High density lipoprotein function is reduced in patients affected by genetic or idiopathic hypogonadism.

Title: High density lipoprotein function is reduced in patients affected by genetic or idiopathic hypogonadism

Short title: High density lipoproteins and testosterone

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Abstract

Background. Low testosterone levels are associated with an increased incidence of cardiovascular (CV) events, but the underlying biochemical mechanisms are not fully understood. The clinical condition of hypogonadism offers a unique model to unravel the possible role of lipoprotein-associated abnormalities in CV risk. In particular, the assessment of the functional capacities of high-density lipoproteins (HDL) may provide novel insights besides traditional risk factors. To determine whether reduced testosterone levels correlate with lipoprotein function, HDL cholesterol efflux capacity (CEC) and serum cholesterol loading capacity (CLC) were evaluated in a series of genetic and rare primary and secondary hypogonadal patients and control subjects. **Methods and Results**. Primary and secondary hypogonadal patients presented with lower HDL ATP-binding cassette transporter A1 (ABCA1)-, ATP binding cassette transporter G1 (ABCG1) - and aqueous diffusion-mediated CEC (-19.6%, -40.9% and -12.9%, respectively), with a 16.2% decrement of total CEC. In the whole series, positive correlations between testosterone levels and both total HDL CEC (r^2= 0.359, p= 0.0001) and ABCG1 HDL CEC (r^2= 0.367, p= 0.0001) were observed. Conversely, serum CLC was markedly raised (+43%) in hypogonadal patients, with primary being increased at a higher extent compared to secondary hypogonadism (18.45 ± 2.78 *vs* 15.15 ± 2.10 µg cholesterol/mg protein) and inversely correlated with testosterone levels (r^2= 0.270, p= 0.001). HDL-C concentrations did not correlate with either testosterone levels, HDL CEC (total, ABCG1 and ABCA1) or serum CLC. **Conclusion(s).** In hypogonadal patients pro-atherogenic lipoprotein-associated changes are associated with lower cholesterol efflux and increased influx, thus offering an explanation for a potentially increased CV risk.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide with a number of over 17 million premature deaths in 2016 (1) and with a high occurrence of cardiovascular (CV) deaths before the age of 65 years in men (2). Among the possible players, the role of sex-steroids is attracting increasing attention. Low levels of testosterone, the key hormone in the control of male puberty, fertility and overall health, correlate, in fact, with both CV risk and incidence of events, but whether this link reflects a causal relationship remains unclear (3). A potential explanation on the role of sex hormones in the control of CV risk can be provided by studies on lipoproteins in patients affected by hypogonadism, a clinical condition characterised by low levels of testosterone. Depending on the anatomical level where the defect occurs, hypogonadism can be classified as primary, when caused by testicular failure, or secondary/central, when due to hypothalamic or pituitary dysfunction (4). If not promptly diagnosed and treated, affected individuals are exposed to various comorbidities, including type 2 diabetes, which might differ according to etiopathogenesis and age of onset (5). Hypogonadal males affected by XXY Klinefelter's syndrome (the most common form of testicular failure) (6) have shown, in a limited number of studies, lower levels of high-density lipoprotein cholesterol (HDL-C) (7) that, from a cross-sectional study may be reduced after testosterone replacement (8).

HDL-C is inversely associated with both CVD and mortality across a wide range of concentrations, thus representing a well-established protective factor (9). While studies conducted in hypogonadal conditions have shown a reduction of HDL-C after testosterone replacement (8, 10), observational studies have also reported that HDL-C concentrations are predominantly lowered in cases reaching supranormal testosterone concentrations (11). Since pharmacological interventions aimed at raising HDL-C levels have not generally supported a beneficial effect on CV outcomes

(12,13), changes occurring after testosterone therapy still have an unclear role in CV occurrence/prevention.

Recent data have shown that HDL-C levels *per se* may not be a reliable indicator of CV risk. Instead, the functional capacity of HDL, *i.e.* the cholesterol efflux capacity (CEC), can provide an independent predictive value (14). Very recently, reduced serum cholesterol efflux capacity was shown to be associated with mortality in patients with –ST elevation myocardial infarction (15). These findings clearly underline the need to focus on HDL function, rather than on HDL-C levels (16). Indeed, the association between HDL CEC and CV risk is well maintained after adjustment for conventional risk factors (17,18). Moreover, since lipid trafficking is a balance between cholesterol efflux and influx, the second leading to direct cholesterol accumulation in arterial macrophages (19), the serum cholesterol loading capacity (CLC) represents another index of atherogenicity. It is in fact raised in pathological conditions leading to a higher CV risk (20).

In the light of present knowledge on CVD risk factors in genetic and non-genetic male hypogonadism and in particular on the uncertain role of testosterone in these processes, the present study aimed at evaluating lipoprotein function, *e.g.* HDL CEC and serum CLC, in a series of hypogonadal men.

2. Methods

2.1 Study participants. Hypogonadal men (n= 20) were selected among patients admitted to our Outpatient Department presenting with signs/symptoms of hypogonadism, associated with low testosterone levels. Specifically, 7 patients were affected by primary hypogonadism as demonstrated by high levels of gonadotropins (4 with Klinefelter syndrome, 1 with congenital primary anorchia and 2 with idiopathic acquired primary hypogonadism with testicular hypoplasia).

104 The remaining 13 were affected by central hypogonadism (21) and low/inappropriately normal
105 levels of gonadotropins (5 with Kallmann syndrome and 8 with isolated hypogonadotropic
106 hypogonadism). None had undergone testosterone replacement therapy. Healthy men (n= 17) age
107 and body mass index (BMI) matched were selected as controls. The study was performed in
108 accordance with the ethical principles in the Declaration of Helsinki for experiments involving
109 humans. The study protocol received approval from the local Ethics Committee (CI_130). Written
110 informed consent was obtained from all study participants or their tutors.

