: 20307520]
10. Rubinow KB, Snyder CN, Hoofnagle AN, Amory JK, Page ST. Acute testosterone deprivation reduces insulin sensitivity in men. *Clin Endocrinol (Oxf)*. 2011 in press.
11. Herbst KL, Coviello AD, Page S, Amory JK, Anawalt BD, Bremner WJ. A single dose of the potent gonadotropin-releasing hormone antagonist acyline suppresses gonadotropins and testosterone for 2 weeks in healthy young men. *J Clin Endocrinol Metab*. 2004; 89:5959–5965. [PubMed: 15579744]
12. Vallance DT, Byrne DJ, Winder AF. Precipitation procedures used to isolate high density lipoprotein with particular reference to effects on apo A-I-only particles and lipoprotein(a). *Clin Chim Acta*. 1994; 229:77–85. [PubMed: 7988057]
13. Kersey PJ, Duarte J, Williams A, Karavidopoulou Y, Birney E, Apweiler R. The International Protein Index: an integrated database for proteomics experiments. *Proteomics*. 2004; 4:1985–1988. [PubMed: 15221759]
14. Yan W, Lee H, Deutsch EW, Lazaro CA, Tang W, Chen E, et al. A dataset of human liver proteins identified by protein profiling via isotope-coded affinity tag (ICAT) and tandem mass spectrometry. *Mol Cell Proteomics*. 2004; 3:1039–1041. [PubMed: 15269248]
15. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem*. 2003; 75:4646–4658. [PubMed: 14632076]
16. Tukey JW, Ciminera JL, Heyse JF. Testing the statistical certainty of a response to increasing doses of a drug. *Biometrics*. 1985; 41:295–301. [PubMed: 4005384]
17. Karlsson H, Leanderson P, Tagesson C, Lindahl M. Lipoproteomics II: mapping of proteins in high-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry. *Proteomics*. 2005; 5:1431–1445. [PubMed: 15761960]
18. Heinecke J. HDL and cardiovascular-disease risk--time for a new approach? *N Engl J Med*. 2011; 364:170–171. [PubMed: 21226584]
19. Suzuki M, Pritchard DK, Becker L, Hoofnagle AN, Tanimura N, Bammler TK, et al. High-density lipoprotein suppresses the type I interferon response, a family of potent antiviral immunoregulators, in macrophages challenged with lipopolysaccharide. *Circulation*. 2010; 122:1919–1927. [PubMed: 20974999]
20. Rader DJ, Alexander ET, Weibel GL, Billheimer J, Rothblat GH. The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *J Lipid Res*. 2009; 50 Suppl:S189–S194. [PubMed: 19064999]
21. Keating NL, O'Malley AJ, Smith MR. Diabetes and cardiovascular disease during androgen deprivation therapy for prostate cancer. *J Clin Oncol*. 2006; 24:4448–4456. [PubMed: 16983113]
22. Keating NL, O'Malley AJ, Freedland SJ, Smith MR. Diabetes and cardiovascular disease during androgen deprivation therapy: observational study of veterans with prostate cancer. *J Natl Cancer Inst*. 2010; 102:39–46. [PubMed: 19996060]
23. The role of niacin in raising high-density lipoprotein cholesterol to reduce cardiovascular events in patients with atherosclerotic cardiovascular disease and optimally treated low-density lipoprotein cholesterol: baseline characteristics of study participants. The Atherothrombosis Intervention in Metabolic syndrome with low HDL/high triglycerides: impact on Global Health outcomes (AIM-HIGH) trial. *Am Heart J*. 2011; 161:538–543. [PubMed: 21392609]
24. Glomset JA. The metabolic role of lecithin: cholesterol acyltransferase: perspectives from pathology. *Adv Lipid Res*. 1973; 11:1–65.
25. Gordon DJ, Rifkind BM. High-density lipoprotein--the clinical implications of recent studies. *N Engl J Med*. 1989; 321:1311–1316. [PubMed: 2677733]
26. Tall AR. Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. *J Intern Med*. 2008; 263:256–273. [PubMed: 18271871]
27. de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler Thromb Vasc Biol*. 2010; 30:796–801. [PubMed: 20075420]

