The effect of transgender hormonal treatment on high density lipoprotein cholesterol efflux capacity

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Abstract:	Background and aims: A decrease in HDL-cholesterol concentrations during transgender hormone therapy has been shown. However, the ability of HDL to remove cholesterol from arterial wall macrophages, termed cholesterol efflux capacity (CEC), has proven to be a better predictor of cardiovascular disease (CVD) largely independently of HDL-concentrations. In addition, the serum capacity to load macrophages with cholesterol (cholesterol loading capacity, CLC) represents an index of pro-atherogenic potential. As transgender individuals are exposed to lifelong exogenous hormone therapy, it becomes of interest to study whether HDL-CEC and serum CLC are affected by HT. HDL-CEC and serum CLC have been evaluated in 15 trans men treated with testosterone and in 15 trans women treated with estradiol and cyproterone acetate at baseline and after 12 months of HT. Methods: Total HDL-CEC from macrophages and its major contributors, the ATP-binding cassette transporters (ABC) A1 and ABCG1 HDL-CEC and HDL-CEC by aqueous diffusion were determined by a radioisotope assay. CLC was evaluated in human THP-1 macrophages. Results : In trans women, total HDL-CEC decreased by 10.8% (95%CI: -14.3;-7.3; p< 0.001), ABCA1 HDL-CEC by 23.8% (-34.7; -12.9; p<0.001) and aqueous diffusion HDL-CEC by 4.8% (-8.4;-1.1; p<0.01). In trans men, only aqueous diffusion HDL-CEC decreased significantly, -9.8% (-15.7;-3.9; p<0.01). ABCG1 HDL-CEC did not change in either group. Serum CLC and HDL subclass distribution were not modified by HT in both groups. Conclusions: Total HDL-CEC decreased during HT in trans women, with a specific reduction in ABCA1 CEC. This finding might contribute to their higher CVD risk.

Highlights

- In trans women, total, ABCA1 and aqueous diffusion HDL-CEC decreased after hormone therapy
- In trans women, changes in HDL-CEC are not associated to plasma HDL-C levels
- Results in trans women may explain the increased CV risk in feminizing hormone therapy
- In trans men only aqueous diffusion HDL-CEC significantly decreased after therapy

Graphical Abstract

C = cholesterol

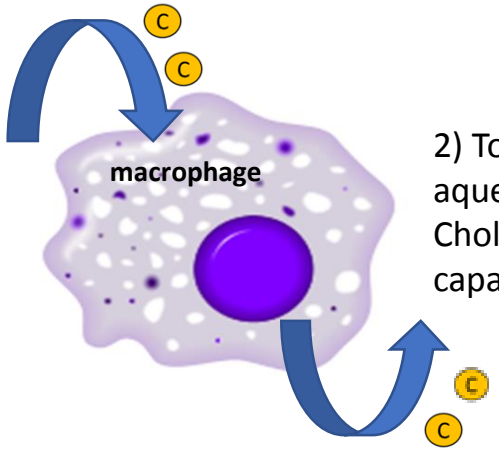
Trans women

M0 → M12

estradiol valerate + Cyproterone acetate

12 months

1) Serum cholesterol loading capacity (CLC) ↔



2) Total, ABCA1 and aqueous diffusion HDL Cholesterol efflux capacity (CEC) ↓

→ The decrease in HDL-CEC is independent of plasma HDL-C levels and could contribute to a higher CV risk.

3) HDL subclasses distribution ↔

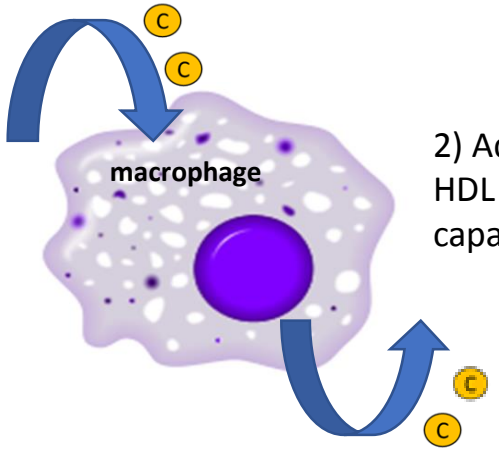
Trans men

M0 → M12

Testosterone or mix of testosterone esters or testosterone undecanoate

12 months

1) Serum cholesterol loading capacity (CLC) ↔



2) Aqueous diffusion HDL Cholesterol efflux capacity (CEC) ↓

→ The decrease in HDL-CEC is related to plasma HDL-C levels

3) HDL subclasses distribution ↔

The effect of transgender hormonal treatment on high density lipoprotein cholesterol efflux capacity

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Abbreviations: CEC, cholesterol efflux capacity; CLC, cholesterol loading capacity; ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; RCT, reverse

cholesterol transport; CHD, coronary heart disease; CETP, cholesteryl ester transfer protein; LC-MS/MS, Liquid Chromatography Mass Spectrometry.

Abstract

Background and aims: A decrease in HDL-cholesterol concentrations during transgender hormone therapy has been shown. However, the ability of HDL to remove cholesterol from arterial wall macrophages, termed cholesterol efflux capacity (CEC), has proven to be a better predictor of cardiovascular disease (CVD) largely independently of HDL-concentrations. In addition, the serum capacity to load macrophages with cholesterol (cholesterol loading capacity, CLC) represents an index of pro-atherogenic potential. As transgender individuals are exposed to lifelong exogenous hormone therapy, it becomes of interest to study whether HDL-CEC and serum CLC are affected by HT. HDL-CEC and serum CLC have been evaluated in 15 trans men treated with testosterone and in 15 trans women treated with estradiol and cyproterone acetate at baseline and after 12 months of HT.