111

112 *2.2 Genetic analyses by targeted Next Generation Sequencing.* Each patient with isolated
113 hypogonadotropic hypogonadism underwent a genetic investigation, using a targeted Next
114 Generation Sequencing (9) technique, to search for rare allelic variants. We extracted the genomic
115 DNA of each patient from peripheral blood lymphocytes using Gene Catcher gDNA 96x10 ml
116 Automated Blood kit (Invitrogen, Life TechnologiesTM). The isolated hypogonadotropic
117 hypogonadism gene panel was designed using Illumina Design Studio (San Diego, CA) and included
118 the following IHH candidate genes: ANOS1(KAL1), FGFR1, PROKR2, PROK2, GNRHR, GNRH1, GNRH2,
119 KISS1, KISS1R, TAC3, TACR3, HS6ST1, FGF8, CHD7, DUSP6, FEZF1, FGF17, FLTR3, IL17, SEMA3A,
120 SEMA3E, SEMA7A, SOX2, SOX10, SPRY4, WDR11, HESX1, NELF. The 28 IHH genes consistently
121 represented in all sequence capture panels were assessed for the purposes of this study. Libraries
122 were prepared using Illumina Nextera Rapid Capture Custom Enrichment kits according to the
123 manufacturer's protocols. All regions not correctly sequenced were recovered with the NexteraVR
124 DNA Library Preparation kit (Illumina, San Diego, CA). For subsequent analyses, we included as "rare
125 variants" all known pathogenic, or rare non-synonymous or splicing-site variants (Minor Allele
126 Frequency, MAF ≤0.01) and novel non-synonymous or splicing-site variants. The frequency and the
127 functional annotation of the identified variants were checked in public and licensed databases

(Ensembl, UCSC Genome browser, 1000 Genome project, ExAC Browser, NCBI, HGMD professional, GnomAD), considering the ethnic groups (Europeans). As previously reported, (22), we excluded common non-synonymous variants with Minor Allele Frequency (MAF) >0.01, synonymous, intronic, and 5' or 3' UTR variants. Each variant found was confirmed by Sanger direct sequencing using BigDyeVR Terminator v. 3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) on a 3100 DNA Analyzer from Applied Biosystems (Foster City, CA).

2.3 Biochemical, hormonal and anthropometric parameters. Fasting blood samples were drawn in the morning before 10 AM and serum stored at −80°C for CEC and CLC assessment. Serum lipids, *e.g.* total cholesterol, low-density lipoprotein (LDL)-C, high density lipoprotein (HDL)-C and triglycerides (TG) were determined by established methodologies, as carried out in the laboratory of the Institution. LDL-C was calculated indirectly according to the Friedewald formula when the TG were < 400 mg/dL. Serum luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone concentrations were measured by the electrochemiluminescence immunoassay "ECLIA" from Roche Diagnostics (Roche Diagnostics, Germany). LH and FSH assays had a lower limit of detection of 0.1 IU/L and a functional sensitivity of 0.2 IU/L. Elecsys® Testosterone II test (Calibrator reference: 05200067 190) had a lower limit of detection of 0.087 nmol/L and a functional sensitivity of 0.4 nmol/L. The inter- or intra-assay coefficients of variation were <5%. Testosterone method was standardized via isotope dilution-gas chromatography/mass spectrometry. BMI and waist circumference were calculated as kg/m^2 and cm, respectively.

2.4. Testosterone and oestradiol evaluation by Mass Spectrometry - LC-MS/MS analysis. Testosterone was purchased from Sigma Aldrich. $^{13}C_3$ -17β-Estradiol (2 ng/sample) was obtained

151 from CDN Isotope Pointe-Claire (Pointe-Claire, Quebec, Canada). SPE cartridges (Discovery DS-C18
152 500 mg) were from Supelco (Milan, Italy). All solvents and reagents were HPLC grade (Sigma Aldrich,
153 Milan, Italy). Positive atmospheric pressure chemical ionization experiments were performed with
154 a linear ion trap – mass spectrometer (LTQ, ThermoFisher Co, San Jose, CA) using nitrogen as sheath,
155 auxiliary and sweep gas. The instrument was equipped with a Surveyor liquid chromatography (LC)
156 Pump Plus and a Surveyor Autosampler Plus (Thermo Fisher Co). The mass spectrometer was
157 employed in a MS/MS mode using helium as collision gas. The LC mobile phases were (A) H_2O/0.1%
158 formic acid and (B) methanol (MeOH)/0.1% formic acid. The gradient (flow rate 0.5 mL/min) was as
159 follows: T0 70%A, T1.5 70%A, T2 55%A, T3 55%A, T35 36%A, T40 25%A, T41 1%A, T45 1%A, T45.2
160 70%A, T55 70%A. The split valve was set at 0–6.99 min to waste, 6.99–43.93 min to source and
161 43.93–55 to waste. The Hypersil Gold column (100 · 3 mm, 3 lm; ThermoFisher Co) was maintained
162 at 40°C. The injection volume was 25 μL and the injector needle was washed with MeOH/water 1/1
163 (v/v). Peaks of the LC-MS/MS were evaluated using a Dell workstation by means of the software
164 Excalibur® Release 2.0 SR2 (ThermoFisher Co). Briefly, serum samples were added with internal
165 standards and mixed in 2 mL of MeOH/acetic acid (99 : 1 v/v). After an overnight extraction at 4°C,
166 samples were centrifuged at 15,300 g for 5 min and the pellet was extracted twice with 1 mL of
167 MeOH/acetic acid (99 : 1 v/v). The organic phases were combined and dried with a gentle stream of
168 nitrogen in a 40°C water bath. The samples were resuspended with 3 mL of MeOH/$H_2$0 (10 : 90 v/v)
169 and passed through a SPE cartridge, previously activated with MeOH (5 mL) and MeOH : H_2O 1 : 9
170 v/v (5 mL); steroids were eluted in MeOH, concentrated and transferred in autosampler vials before
171 the LC-MS/MS analysis. Quantitative analysis was performed on the basis of calibration curves
172 prepared and analyzed daily: blank samples (6% albumin in phosphate-buffered saline) were spiked
173 with internal standard. Increasing amounts (0.05–5 ng/sample) of T were added. Standard curves
174 (eight concentration levels) were fitted by linear regression equation: y = ax+b. Calibration curves

were extracted and analyzed as described above for samples. Limits of quantification, precision and accuracy of the assays have been previously reported (23).

2.5 HDL cholesterol efflux capacity. The HDL fraction was isolated from serum by precipitating apoB-containing lipoproteins with polyethylene glycol (24)**.** In order to avoid any lipoprotein remodelling, sera were slowly defrosted in ice immediately before this procedure (20). HDL cholesterol efflux capacity (CEC), occurring through the main pathways (25,26), was evaluated by a standardized and widely used radioisotopic technique as previously described (15,16,27).