28. Langer C, Gansz B, Goepfert C, Engel T, Uehara Y, von Dehn G, et al. Testosterone up-regulates scavenger receptor BI and stimulates cholesterol efflux from macrophages. *Biochem Biophys Res Commun.* 2002; 296:1051–1057. [PubMed: 12207878]
29. Mikkola TS, Anthony MS, Clarkson TB, St Clair RW. Serum cholesterol efflux potential in postmenopausal monkeys treated with tibolone or conjugated estrogens. *Metabolism.* 2002; 51:523–530. [PubMed: 11912565]
30. von Eckardstein A, Crook D, Elbers J, Ragoobir J, Ezech B, Helmond F, et al. Tibolone lowers high density lipoprotein cholesterol by increasing hepatic lipase activity but does not impair cholesterol efflux. *Clin Endocrinol (Oxf).* 2003; 58:49–58. [PubMed: 12519412]
31. Behre HM, Bockers A, Schlingheider A, Nieschlag E. Sustained suppression of serum LH, FSH and testosterone and increase of high-density lipoprotein cholesterol by daily injections of the GnRH antagonist cetrorelix over 8 days in normal men. *Clin Endocrinol (Oxf).* 1994; 40:241–248. [PubMed: 8137524]
32. Buchter D, Behre HM, Kliesch S, Chirazi A, Nieschlag E, Assmann G, et al. Effects of testosterone suppression in young men by the gonadotropin releasing hormone antagonist cetrorelix on plasma lipids, lipolytic enzymes, lipid transfer proteins, insulin, and leptin. *Exp Clin Endocrinol Diabetes.* 1999; 107:522–529. [PubMed: 10612483]
33. von Eckardstein A, Kliesch S, Nieschlag E, Chirazi A, Assmann G, Behre HM. Suppression of endogenous testosterone in young men increases serum levels of high density lipoprotein subclass lipoprotein A-I and lipoprotein(a). *J Clin Endocrinol Metab.* 1997; 82:3367–3372. [PubMed: 9329370]
34. Bagatell CJ, Knopp RH, Rivier JE, Bremner WJ. Physiological levels of estradiol stimulate plasma high density lipoprotein2 cholesterol levels in normal men. *J Clin Endocrinol Metab.* 1994; 78:855–861. [PubMed: 8157711]
35. Purnell JQ, Bland LB, Garzotto M, Lemmon D, Wersinger EM, Ryan CW, et al. Effects of transdermal estrogen on levels of lipids, lipase activity, and inflammatory markers in men with prostate cancer. *J Lipid Res.* 2006; 47:349–355. [PubMed: 16299398]
36. Movva R, Rader DJ. Laboratory assessment of HDL heterogeneity and function. *Clin Chem.* 2008; 54:788–800. [PubMed: 18375481]
37. July LV, Akbari M, Zellweger T, Jones EC, Goldenberg SL, Gleave ME. Clusterin expression is significantly enhanced in prostate cancer cells following androgen withdrawal therapy. *Prostate.* 2002; 50:179–188. [PubMed: 11813210]
38. Nieminen T, Kahonen M, Lehtimäki T. The effects of apoA-I/C-III/A-IV, apoE and apoB polymorphisms on carotid artery intima-media thickness. *Future Cardiol.* 2006; 2:179–186. [PubMed: 19804074]
39. Dallinga-Thie GM, Dullaart RP, van Tol A. Concerted actions of cholesteryl ester transfer protein and phospholipid transfer protein in type 2 diabetes: effects of apolipoproteins. *Curr Opin Lipidol.* 2007; 18:251–257. [PubMed: 17495597]
40. Duverger N, Tremp G, Caillaud JM, Emmanuel F, Castro G, Fruchart JC, et al. Protection against atherogenesis in mice mediated by human apolipoprotein A-IV. *Science.* 1996; 273:966–968. [PubMed: 8688083]
41. Srivastava RA, Kitchens RT, Schonfeld G. Regulation of the apolipoprotein AIV gene expression by estrogen differs in rat and mouse. *Eur J Biochem.* 1994; 222:507–514. [PubMed: 8020488]