Methods: Total HDL-CEC from macrophages and its major contributors, the ATP-binding cassette transporters (ABC) A1 and ABCG1 HDL-CEC and HDL-CEC by aqueous diffusion were determined by a radioisotope assay. CLC was evaluated in human THP-1 macrophages.

Results: In trans women, total HDL-CEC decreased by 10.8% (95%CI: -14.3;-7.3; $p<0.001$), ABCA1 HDL-CEC by 23.8% (-34.7; -12.9; $p<0.001$) and aqueous diffusion HDL-CEC by 4.8% (-8.4;-1.1; $p<0.01$). In trans men, only aqueous diffusion HDL-CEC decreased significantly, -9.8% (-15.7;-3.9; $p<0.01$). ABCG1 HDL-CEC did not change in either group. Serum CLC and HDL subclass distribution were not modified by HT in both groups.

Conclusions: Total HDL-CEC decreased during HT in trans women, with a specific reduction in ABCA1 CEC. This finding might contribute to their higher CVD risk.

Keywords: HDL function, cholesterol efflux capacity, cholesterol loading capacity, ABCA1, macrophages, cardiovascular disease, testosterone, estrogen.

1. Introduction

Sex hormones have long been associated with cardiovascular disease (CVD) and CV risk factors[1]. Estrogen was previously considered to have protective effects on CVD, whereas detrimental effects on CVD were ascribed to testosterone. Currently, long-term data on CVD risk in estrogen replacement therapy is conflicting. Supplementation in younger postmenopausal women may be beneficial, but CVD risk may increase when hormone therapy is initiated in late menopause [2]. Regarding testosterone replacement therapy, long-term data on CVD risk is lacking [3, 4]. Over the years, the relationship between sex hormones and CVD has turned out to be even more complex, as androgens and estrogens have been shown to exert a number of direct and indirect effects on different biological processes and may act in a sex-specific manner. In studies on sex disparities in health and disease, it remains difficult to differentiate sex hormone specific effects from other sex specific factors, such as genetic and epigenetic aspects [5].

For transgender individuals who receive lifelong exogenous hormone therapy [6] to match physical characteristics of their gender identity [7], studying the effects of sex hormones on CV risk factors is especially important, as effects described in the cisgender population do not necessarily apply to transgender HT. Trans men (female sex recorded at birth, male gender identity) are treated with testosterone, whereas trans women (male sex recorded at birth, female gender identity) are treated with estrogens and, in general, with an anti-androgen agent (*e.g.*, cyproterone acetate).

In a previous report, our study group reported changes in blood lipids during HT in transgender individuals[8]. In trans women, a decrement in LDL-cholesterol (LDL-C), total cholesterol [9] and triglycerides was found, whereas increased LDL-C, total cholesterol and triglycerides were observed in trans men. In both trans men and trans women, the concentrations of HDL-cholesterol (HDL-C) were reduced.

Concerning HDL and CV risk, there is increasing attention to the measurement of HDL function rather than concentration, as the former appears to be a better predictor of CVD risk. The interest in HDL function was prompted by studies with cholesteryl ester transfer protein (CETP)-inhibitors leading to a raise in HDL-C concentrations with no reduction in CV events[10], despite opposite conclusions were reported by epidemiological studies[11]. The main atheroprotective function of HDL is presumed to be

the promotion of reverse cholesterol transport (RCT), defined as the removal of cholesterol from peripheral cells and transfer back to the liver.

Cholesterol efflux capacity (HDL-CEC) is a functional assessment of HDL, measuring the first step of RCT, namely the ability of HDL to remove cholesterol from peripheral cells[12]. HDL-CEC is an independent predictor of CVD[13] and a better predictor of coronary heart disease (CHD) risk compared to plasma HDL-C concentrations[14]. HDL-CEC can be divided into different components: a passive process termed aqueous diffusion, usually correlated to the HDL-C concentrations, and an active process regulated by the ATP-binding cassette (ABC) A1 and ABCG1 proteins. The ABCA1-mediated cholesterol efflux is a process generally independent of the HDL-C concentration[15]. The ABCA1 protein is the major regulator of cholesterol efflux and can contribute up to 80% of total HDL-CEC[16]. Several studies have reported associations between sex hormones and HDL-CEC, but the direction and magnitude of these have varied markedly among different populations. HDL-CEC was decreased in hypogonadal males[17], but it was also reduced in body builders using anabolic steroids[18]. A single study reported small reductions in HDL-CEC in trans women during hormone therapy, but the hormone treatment regimen differed from standard practice and the individual components of HDL-CEC were not assessed[19].

In this scenario, it is worth mentioning that lipid trafficking is the end result of cholesterol efflux and influx, the latter leading to direct cholesterol accumulation in arterial wall macrophages[20]. The serum cholesterol loading capacity (CLC) offers another functional index of the proatherogenic potential, since it is raised in pathological conditions leading to a higher CV risk [21].

Thus, with increasing evidence of the relationship between HDL-CEC and CV risk and previous associations of HDL-CEC with testosterone and estrogen treatments, the primary objective of this study focused on the effects of transgender HT treatment on HDL-CEC. In order to investigate whether hormonal treatment of trans women and trans men may lead to changes in HDL function, the overall CEC and the individual pathways have been assessed. In view of the critical role of cholesterol loading in macrophages, an additional objective has been the assessment of serum CLC.