2.5.1 Total, aqueous diffusion and ABCA1 CEC. We evaluated total CEC (tCEC) and its major contributors, aqueous diffusion and ATP-binding cassette transporter A1 (ABCA1)-mediated efflux, in the J774 murine macrophage cell model. In particular, cells in basal conditions were used to evaluate aqueous diffusion, whereas cells incubated with a cAMP analogue (cpt-cAMP 0.3 mM; Sigma-Aldrich) inducing ABCA1 expression were used to measure HDL tCEC. The specific ABCA1-mediated efflux contribution was calculated as the difference between tCEC and aqueous diffusion CEC. J774 macrophages were grown in 10% FCS-containing DMEM (both FCS and DMEM from Lonza, Verviers, Belgium) in the presence of antibiotics (Penicillin-Streptomycin from Thermo Fisher Scientific, CA). Cells were then labelled with [1,2-^{3}H] cholesterol at 2μCi/ml (Perkin Elmer, Waltham, MA) for 24 hours in the presence of 2 μg/ml of an inhibitor of the cholesterol esterifying enzyme acyl-coenzyme A:cholesterol acyltransferase (Sandoz 58035; Sigma-Aldrich) to prevent accumulation of cholesteryl esters. J774 cells were incubated in the absence or presence of the cAMP analogue in 0.2% BSA-containing medium for 18 hours (BSA from Sigma-Aldrich). Cells were then exposed for 4 hours to the HDL fraction of sera from either control and hypogonadal subjects

198 at 2% (v/v) in medium. HDL CEC was expressed as the percentage of radiolabelled cholesterol
199 released into the medium over total radioactivity incorporated by cells. To check for adequate cell
200 responsiveness, lipid-free human Apolipoprotein A-I (apoA-I) (Sigma-Aldrich) and the HDL fraction
201 of a standard serum obtained from a pool of normolipidemic subjects were tested together with
202 serum samples in each assay. The relative CEC values were used to normalize the different
203 experiments in order to correct for the inter-assay variability. Intra-assay variation coefficients for
204 HDL CEC assays were < 10%.
205
206 *2.5.2 ABCG1 CEC*. Serum HDL CEC mediated by the ATP binding cassette transporter G1 (ABCG1)
207 was evaluated by using Chinese hamster ovary (CHO) cells transfected and not transfected with the
208 human ABCG1 gene. The specific ABCG1 contribution was calculated as the difference between CEC
209 obtained in ABCG1-transfected cells and CEC obtained in non-transfected cells. Specifically, CHO
210 cells were cultured in 10% FCS-containing Ham's F-12 (both from Lonza, Verviers, Belgium) in the
211 presence of antibiotics (Zeocin and Geneticin from Waltham, MA). CHO cells after labelling with
212 [1,2-^{3}H] cholesterol at 1μCi/ml underwent an equilibration period in 0.2% BSA-containing medium
213 for 90 minutes. Cells were successively exposed for 6 hours to the HDL fraction of sera from either
214 control or hypogonadal subjects at 1% (v/v) in medium. HDL CEC was expressed as the percentage
215 of radiolabelled cholesterol release to the medium over total radioactivity incorporated by cells. To
216 check for adequate cell responsiveness, human isolated HDL and HDL fractions of a standard serum
217 obtained from a pool of normolipidemic subjects were tested together with the serum samples in
218 each assay. Human isolated HDL (d = 1.063–1.21 g/ml) were purified by sequential
219 ultracentrifugation from the plasma of healthy volunteers. The relative CEC values were used to
220 normalize the different experiments in order to correct for the inter-assay variability. Intra-assay
221 variation coefficients for HDL CEC assays were < 10%.

222

223 *2.6 Serum cholesterol loading capacity.* To avoid any lipoprotein remodelling, sera were slowly
224 defrosted in ice immediately before cholesterol loading capacity (CLC) measurement (20). Whole
225 serum CLC was evaluated on human monocyte-derived macrophages THP-1 with a fluorometric
226 technique (28). Human THP-1 monocytes were grown in 10% FCS-containing RPMI (both from
227 Lonza) in the presence of antibiotics (Penicillin-Streptomycin). Cells were plated in the presence of
228 100 ng/ml PMA (Sigma-Aldrich, Milan, Italy) for 72 hours to allow differentiation into macrophages.
229 Cells were then incubated with 5% lipoprotein deficient serum (LPDS; Sigma-Aldrich) for 24 hours
230 and exposed for 24 hours to 10% (v/v) of whole serum from controls and from hypogonadal
231 patients. At the end of incubation, cell monolayers were lysed in 1% sodium cholate solution (Sigma-
232 Aldrich) supplemented with 10 U/ml DNase (Sigma-Aldrich). Cholesterol was then measured
233 fluorometrically using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR)
234 following manufacturer's instructions. An aliquot of cell lysates was used to measure cell proteins
235 by the Bicinchoninic Acid assay (Thermo Fisher Scientific, CA). CLC was expressed as micrograms of
236 cholesterol per milligram of protein. To check for adequate cell responsiveness sera obtained from
237 pools of normolipidemic and hypercholesterolemic subjects were tested together with serum
238 samples in each assay. The relative CLC values were used to normalize the different experiments in
239 order to correct for inter-assay variability. Intra-assay variation coefficients for the CLC assays were
240 < 10%.

241

242 *2.7 Statistical analyses.* Sample size was calculated *a priori* by using the G*Power software selecting:
243 two-tailed t-test, the difference between two independent means and *a priori* power analysis, fixing
244 a power of 0.95 and a significance level of 0.05. Sample size was found to be at least 10 subjects per

group (26). Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, CA). Each sample was run in triplicate. Data were expressed as mean ± SEM. Differences in CEC and CLC between control and hypogonadal groups were evaluated by using the unpaired two-tailed Student t-test or Mann Whitney test for parameters with normal and skewed distribution, respectively. When necessary, differences between groups were evaluated by non-parametric Kruskal-Wallis one-way ANOVA analysis. The relationship between parameters was assessed by linear regression analysis and the correlation coefficient (r^2) was reported. Significant differences were defined as $p < 0.05$.