Figure 1.
An increase in HDL-C was apparent across Group 1 study subjects. * $p < 0.036$ compared with baseline

Figure 2.
HDL cholesterol efflux capacity increased in 6 of 7 Group 1 subjects. * $p < 0.05$ compared with baseline

Figure 3. Clusterin associated with HDL increased in 6 of 7 Group 1 subjects. This increase persisted as a trend subsequent to sex steroid normalization.

Table 1

	Group 1 (n=7)					Group 2 (n=6)					Group 3 (n=8)				
	D0	D28	p (v. Day 0)	D56	p (v. Day 0)	D0	D28	p (v. Day 0)	D56	p (v. Day 0)	D0	D28	p (v. Day 0)	D56	p (v. Day 0)
Total Cholesterol (mmol/L)	4.97 (1.1)	5.44 (1.2)	0.06	4.95 (1.1)	1.0	4.48 (0.4)	4.51 (0.3)	0.06	4.14 (0.6)	0.25	4.56 (1.5)	4.56 (0.9)	0.67	4.27 (1.2)	0.18
LDL Cholesterol (mmol/L)	2.95 (0.8)	3.29 (0.7)	0.35	2.87 (0.5)	0.87	2.77 (0.3)	2.80 (0.2)	0.84	2.49 (0.5)	0.35	2.67 (1.3)	2.75 (0.8)	0.57	2.51 (1.0)	0.56
HDL Cholesterol (mmol/L)	1.19 (0.2)	1.37 (0.2)	0.02*	1.19 (0.2)	0.61	1.32 (0.2)	1.32 (0.1)	0.67	1.32 (0.2)	0.39	1.40 (0.2)	1.32 (0.2)	0.53	1.30 (0.2)	0.32
Triglycerides (mmol/L)	1.79 (2.1)	1.73 (1.4)	0.55	1.89 (2.3)	0.27	0.82 (0.2)	0.86 (0.3)	0.92	0.80 (0.4)	0.67	1.08 (0.5)	1.08 (0.4)	0.89	1.02 (0.3)	0.67

*
p<0.036

Table 2

Apo-B deplete serum cholesterol efflux capacity, with values expressed as mean fraction of total [^3H] cholesterol released into the medium (standard deviation).

	Cholesterol Efflux (%)				
	Day 0	Day 28	p (v. Day 0)	Day 56	p (v. Day 0)
Group 1 (n=7)	6.7 (1.0)	7.4 (0.7)	0.03*	7.0 (0.2)	0.40
Group 2 (n=6)	6.5 (0.6)	6.8 (0.6)	0.15	6.4 (0.9)	0.92
Group 3 (n=8)	6.8 (0.8)	7.2 (0.5)	0.40	6.7 (0.5)	0.67

* p<0.05

Table 3

Changes in HDL-associated proteins evident with sex steroid withdrawal (n=7), with values expressed as mean spectral count (standard deviation).

Protein	Day 0	Day 28	p value
Clusterin	9.6 (2.2)	12.6 (2.0)	0.007*
ApoA-IV	31.9 (8.3)	38.4 (6.1)	0.04
SAA2	3.9 (2.1)	6.6 (3.4)	0.09
β 2 glycoprotein 1	2.5 (0.5)	2.0 (0.6)	0.08
SERPINA	10.7 (2.9)	12.7 (1.5)	0.06
ApoA-I	1332 (381)	1399 (204)	0.56
ApoA-II	299 (71)	321 (31)	0.53
ApoE	51 (15)	51 (17)	0.77

*
p<0.015