2. Patients and methods

2.1 Study design

The European Network for the Investigation of Gender Incongruence (ENIGI) is a partnership of five European gender identity clinics in Amsterdam, the Netherlands; Ghent, Belgium; Oslo, Norway; Florence, Italy and Tel Aviv, Israel. ENIGI was initiated in order to obtain more insight into the potential diversity in diagnostics and treatment of transgender individuals. The ENIGI started in 2010. Participants were included in the ENIGI endocrine study when they started HT. Subjects were eligible to participate if they had not used HT before and if they had sufficient knowledge of the native languages. At the start of HT, subjects received oral and written information on the ENIGI endocrine protocol from their physician and informed consent was obtained according to the institutional guidelines. A full overview of the ENIGI endocrine protocol has been published previously [6, 22].

2.2 Study population

In this study, a random sample of 15 trans men and 15 trans women was drawn from participants included in the ENIGI endocrine protocol from June 2010 to November 2017 in Amsterdam. Participants were free of CV disease and medications altering cholesterol levels (e.g. statins or beta-blockers) [23]. Functional measurements were performed at baseline and after 12 months of HT. Based on the preference of each participant, trans men were treated with: (i) testosterone gel (Androgel®) at a daily dose of 50mg, [24] a mix of testosterone esters (Sustanon®) in 250 mg injections once per three weeks or (iii) 1000 mg injections of testosterone undecanoate (Nebido®) once per twelve weeks. Trans women were treated with twice daily oral estradiol valerate (Progynova®) 2 mg tablets (total dose 4 mg daily), or a transdermal preparation in the form of patches in a twice weekly dose of 100 µg/day (Systen®). Cyproterone acetate (daily dose of 50 mg), was prescribed as a testosterone-blocking agent to all trans women. There was no change in dose and route of administration of all hormones during the 12 months of treatment. No participant had undergone gender-affirming genital surgery.

2.3 Data collection

All measurements were performed during out-patient clinic visits. Data regarding lifestyle habits, medical history and use of other types of medications were recorded by the treating physician. Body weight was measured at baseline and after 12 months of therapy in light indoor clothing without shoes. Blood pressure was measured using an electronic blood pressure monitor with the patient in the sitting position. Body mass index (BMI) was calculated by dividing body weight in kilograms by height in meters squared. Venous blood samples were obtained after overnight fasting. Measurements were performed before the start of HT (baseline) and after 12 months of follow-up. Serum measurements included TC, triglycerides, LDL-C and HDL-C. TC, triglyceride and HDL-C levels, were measured using enzymatic methods (Roche Cobas 8000 module c502, Roche Diagnostics, Mannheim, Germany), with an inter-assay coefficient of variation (CV) of 1.6%, 1.9% and 1.3%, respectively. LDL-C values were calculated using the Friedewald formula.

Estradiol was determined using liquid chromatography tandem mass spectrometry (Amsterdam University Medical Center, location VUmc, Amsterdam, the Netherlands) with an inter-assay coefficient of variation of 7% and limit of quantification (LOQ) of 20 pmol/L. Testosterone was measured using a competitive immunoassay (Architect; Abbott, Abbott Park, IL) with an inter-assay CV range of 6% to 16% and a LOQ of 0.1 nmol/L. Comparability between the measurement of testosterone by the Architect immunoassay and LC-MS/MS is excellent and has been previously published (slope 1.05, r 0.97)[25, 26]. Luteinizing hormone (LH) was measured using an immunometric assay (Architect, Abbott) with an interassay CV <6% and LOQ of 2 U/L.

2.4 HDL-CEC

HDL-CEC was evaluated on the HDL fraction, isolated from whole serum by precipitating the apoB-containing lipoproteins with polyethylene glycol[27].This procedure, that allows to obtain biological samples containing only HDL, is comparable to isolation of HDL by ultracentrifugation for the study of CEC [28].

In order to avoid any lipoprotein remodeling, sera were slowly defrosted in ice immediately before the procedure. HDL-CEC by the main cholesterol efflux pathways was evaluated by a standardized and widely used radioisotopic cell-based technique [17, 29].

2.5 Total, aqueous diffusion and ABCA1 HDL-CEC

Total HDL-CEC, the parameter inversely associated to CV risk, and its major contributors, the aqueous diffusion process and the efflux mediated by the ATP-binding cassette transporter A1 (ABCA1) was evaluated in the J774 murine macrophage cell model. In particular, J774 in basal conditions were used to evaluate aqueous diffusion, whereas J774 cells incubated with a cAMP analogue (cpt-cAMP 0.3 mM; Sigma Aldrich, Milano, Italy) inducing ABCA1 expression [30] were used to measure total HDL-CEC. The specific ABCA1-mediated efflux contribution was calculated as the difference between total and aqueous diffusion HDL-CEC. J774 macrophages were seeded in 10% fetal calf serum (FCS) containing DMEM (both FCS and DMEM from Lonza, Verviers, Belgium) in the presence of antibiotics (penicillin–streptomycin from Thermo Fisher Scientific, MA, USA). Cells were then labelled with [1,2-^{3}H] cholesterol (PerkinElmer, MA) at 2µCi/ml for 24 hours in the presence of 2 µg/ml of an inhibitor of the 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 trans women and trans men at baseline and after 12 months of HT at 2% (v/v) in medium. HDL-CEC was expressed as the percentage of radiolabeled cholesterol released into the medium over total radioactivity incorporated by cells. To check for adequate cell responsiveness, lipid-free human apolipoprotein A-I (Sigma-Aldrich) and the HDL fraction of a standard serum obtained from a pool of normolipidemic subjects, not using HT, were tested together with serum samples in each assay. The relative HDL-CEC values were used to normalize the different experiments in order to correct for the inter-assay variability. Intra-assay CV for HDL-CEC assays were < 10%.