3. Results

3.1 Hypogonadal patients show a normal lipid profile. The hypogonadal patients underwent genetic characterization by NGS for causative genes related to isolated hypogonadotropic hypogonadism (Table 1). In order to assess whether hypogonadal men had an altered lipid profile, clinical and biochemical parameters were evaluated (Table 2). All hypogonadal subjects presented with an average of low testosterone levels (4.21 ± 0.86 nmol/L; normal range 9.9-27.8), compared to controls with levels in the eugonadal range (15.96 ± 1.50 nmol/L) (4). Testosterone quantification was confirmed by LC-MS/MS analysis (Table 2) and the direct correlation with data obtained with immunoassay was: $r^2 = 0.833$, $p < 0.0001$. No differences were found in oestradiol circulating levels between hypogonadals and eugonadals, *i.e.* 45.4 ± 0.21 pmol/L *vs* 52.9 ± 0.20 pmol/L, respectively. Normal/low gonadotropins were detected in patients with ~~central~~secondary hypogonadism, such as Kallmann's syndrome or isolated hypogonadotropic hypogonadism (LH 1.34 ± 0.42 IU/L; normal range 1.7-8.6; FSH 1.81 ± 0.50 IU/L; normal range 1.5-12.4) (Table 3). Levels were instead high, as expected, in patients with primary hypogonadism, such as Klinefelter syndrome or other primary hypogonadisms (LH 27.8 ± 7.2 IU/L; normal range 1.7-8.6; FSH 41.7 ± 9.23 IU/L; normal range 1.5-

12.4) (Table 3). When the lipid profile (total cholesterol, HDL-C, LDL-C and triglycerides) was evaluated, no statistically significant differences were found between the hypogonadal patients and controls. When examining the primary and secondary forms (Table 3), significantly higher total (not LDL) cholesterol was noted in the former, however within normal limits. No patient had a diagnosis of diabetes (mean blood glucose 94 mg/dL); mean PSA was 0.52 (ng/mL) and hematocrit 44%.

3.2 Hypogonadism reduces cholesterol efflux capacity (CEC). To assess whether hypogonadism compromises HDL function, despite the normal lipid profile, HDL total CEC (tCEC) of sera from hypogonadal patients *vs* eugonadal controls was evaluated by using a model of cAMP-stimulated J774 macrophages. The presence of hypogonadism was associated with 16.2 % lower HDL tCEC ($p < 0.0001$; Figure 1A). Accordingly, when the two main contributors to tCEC, *i.e.* ABCA1 and aqueous diffusion, were considered, the hypogonadal status induced a significant -19.6% drop in ABCA1-mediated CEC ($p= 0.0012$; Figure 1B) and a -12.9% reduction in aqueous diffusion CEC ($p = 0.0128$; Figure 1D). Moreover, hypogonadal patients showed a marked impairment in the ABCG1-mediated CEC compared to controls (-40.9%, $p<0.0001$; Figure 1C), as evaluated in Chinese hamster ovary (CHO) cells overexpressing or not expressing the ABCG1 transporter. By separately analysing controls, primary and secondary hypogonadal patients, CEC values in both cases were significantly reduced (Figure 2, A-C), except for aqueous diffusion CEC. Comparison of CECs between primary and secondary hypogonadal patients did not show any significant difference (Figure 2, A-D).

3.3. Hypogonadism raises cholesterol loading capacity (CLC). Since cell cholesterol content is the end result of cholesterol efflux and influx, evaluation of the pro-atherogenic potential of whole serum was evaluated by determining CLC in human monocyte-derived macrophages THP-1. CLC was found to be markedly and significantly raised (+43%, $p <0.0001$) in patients with hypogonadism

compared to controls (Figure 3A)**.** Then, by separately analysing CLC in controls, primary and secondary hypogonadisms, we found a significant increase in both cases compared to controls (Figure 3B). Further, sera from patients with primary hypogonadisms had a significant higher CLC compared to patients with secondary hypogonadisms (18.45 ± 2.78 *vs* 15.15 ± 2.10 µg cholesterol/mg protein, respectively; p=0.019 (Figure 3B).

3.4 Testosterone levels correlate with HDL CEC and CLC. To evaluate a possible relationship among lipoprotein function and testosterone levels, a comparative analysis of the two groups, *i.e.* controls and hypogonadal men, was performed. A direct correlation between testosterone and both HDL tCEC and ABCG1 CEC (tCEC: r^2= 0.359, p= 0.0001; ABCG1 CEC: r^2= 0.367, p= 0.0001) was observed (Figures 4, A-B). HDL-C concentrations did not instead correlate either with total CEC (r^2= 0.017, p= 0.439), ABCA1 CEC (r^2= 0.065, p= 0.138), ABCG1 CEC (r^2= 0.007, p= 0.872) and testosterone levels (r^2 = 0.074; p = 0.113) or CLC (r^2 = 0.009, p = 0.565). Conversely, HDL levels correlated with aqueous diffusion CEC (r^2= 0.269, p= 0.001). Finally, the raised serum CLC ~~activity~~ appeared to be inversely correlated with testosterone (r^2= 0.270; p= 0.001; Figure 4 C), but not with LDL-C levels (r^2= 0.015; p = 0.487). Similar results were found when testosterone levels were determined by LC-MS/MS analysis (Figure 4 D-F).

4. Discussion

The present study, aimed at evaluating possible differences in HDL function between genetic/rare hypogonadal conditions and healthy controls, showed marked alterations in the efflux/loading capacities, *i.e.* HDL CEC and serum CLC. The HDL ABCA1-, ABCG1- and aqueous diffusion-mediated CECs were reduced by -19.6%, -40.9% and -12.9%, respectively, and total CEC by

-16.2%. CEC is a measure of the HDL anti-atherogenic activity, generally identified as a CV risk biomarker, independent of HDL-C concentrations (29). CLC, a marker of the serum cholesterol loading capacity of macrophages, linked to an enhanced atherosclerosis risk, was instead raised by 43% (19,20).

Our studied population of hypogonadal patients had a lipoprotein profile within the range of normality, similar to controls; indeed, hypogonadal males presented with non-statistically higher HDL-C levels. These findings are in contrast with previous cross-sectional and prospective observational studies showing that hypogonadism or low testosterone levels are accompanied by a pro-atherogenic lipid profile, with higher LDL-C and lower HDL-C, compared to healthy controls (30). The lipid profile within normal limits observed in our patients may be related to the absence of clear-cut obesity (Table 2). Obesity in itself can, in fact, reduce circulating testosterone, because of raised adipose tissue aromatase activity, leading to an enhanced conversion of testosterone to oestradiol (31). In this series of patients with Kallmann, Klinefelter syndromes, anorchia, and other acquired primary hypogonadism, the absence of obese individuals was associated with not significant differences in oestrogen levels, ruling out any significant influence of raised aromatase (31). In this series, abdominal circumferences were also within normal limits.