2.6 ABCG1 HDL-CEC

Serum HDL-CEC mediated by the ATP binding cassette transporter G1 (ABCG1) was evaluated by using Chinese hamster ovary (CHO) cells transfected and not transfected with the human ABCG1 gene. The specific ABCG1 contribution was calculated as the difference between HDL-CEC obtained in ABCG1-transfected cells and HDL-CEC obtained in non-transfected cells. Specifically, CHO cells were seeded in 10% FCS-containing Ham's F-12 (both from Lonza) in the presence of antibiotics (penicillin-streptomycin and zeocin from Thermo Fisher Scientific). CHO cells, after labelling with [1,2-^{3}H] cholesterol at 1μCi/ml, underwent an equilibration period in 0.2% BSA-containing medium for 90 minutes. Cells were then exposed for 6 hours to the HDL fraction of sera from trans women and trans men-before and after HT at 1% (v/v) in the medium. HDL-CEC was expressed as the percentage of radiolabeled cholesterol released into the medium over total radioactivity incorporated by cells. To check for adequate cell responsiveness, human isolated HDL and the HDL fraction of a standard serum from a pool of normolipidemic subjects, not using HT were tested together with the serum samples in each assay.

Human isolated HDL (d 1.063-1.21 g/mL) was purified by sequential ultracentrifugation from the plasma of healthy volunteers not using HT. The relative HDL-CEC values were used to normalize the different experiments to correct for the inter-assay variability. Intra-assay CV for HDL-CEC assays were < 10%.

2.7 Serum CLC

To avoid lipoprotein remodeling, sera were slowly defrosted in ice immediately before CLC measurement [31]. Whole serum CLC was evaluated on human monocyte-derived THP-1 macrophages with a fluorometric technique [32]. Human THP-1 monocytes were grown in 10% FCS containing RPMI (both from Lonza) in the presence of antibiotics (penicillin–streptomycin). Cells were plated in the presence of 100 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 72 hours to allow differentiation into macrophages. Cells were then incubated with 5% lipoprotein-deficient serum (Sigma-Aldrich) for 24 hours and exposed for 24 hours to 10% (v/v) whole serum from trans women and trans men before and after HT. At the end of the incubation, cell monolayers were lysed in 1%

sodium cholate solution (Sigma-Aldrich), supplemented with 10 U/mL DNase (Sigma-Aldrich). Cholesterol was then measured fluorometrically using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR) following manufacturer's instructions. An aliquot of cell lysates was used to measure cell proteins by the bicinchoninic acid assay (Thermo Fisher Scientific). CLC was expressed as micrograms of cholesterol/milligram of protein. To check for adequate cell responsiveness, sera obtained from pools of normolipidemic and hypercholesterolemic subjects were tested together with serum samples in each assay. The relative CLC values were used to normalize the different experiments in order to correct for inter-assay variability. Intra-assay CV for the CLC assays was < 10%.

2.8 HDL subclass distribution

HDL subclass distribution was evaluated on a subset of samples (n= 6 trans women and n= 6 trans men all with significantly impaired CEC pathways) by two-dimensional electrophoresis. The first dimension was in a 0.5% agarose gel electrophoresis (Hydragel protein(e) kit, Sebia PN4120) in which 10 µl of plasma were separated by charge. Afterwards, agarose gel strips containing the pre-separated lipoproteins were transferred to a home-made nondenaturing 3–20% polyacrylamide gradient gel, where separation by size was performed at 30 mA for three hours. Separated particles were then blotted onto a nitrocellulose membrane and incubated with a human anti-apoA-I antibody (Sigma Aldrich) [33]. Densitometric analysis was performed with a GS-690 Imaging Densitometer and the Multi-Analyst software (Bio-Rad Laboratories, Hercules, CA, USA). The relative content of distinct HDL subclasses was calculated by using the Bio Rad Multi-Analyst /PC Software, and expressed as percentage of total apoA-I.

2.9 Statistical analysis

G*Power software (Düsseldorf, Germany) was used for *a priori* sample size estimation. Based on data from a previous study [34], a baseline level of total HDL-CEC of 8.0±1.5 was assumed, with a within-person correlation of 0.8. An effect size of 10% was assumed as clinically relevant, based on data from

Salaheen et al.[14]. With an alpha of 0.05 and power of 80%, a sample size of at least 14 individuals per group was required.

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA). Each sample was run in triplicate. Data are reported as mean ± SD or median with interquartile (IQR) range (25^{th} to 75^{th} percentile) for parameters with normal and skewed distribution, respectively. Differences between baseline and treatment were evaluated by using the paired two-tailed Student t test or the Wilcoxon matched-pairs signed rank test for parameters with normal and skewed distribution, respectively. The analyses were repeated after stratifying for different formulations of estrogen and testosterone. Independent samples t test, one way ANOVA, Wilcoxon rank sum test or Kruskal-Wallis test were used to test for differences in measures of HDL-CEC and serum hormone concentrations between treatment modalities. The relationship between parameters was assessed by correlation analysis using an univariate logistic regression. Pearson or Spearman correlation coefficient (r) were reported for data with normal and skewed distribution, respectively. Statistical significance was defined as $p < 0.05$.

3. Results

3.1 Patient characteristics

The baseline characteristics of the study population are displayed in Table 1. It consisted of 15 trans women and 15 trans men. Mean age of trans women was 33±12 years and mean BMI 23.3±4.5. They were treated with estradiol valerate (N=4, 27%) or estradiol patches (N=11, 73%). All trans women were prescribed cyproterone acetate at a dose of 50 mg once a day. In these individuals, plasma testosterone dropped from 22 nmol/L to 0.6 nmol/L and LH decreased from 3.4 to 0.1 UI/L, whereas estradiol rose from 93 pmol/L to 157 pmol/L.