Low HDL-C levels are an established CV risk marker, as reported in the major CV Guidelines (9,32). However, the non-significant CV preventive efficacy of treatments raising HDL-C levels (33), in spite of multiple drug effects involving not only HDL-C raising but also pleiotropic effects on fibrinolysis (34,35), inflammation and others (36), has clearly indicated that HDL-C *per se* may not be a major determinant of CV risk. HDL function is now rated as a more meaningful approach to risk evaluation (16). Our hypogonadal patients, characterized by very low testosterone, with HDL-C levels in the normal range, showed a marked reduction in HDL CEC as well as raised serum CLC.

Impairment of tCEC has been associated with the highest inverse correlation with incidence and prevalence of CVD (16). Hypogonadal patients showed lower HDL CEC by both the ABCA1 and ABCG1 pathways, despite HDL-C levels in the normal range, thus confirming that a mere measurement of HDL-C levels has clear limitations in assessing function. In a previous report from hypogonadal men, testosterone replacement did not alter total HDL-C, but clearly modified the HDL proteome, raising paroxonase-1 and fibrinogen-α-chain, as well as reducing apoA-IV, this last a change of unclear significance (37). The impairment of HDL CEC in our population may therefore be related to specific hypogonadism-associated compositional changes in the protein or phospholipid cargo of HDL, known to affect HDL CEC (38).

The controversial correlation between testosterone deficiency and lipoprotein-associated CV risk reported in epidemiological studies is not well supported by deprivation findings (39). Middle-aged subjects, medically-castrated by the GnRH antagonist acyline, had significant HDL-C rises, without changes in efflux capacity (40); the same procedure, instead, increased both HDL-C and CEC in young healthy men (41). Our findings, from *naïf* hypogonadal patients, indicate a reduction in HDL CEC in a clearly different experimental setting, *i.e.* a natural model of hypogonadism *vs* pharmacological castration. This implies a different timing of exposure to low testosterone (chronic exposure *vs* a relatively acute exposure in pharmacological castration) with a consequently different impact on the lipoprotein profile. Moreover, mean levels of circulating testosterone in our hypogonadal patients were markedly reduced, compared to the pharmacological deprivation study (4.2 nmol/L compared to 7.7 nmol/L). The observed impairment of ABCA1 and ABCG1 CEC could also reflect a relative reduction of the amounts and/or efficiency of either small or large HDL (42,43). The significant decrement in HDL function may thus suggest that an additional HDL remodelling (44) may occur in hypogonadism. Indeed, testosterone treatment is associated with a specific increase in the mature subfraction of HDL, *i.e.* mostly HDL_2 (45,46),

responsible for the CEC ~~activity~~ mainly through ABCG1 (43). Since macrophage cholesterol accumulation is the end result of efflux-influx processes, determination of CLC is essential, in order to evaluate the net impact of hypogonadism on the overall serum atherogenic properties. The observed significant increase in CLC from hypogonadal patients clearly indicates that the likely rise of arterial wall foam cell formation may contribute to a higher CV risk (19,20).

Hypogonadisms can be classified into primary or secondary, caused by either testicular failure, or hypothalamic/pituitary dysfunction, respectively (4). Despite no differences in HDL CEC between the two hypogonadisms, sera from the primary hypogonadal patients were associated with a higher pro-atherogenic potential, compared to sera from secondary hypogonadal patients. The higher CLC levels in Klinefelter patients are most likely associated with a pro-atherogenic lipoprotein pattern, as also supported by a recent review (47), indicating that these patients have an increased risk of developing leg thrombosis or ulcers, even in the absence of any triggering factor. In a larger cohort of Klinefelter syndrome (832 subjects), a more frequent incidence of ischemic heart disease, peripheral vascular disease, pulmonary embolism and even intestinal thrombosis was reported (48). There is, conversely, no increased CV risk in Kallmann patients (49), although a late diagnosis might expose patients to co-morbidities related to chronic hypogonadism leading to the need of rehabilitation programmes (50).

The well-established impact of non-drug related hypogonadism on HDL CEC and serum CLC shown in the present study provides an important indication for further confirmatory evaluations, also in other populations. While it is reasonable to hypothesize that structural changes in HDL might be responsible for the alterations of HDL function, our study had the limit of not having tested HDL structure/composition. The evaluated patients are now ongoing testosterone replacement therapy and cholesterol fluxes will be re-evaluated after hormonal normalization.

In conclusion, these findings demonstrate that both central and primary hypogonadisms are associated with an impaired HDL function, measured as CEC, and with an increased proatherogenic potential of serum, as witnessed by a raised CLC. These effects occurred in the absence of any evident change in serum lipoprotein profile. Derangements in lipoprotein function may translate into an increased atherogenic risk, adding important insights into possible preventive strategies aimed at reducing CV risk in hypogonadal patients, at least in those of genetic and idiopathic origins.

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Figure legends.

Figure 1. *HDL cholesterol efflux capacity (CEC) was reduced in hypogonadal patients*. In control subjects and in patients with hypogonadism, total HDL CEC (panel A), ABCA1-mediated CEC (panel B), ABCG1-mediated CEC (panel C) and aqueous diffusion CEC (panel D) have been assessed by using J774 murine macrophages (panels A, B, D) and CHO cells (panel C). Each point of the scatter plot represents the mean of triplicate analyses of each serum sample. Empty triangles (Δ) and solid squares (■) refer to CEC values of primary and secondary hypogonadisms, respectively. The horizontal solid line is the mean of each group. Differences in CEC between control and hypogonadal groups were evaluated by using the unpaired two-tailed Student t-test or Mann Whitney test for parameters with normal and skewed distribution, respectively. Significant differences were defined as $p < 0.05$.

Figure 2. *HDL cholesterol efflux capacity (CEC) by separately analysing controls, primary and secondary hypogonadal patients.* In control subjects and in patients with hypogonadism, total HDL CEC (panel A), ABCA1-mediated CEC (panel B), ABCG1-mediated CEC (panel C) and aqueous diffusion CEC (panel D) have been assessed by using J774 murine macrophages (panels A, B, D) and CHO cells (panel C). Empty triangles (Δ) and solid squares (■) refer to CEC values of primary and secondary hypogonadism, respectively. The horizontal solid line is the mean of each group. Each point of the scatter plot represents the mean of triplicate analysis of each sample. Differences between groups were evaluated by non-parametric Kruskal-Wallis one-way ANOVA analysis. Significant differences were defined as $p < 0.05$.

570 **Figure 3**. *Cholesterol loading capacity (CLC) was increased in hypogonadal patients.* In control
571 subjects and in patients with hypogonadism serum CLC was evaluated by using human monocyte-
572 derived macrophages THP-1. Each point of the scatter plot represents the mean of triplicate
573 analyses of each serum sample. Empty triangles (Δ) and solid squares (■) refer to CLC values of
574 primary and secondary hypogonadisms, respectively. The horizontal solid line is the mean of each
575 group. Panel A) Differences in CLC between control and hypogonadal groups were evaluated by
576 using the unpaired two-tailed Student t-test. Significant differences were defined as $p< 0.05$. Panel
577 B) Differences between groups were evaluated by non-parametric Kruskal-Wallis one-way ANOVA
578 analysis. Significant differences were defined as $p< 0.05$.

579

580 **Figure 4**. *Testosterone serum levels correlate with HDL cholesterol efflux capacity (CEC) and*
581 *cholesterol loading capacity (CLC)*. Results of linear regression analyses of total and ABCG1 HDL CEC
582 and serum CLC with testosterone serum levels evaluated by immunoassay (panels A-C) and LC-
583 MS/MS (panels D-F) are reported in controls and patients with hypogonadisms (● control subjects,
584 ○ Klinefelter syndrome patients, □ other primary hypogonadal patients, ■ secondary hypogonadal
585 patients). The relationship between serum lipoprotein functions and testosterone levels was
586 assessed by linear regression analysis and the correlation coefficient (r^2) is reported. Significance
587 was defined as $p< 0.05$.

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Table 1. Genetic characterization of the patient cohort

Diagnosis	Karyotype	Next Generation Sequencing for IHH known causal genes				
		Gene	**Allelic Variant**	**Exon**	**cDNA**	**Protein**
Klinefleter	47,XXY	n/a				
Klinefleter	47,XXY	n/a				
Klinefleter	47,XXY	n/a				
nIHH	46,XY	None rare allelic variant				
KS	46,XY	PROKR2	heterozygous	2	472G>A	V158I
KS	46,XY	GnRHR	homozygous	1	35delA	N12IfsX11
KS	46,XY	FGFR1	heterozygous	16	2153G>A	R718H
KS	46,XY	FGFR1	heterozygous	9	1203insT	G402WfsX6
nIHH	46,XY	None rare allelic variant				
nIHH	46,XY	None rare allelic variant				
nIHH	46,XY	CHD7	heterozygous	3	2053_2058dupGCAAAA	K686_T687insAK
nIHH	46,XY	SPRY4 SEMA3A	Heterozygous heterozygous	3 17	653A>G 1910G>A	N218S R637H
KS	46,XY	None rare allelic variant				
CA	46,XY	n/a				
nIHH	46,XY	FLRT3	heterozygous	3	1255A>T	T419S
nIHH	46,XY	PROK2	heterozygous	1	2T>G	M1R
nIHH	46,XY	None rare allelic variant				
IAPH	46,XY	n/a				
Klinefleter	47,XXY	n/a				
IAPH	46,XY	n/a				

n/a, not applicable; Klinefelter= Klinefelter's syndrome; nIHH= normosmic Idiopathic Hypogonadotropic Hypogonadism; KS= Kallmann's syndrome; CA= congenital idiopathic anorchia; IAPH= idiopathic acquired primary hypogonadism

Table 2. Clinical and biochemical characteristics of hypogonadal patients and control subjects

	Control subjects	Hypogonadal subjects	p-value
Age (yrs)	41 ± 6	36 ± 4	0.287
BMI (Kg/m^2)	22.8 ± 0.8	24.7 ± 7.8	0.137
Waist circumference (cm)	84.04 ± 3.04	91.45 ± 3.52	0.118
LH (IU/L)	5.97 ± 0.96	11.70 ± 4	0.641
FSH (IU/L)	4.95 ± 0.78	17.10 ± 5.1	0.933
TTe immunoassay (nmol/L)	15.96 ± 1.50	4.21 ± 0.86	**<0.0001**
TTe LC-MS/MS (nmol/L)	11.78 ± 1.04	5.00 ± 0.67	**<0.0001**
TC (mg/dL)	197 ± 8	180 ± 7	0.473
HDL-C (mg/dL)	54 ± 12	63 ± 20.1	0.101
LDL-C (mg/dL)	107 ± 28	89 ± 8	0.114
TGs (mg/dL)	110 ± 15	115 ± 13	0.525

629 Values are indicated as mean ± SEM. Significant differences were reported in bold. BMI, Body Mass
630 Index; LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone; TC, Total Cholesterol; HDL-C,
631 High-Density Lipoprotein Cholesterol; LDL-C, Low-Density Lipoprotein Cholesterol; TG, Triglycerides
632
633
634 **Table 3.** Clinical and biochemical characteristics of hypogonadal patients according to the diagnosis and levels
635 of TTe.

	Primary Hypogonadism (range TTe= 0.6 - 11.50 nmol/L)	**Secondary Hypogonadism (range TTe= 0.15 – 6.90 nmol/L)**	**p-value**
Numerosity	7	13	
Age (yrs)	42 ± 7	33 ± 4	0.601
BMI (Kg/m^2)	26.30 ± 0.93	23.5 ± 1.5	0.391
Waist circumference (cm)	97.57 ± 3.08	88.15 ± 5.01	0.361
LH (IU/L)	27.8 ± 7.2	1.34 ± 0.42	**<0.0001**
FSH (IU/L)	41.7 ± 9.23	1.81 ± 0.50	**<0.0001**
TC (mg/dL)	191 ± 11	166 ± 9	**0.044**
HDL-C (mg/dL)	56 ± 21	65 ± 20	0.382
LDL-C (mg/dL)	109 ± 13	83 ± 12	0.118
TG (mg/dL)	107 ± 16	116 ± 21	0.832

636 Values are indicated as mean ± SEM. Significant differences were reported in bold. BMI, Body Mass Index;
637 LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone; TC, Total Cholesterol; HDL-C, High-Density
638 Lipoprotein Cholesterol; LDL-C, Low-Density Lipoprotein Cholesterol; TG, Triglycerides; TTe, testosterone.
639