The mean age of trans men was 28±13 years and mean BMI 24.8±4.2. Trans men were treated with testosterone gel (N=4, 27%), testosterone esters (N=6, 40%) or testosterone undecanoate (N=5, 33%). After treatment, testosterone rose from 1.4 nmol/L to 24 nmol/L, whereas no significant changes were found for LH and estradiol. No statistically significant differences were observed between the different treatment modalities in terms of estrogen and testosterone levels in trans women and trans men, respectively (Table 2).

3.2 Effect of hormone therapy on BMI and lipid profile

The effects of HT, in both groups, are described in Table 1. In trans women, HT led to a slight increase of BMI. In the case of lipids, a reduction of TC and LDL-C, but no changes in triglycerides were observed in trans women. Additionally, HDL-C decreased by 14.3% (95%CI: -22.2;-6.4). No differences in BMI were found in trans men before and after 12 months of treatment. Changes in lipids and lipoproteins were modest, with non-significant variations in TC and LDL-C and a significant rise in triglycerides. HDL-C was reduced by 19.6% (95%CI: -33.5; -5.6;).

3.3 Effect of hormone therapy on cholesterol efflux capacity

In trans women, 12 months of HT led to a decrement in total HDL-CEC by 10.8% (95%CI: -14.3;-7.4), with a 23.8% (95%CI: -34.7;-12.9) reduction of ABCA1 mediated HDL-CEC and a -4.8% (95%CI: -8.4;-1.1) of aqueous diffusion mediated HDL-CEC (Fig. 1A, B and D, respectively). Non-significant changes in total HDL-CEC, *i.e.*, -6.7% (95%CI: -13.7;0.2; p =0.06), and ABCA1 mediated

HDL-CEC -0.7% (95%CI: -15.0;13.7) were observed in trans men. Conversely, aqueous diffusion mediated HDL-CEC significantly dropped by -9.8% (95%CI: -15.7;-3.9; p <0.01) (Fig. 1E, F and H, respectively). In both trans women and trans men, no changes were found in ABCG1 mediated HDL-CEC (Fig. 1C and G, respectively). Fig. 1 graphically displays absolute changes in HDL-CEC related parameters in trans women and trans men. Table 2 reports the absolute changes in HDL-CEC related parameters stratified by different formulations of estrogen and testosterone. No statistically significant differences were found between different treatment modalities.

3.4 Effect of hormone therapy on serum cholesterol loading capacity

As cell cholesterol content is the resultant of cholesterol efflux and influx, evaluation of the proatherogenic potential of whole serum before and after HT was evaluated by the determination of CLC in human THP-1 monocyte-derived macrophages. CLC did not change after HT in both trans women and trans men. Specifically, at baseline, CLC was 12.78±5.19 μg cholesterol/mg protein in trans women *vs.* 11.9±4.33 μg cholesterol/mg protein after 12 months of treatment (Fig. 2A). In trans men, CLC was 12.57±2.9 μg cholesterol/mg protein at baseline and 11.91±2.94 μg cholesterol/mg protein after 12 months of treatment (Fig. 2B).

3.5 Effect of hormone therapy on HDL subclasses

In trans women, percentages of both large α-migrating HDL particles and small preβ-HDL did not differ after 12 months of hormonal treatment. Percentage of preβ-particles before treatment was 9.24±6.88 and after treatment was 7.33±4.22 (Fig. 3A). Similar conclusions were reached in trans men: no significant changes of HDL subclass distribution were observed after HT. The percentage of preβ-particles before treatment was 13.65±10.26 and after treatment was 17.51±9.38 (Fig. 3B).

3.6 Correlation of HDL-CEC with HDL-C concentrations, hormone levels and body weight

The relationship between HDL-CEC, HDL-C concentrations, sex hormones as well as indexes of body weight was evaluated. In trans women, HDL-C concentrations were correlated with aqueous diffusion HDL-CEC (r= 0.408, p= 0.025; Fig. 4B), not with total HDL-CEC, ABCA1 HDL-CEC or ABCG1

HDL-CEC (Fig. 4A, C and D). Although weak, total HDL-CEC was positively correlated with testosterone (r= 0.357, p= 0.05) and LH (r= 0.374, p = 0.045) and inversely associated with estradiol (r = -0.474; p = 0.008); (Supplementary Figure 1S, panels A, E and I, respectively). Additionally, ABCA1 mediated HDL-CEC was directly correlated with LH (r= 0.460, p= 0.012) and inversely with estradiol (r= -0.463, p= 0.010) **(**Supplementary Fig. 1S panels G and J, respectively**).**

In trans men, total and aqueous diffusion HDL-CEC were positively correlated with absolute HDL-C levels (r = 0.533, p <0.01 and r = 0.624, p <0.01) (Figure 4E and F, respectively). No correlation was instead present between either ABCA1 HDL-CEC or ABCG1 HDL-CEC and HDL-C levels (Fig. 4G and H). In trans men, no correlations between HDL-CEC and testosterone and LH were found (data not shown).

Finally, in both trans women and trans men, CEC measurements did not correlate either with BMI or body weight (data not shown).

3.7 Correlation among HDL-CEC pathways

The possible correlations between total HDL-CEC and each HDL-CEC pathway were also analyzed. In both trans women and trans men, total HDL-CEC was strongly and positively associated with aqueous diffusion CEC (r = 0.593; p = 0.0005 in trans women; r = 0.720; p <0.0001 in trans men) and with the ABCA1-mediated HDL-CEC (r = 0.682; p <0.0001 in trans women; r = 0.677; p <0.0001 in trans men). No significant correlations were found between the other cholesterol efflux mechanisms (data not shown).

4. Discussion

The current study evaluated whether HDL function measured as HDL-CEC was affected by transgender HT and whether these effects were dependent on the decrease in HDL-C concentrations. In trans women, HT significantly reduced total HDL-CEC (11%), an effect mainly driven by the ABCA1 pathway. This last is the major regulator of cholesterol efflux and is independent of HDL-C concentration [35]. However, no clear differences were observed among treatment modalities in trans women. Total HDL-CEC and ABCA1-mediated HDL-CEC seemed to decrease slightly more with

transdermal compared to oral administration of estradiol, but this may also reflect the larger change in estradiol levels in trans women treated with patches compared to tablets. Different conclusions were reached in trans men, namely, a non-significant decrement in total HDL-CEC and a significant reduction only in the aqueous diffusion mediated HDL-CEC. The latter could be simply consequent to the reduced concentrations of HDL-C, as previously reported [15], an evidence in line with the positive correlation that we found between aqueous diffusion mediated HDL-CEC and HDL-C. No differences between treatment modalities were observed in trans men.

Concerning the blood lipid profile, similar directional changes were found as described in our previous report [8], with the exception of a lack of triglyceride reduction in trans women. This is possibly attributable to a lack of power in the current study, as we observed a broader range of triglyceride changes in trans women in our previous report.

Relative to body weight, in trans women, it generally increases during HT[36], as found in the present study. This coincided with the HDL-CEC changes. In prior studies, body weight and HDL-CEC were associated, although no clear causal mechanism was apparent. Consistently, weight loss after bariatric surgery raised total HDL-CEC from ABCA1-overexpressing macrophages [37, 38]. Further, in non-obese, non-diabetic individuals, increased BMI is associated with changes in the protective functions of HDL, such as reduced HDL-CEC and increased antioxidant activity [39]. Despite the above described association between body weight and HDL-CEC, in the present study no correlations between BMI/body weight with cholesterol efflux capacity were found. This suggests that the effects on cholesterol efflux are independent of weight gained during the 12 months of HT.

4.1 Cholesterol efflux capacity and sex hormones.

HDL-CEC appears to be a more sensitive index of the CV risk versus HDL-C or other biochemical risk markers. Although the seminal study by Rohatgi et al. [13] showed that the lowest quartile of HDL-CEC was associated with a 67% higher risk of CVD, comparison with the present findings are difficult due to methodological differences in the assessment of HDL-CEC. To put the present evidence into a clinical perspective however, a better comparison can be made with the nested case-control study from the prospective Epic-Norfolk cohort, also using the validated *ex-vivo*

radiotracer method adopted in our study. The Epic-Norfolk study indicated that a 10% reduction of total HDL-CEC, as found in our trans women, is associated with an approximate 15% rise of the odds ratio (OR) for the incidence of coronary heart disease (CHD) [14]. However, controversy still exists on the role of HDL-CEC as an independent CVD predictor. In a recent Health Professionals Follow-Up Study, that included middle-aged and older cisgender men, HDL-CEC was not associated with CHD after adjustment for HDL-C concentrations [40]. In addition, in a nested case-control study within the JUPITER-trial with rosuvastatin, no significant association was found between baseline CEC and incidence of CV events [41]. These contrasting results highlight the need for additional studies (*e.g.*, studying sex-specific effects) to better understand the underlying pathophysiological mechanisms of HDL-CEC in CVD.

Studies evaluating the influence of sex hormones on HDL-CEC have provided mixed results. In the case of estrogens, HDL-CEC was correlated with endogenous estrogens in pre-menopausal women, but the correlation was lost after menopause [9]. Increased HDL-CEC was instead reported after administration of conjugated equine estrogen and medroxyprogesterone in a different study [42]. The unclear association between estrogens and HDL-CEC suggests that the reduced total HDL-CEC, that we observed in trans women, is more likely to be ascribed to the drop in testosterone levels. This is supported by a previous observation by our group, showing a markedly decreased HDL-CEC comparing primary or secondary hypogonadal patients to controls [17].

Studies on the association between testosterone and HDL-CEC have not provided conclusive information, *e.g.*, no differences were observed in HDL-CEC before and after treatment in chemically castrated healthy adult males or older hypogonadal men [43, 44]. This finding is similar to the absence of changes in HDL-CEC in trans men in the present study. Wultsch et al evaluated changes in HDL-CEC during transgender HT [19], with findings comparable to the current study (decreased HDL-CEC in trans women but not in trans men), despite the addition of finasteride to the treatment protocol for trans women, which is a non-standard procedure in practice [7]. The authors also used a different cell model and did not specify the contribution of different pathways of HDL-CEC.

The contrasting results described above underline the fact that association between sex hormones and HDL-function may vary among different populations and are thus not easily

comparable, as reviewed by Schiffer et al [45]. The addition of the anti-androgen cyproterone acetate (or other agents) to the HT of trans women makes it difficult to isolate effects driven by estrogen, consequent to testosterone suppression or to specific side-effects of the anti-androgen. Indeed, HDL-C concentrations are reduced in trans women treated with cyproterone acetate, whereas they increase with other anti-androgens (*e.g.*, spironolactone or a GnRH-analog)[46, 47]. In our study the decreased HDL-CEC in trans women is likely to be attributed to the use of cyproterone acetate; which, aside from the anti-androgen activity also exert progestogen-like effects. This hypothesis is in line with the data by Wultsch et al[19] who found a smaller decrease in HDL-CEC in trans women, despite estradiol levels being higher than in the current study. These differences highlight the need for randomized studies comparing different treatment modalities to study CV risk in transgender endocrine care.