Figure 1

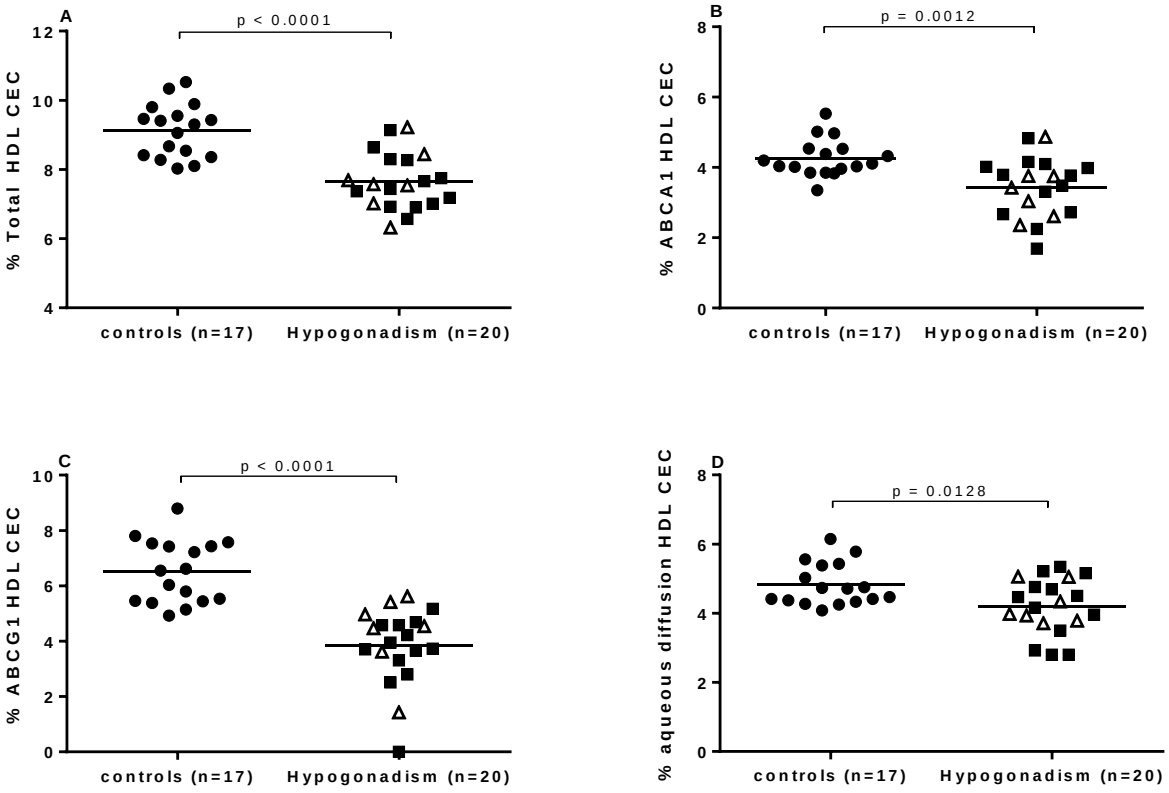

A
p < 0.0001
% Total HDL CEC
12
10
8
6
4
controls (n=17)
Hypogonadism (n=20)
B
p = 0.0012
% ABCA1 HDL CEC
8
6
4
2
0
controls (n=17)
Hypogonadism (n=20)
C
p < 0.0001
% ABCG1 HDL CEC
10
8
6
4
2
0
controls (n=17)
Hypogonadism (n=20)
D
p = 0.0128
% aqueous diffusion HDL CEC
8
6
4
2
0
controls (n=17)
Hypogonadism (n=20)