In this complex scenario, in order to investigate whether impairment in ABCA1 HDL-CEC found in trans women was linked to a depletion in lipid-poor pre-β HDL, a two-dimensional electrophoretic separation was performed in a subset of participants. Indeed, it is believed that lipid-poor pre–β HDL particles are the primary acceptors of cholesterol efflux from macrophages through ABCA1 [48]. Neither the percentage of mature α-HDL nor that of pre-β HDL was affected by 12 months HT in both trans women and trans men. Thus, the ABCA1 HDL-CEC changes observed in trans women likely reflect possible HDL compositional modifications that occur after HT [44] and may impact cholesterol efflux pathway without affecting HDL subclass distribution [34]**.**

It has been shown that LDL can induce cholesterol efflux from cultured macrophage foam cells via the ABCA1 transporter [49]. In our experimental setting we may exclude the contribution of LDL to cholesterol efflux since CEC studies have been performed by incubating cells with the serum fraction containing only HDL. However, in order to evaluate the contribution of LDL, we analyzed the CLC of whole sera from all transgender individuals before and after HT. Although this was an indirect method to exclude the involvement of LDL in RCT, HT did not affect CLC either in trans women or in trans men. Moreover, it should be underlined that HT did not significantly increase LDL-C in either groups, instead in trans women LDL-C was reduced.

4.2 Limitations

The present results should be interpreted within the context of potential limitations, *e.g.*, its observational nature. While the study was sufficiently powered to support the overall changes in total HDL-CEC, additional analyses comparing treatment modalities were underpowered and should be interpreted with caution. Moreover, the international variations in treatment modalities limit the ability to generalize results to other populations. Secondly, the contribution of SR-BI on HDL CEC was not evaluated. SR-BI-promoting cholesterol efflux from macrophages is generally associated to atheroprotection [50]. Considering that the main activity of SR-BI is to facilitate the AD efflux [51], the impact of HT on SR-BI HDL-CEC is expected to be quite similar to that observed in the case of AD HDL-CEC. Finally, lipid composition of the HDL particles [24], as well as HDL proteome, or HDL paroxonase-1 have not been evaluated [44]. However, it is worth mentioning that the current study is the second to study HDL-CEC during transgender HT and the first to specify pathways through which HDL-CEC is mediated in these individuals.

4.3 Conclusions

As recently summarized by Connelly et al.[52], the pathways by which sex hormones affect CV risk are numerous and complex and the net risk change induced by transgender HT can be interpreted as the resultant of effects on all these different pathways. In this context, the current findings suggest that, especially in trans women, a decrease in HDL-CEC during feminizing HT could contribute to a higher CV risk. Indeed, while HDL-C concentrations decrease in both trans men and trans women, HDL-CEC is independently lowered in trans women through a decrease in ABCA1-mediated HDL-CEC.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Conceptualization, D.M.v.V., C.R.S., M.d.H. and M.R.; methodology, D.M.v.V, M.P.A., F.Z., S.S. and M.R.; validation, D.M.v.V., M.P.A., M.d.H. and M.R.; formal analysis, D.M.v.V., M.P.A. F.Z. and A.S. investigation, D.M.v.V., M.P.A., F.Z. and M.R.; resources, D.M.v.V, S.S. and M.d.H; data curation, D.M.v.V, M.P.A., F.Z. and M.R.; writing—original draft preparation, D.M.v.V., M.P.A., F.Z., M.R.M.; writing—review and editing, D.M.v.V., M.P.A., F.Z., M.d.H., A.S. and M.R.; supervision, C.R.S. and M.d.H. All authors have read and agreed to the submitted version of the manuscript.

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Table 1. Baseline characteristics of the study population

	Trans women (N = 15)		**Trans men (N = 15)**	
	Baseline	**12 months HT**	**Baseline**	**12 months HT**
Age (years)	33 ± 12		28 ± 13	
Testosterone formulation (N, %)	n/a		Testosterone gel (4, 27%) Testosterone esters (6, 40%) Testosterone undecanoate (5, 33%)	
Estrogen formulation (N, %)	Estradiol valerate (4, 27%) Estradiol patches (11, 73%)			
Anti-androgen (N, %)	Cyproterone acetate 1dd 50 mg (15, 100%)			
Medical history	1x Depression, 1x hypertension, 1x ADHD		1x Depression, 1x ADHD	
Co-medication	1x ACE-inhibitor, 1x proton pump inhibitor, 1x methylphenidate		1x methylphenidate 1x SSRI 1x iron supplement	
Smoking (%)	33%		20%	
Alcohol (units/week)	1 (0-2)		0 (0-4)	
BMI (kg/m^2)	23.3 ± 4.5	24.3 ± 4.5 *	24.8 ± 4.2	24.4 ± 4.2
Testosterone (nmol/L)	22 (16-34)	0.6 (0.5-1.1)*	1.4 (1.1-1.7)	24 (18-40)*