Figure 2

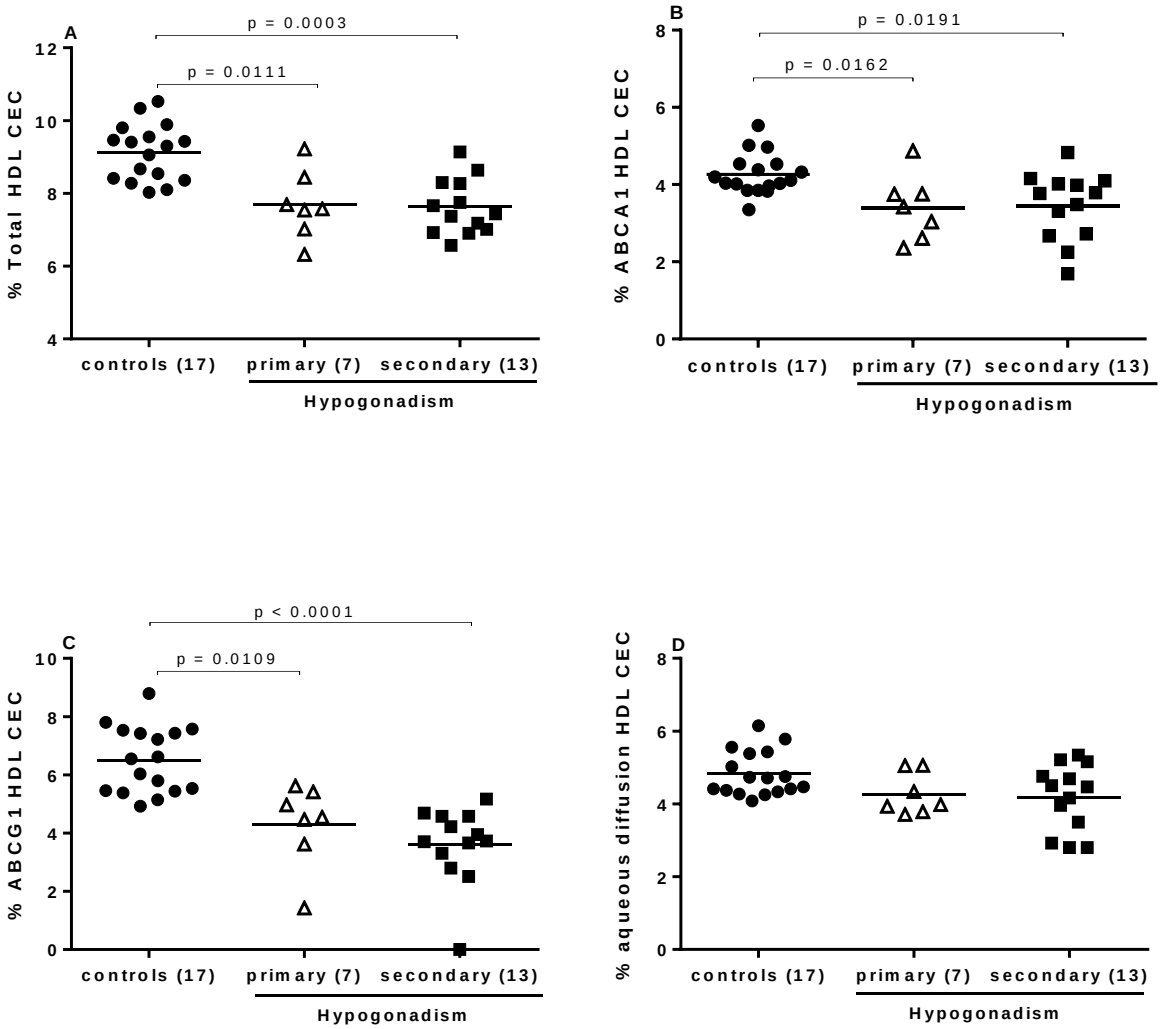

A
p = 0.0003
p = 0.0111
% Total HDL CEC
12
10
8
6
4
controls (17)
primary (7)
secondary (13)
Hypogonadism
B
p = 0.0191
p = 0.0162
% ABCA1 HDL CEC
8
6
4
2
0
controls (17)
primary (7)
secondary (13)
Hypogonadism
C
p < 0.0001
p = 0.0109
% ABCG1 HDL CEC
10
8
6
4
2
0
controls (17)
primary (7)
secondary (13)
Hypogonadism
D
% aqueous diffusion HDL CEC
8
6
4
2
0
controls (17)
primary (7)
secondary (13)
Hypogonadism

Figure 3

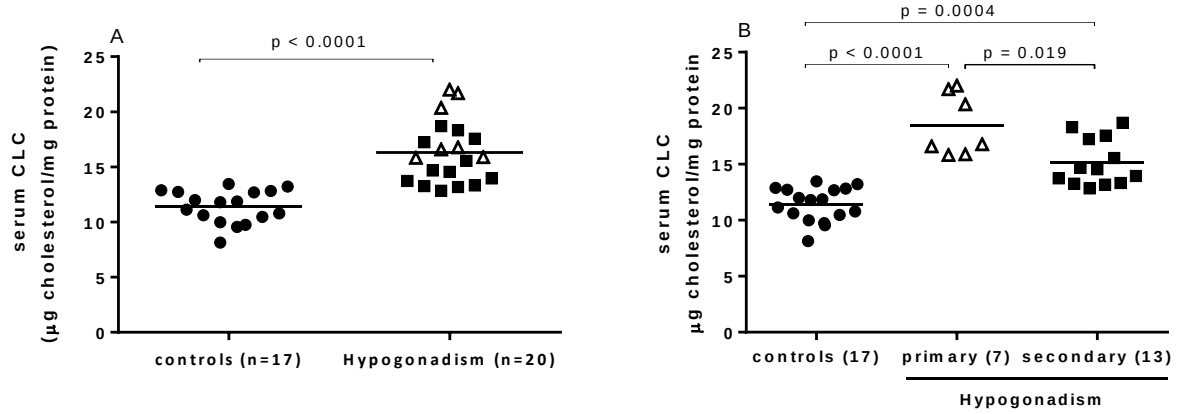
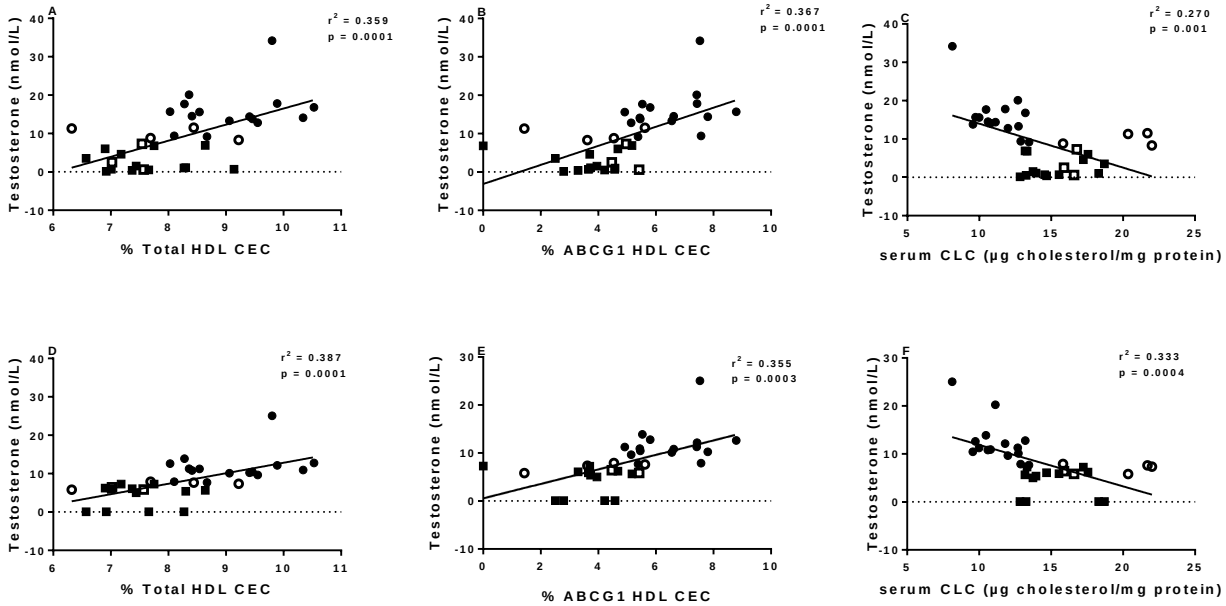

A
p < 0.0001
serum CLC
(µg cholesterol/mg protein)
25
20
15
10
5
0
controls (n=17)
Hypogonadism (n=20)
B
p = 0.0004
p < 0.0001
p = 0.019
µg cholesterol/mg protein
controls (17)
primary (7)
secondary (13)
Hypogonadism

Figure 4


A
r² = 0.359
p = 0.0001
Testosterone (nmol/L)
% Total HDL CEC
B
r² = 0.367
p = 0.0001
Testosterone (nmol/L)
% ABCG1 HDL CEC
C
r² = 0.270
p = 0.001
Testosterone (nmol/L)
serum CLC (µg cholesterol/mg protein)
D
r² = 0.387
p = 0.0001
Testosterone (nmol/L)
% Total HDL CEC
E
r² = 0.355
p = 0.0003
Testosterone (nmol/L)
% ABCG1 HDL CEC
F
r² = 0.333
p = 0.0004
Testosterone (nmol/L)
serum CLC (µg cholesterol/mg protein)