Estradiol (pmol/L)	93 (78-116)	157 (103-505)*	141 (76-323)	158 (103-214)
LH (IU/L)	3.4 (2.7 – 4.3)	0.1 (0.1-0.1)	4.5 (2.9 – 12.7)	3.3 (0.3-5.1)
Total cholesterol (mmol/L)	4.80 (4.33 – 5.70)	4.47 (4.06 – 4.80) *	4.40 (3.68 – 5.00)	4.36 (3.93 - 4.90)
LDL cholesterol (mmol/L)	2.97 (2.70 – 3.67)	2.84 (2.15 – 3.26) *	2.06 (1.70 – 2.91)	2.53 (2.09 – 2.90)
HDL cholesterol (mmol/L)	1.32 (1.13 – 1.66)	1.17 (1.00- 1.44) *	1.61 (1.34 – 1.99)	1.31 (1.16 – 1.57) *
Triglycerides (mmol/L)	0.70 (0.60 – 0.91)	0.84 (0.58 – 0.94)	0.72 (0.59 – 1.00)	0.90 (0.71 - 1.10) *

Data are presented as mean ± SD or median with interquartile range (25^{th} to 75^{th} percentile). * $p< 0.05$.
ADHD, attention deficit hyperactivity disorder; HT, hormone therapy; SSRI, selective serotonin reuptake inhibitor; BMI, body mass index; LH, luteinizing hormone.

Table 2. Changes in functional components of HDL cholesterol and hormone levels by different treatment modalities

	Measurements of HDL cholesterol function (relative change in %)				Change in hormone levels	
	Total CEC	ABCA1 CEC	ABCG1 CEC	Aqueous diffusion CEC	Delta estradiol (pmol/L)	Delta testosterone (nmol/L)
Trans women						
Total (n=15)	-10.8 (-14.3; -7.3)	-23.8 (-34.7; -12.9)	0.7 (-10.4; 11.9)	-4.8 (-8.4; -1.1)	79 (36 – 388)	-21.5 (-32.6 – -15.4)
Patches (n=11)	-12.5 (-15.6; -9.3)	-26.2 (-34.2; -18.1)	3.8 (-8.4; 16.1)	-6.5 (-9.9; -3.1)	106 (47 – 554)	-17.5 (-34.9 – -14.0)
Tablets (n=4)	-6.0 (-12.4; 0.4)	-17.5 (-50.0; 14.9)	-7.0 (-22.7; 8.8)	0.3 (-6.2; 6.8)	30 (-17 – 74)	-24.7 (-28.8 – -20.0)
p-value for difference	0.07	0.16	0.33	0.07	0.11	0.43
Trans men						
Total (n=15)	-6.7 (-13.7; 0.2)	-0.7 (-15.0; 13.7)	-3.6 (-13.8; 6.7)	-9.8 (-15.7; -3.9)	11 (-206 – 129)	21.3 (16.4 – 33.4)
Gel (n=4)	-5.6 (-22.7; 11.4)	5.8 (-28.9; 33.3)	-6.3 (-23.5; 10.9)	-10.2 (-26.3; 6.0)	15 (-147 – 148)	28.7 (22.5 – 35.1)
Esters mix (n=6)	-4.7 (-15.3; 5.9)	-4.5 (-28.3; 19.3)	5.8 (-9.0; 20.8)	-6.7 (-12.8; -0.5)	13 (-206 – 129)	20.8 (13.9 – 33.4)
Undecanoate (n=5)	-9.9 (-17.8; -2.1)	-2.0 (-30.8; 26.8)	-17.8 (-30.7; -4.9)	-13.1 (-21.5; -4.7)	6 (-290 – 83)	20.8 (16.4 – 22.7)
p-value for difference	0.37	0.73	0.27	0.50	0.57	0.65

Relative changes in HDL components were expressed as percentage change (±95% CI). Absolute changes in hormone levels were presented as median (IQR).
CEC, cholesterol efflux capacity; ABCA1, ATP-binding cassette.

Figure legends

Figure 1. Cholesterol efflux capacity in trans women and trans men at baseline and after hormone therapy.

Each point of the scatter plot represents the mean percentage of triplicate analyses of each serum sample. The horizontal, solid line is the mean of each group. Changes in (A and E) total HDL-CEC, (B and F) ABCA1-mediated HDL-CEC, (C and G) ABCG1-mediated HDL-CEC and (D and H) aqueous diffusion mediated HDL-CEC. CEC, cholesterol efflux capacity; M0, baseline; M12, twelve-month follow-up; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1

Figure 2. Cholesterol loading capacity in trans women and trans men at baseline and after hormone therapy.

Each point of the scatterplot represents the mean percentage of triplicate analyses of each serum sample. The horizontal, solid line is the mean of each group. M0, baseline; M12, twelve months follow-up.

Figure 3. 2D electrophoretic analysis of HDL of trans women and trans men at baseline and after hormone therapy.

In a subset of trans women (n = 6; panel A) and trans men (n = 6; panel B), α- and preβ-migrating HDL subclasses have been separated by 2D electrophoresis and immunodetected with an anti apoA-I antibody. Red circles indicate preβ-HDL subclasses. A representative image is shown.

Figure 4. Correlations between cholesterol efflux capacity and serum HDL-cholesterol levels in trans women and trans men at baseline and after hormone therapy.

Baseline measurements (●) and follow up measurements (○) are combined. Pearson or Spearman correlation coefficient (r) was reported for parameters with normal and skewed distribution,

respectively. CEC, cholesterol efflux capacity; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1.

Reviewer #1: ATH-D-20-01166- R2.
The effect of transgender hormonal treatment on high density lipoprotein cholesterol efflux capacity by Daan M. van Velzen et al.
Major flaws, which I previously highlighted, remain, unfortunately, also after the revision. The novelty of the article is very limited as more than one paper have been already published investigating effects of gender affirming hormonal therapy (HT) and cholesterol efflux capacity, see for instance. J Sex Med . 2015 Jun;12(6):1436-9. doi: 10.1111/jsm.12878. In this paper indeed whole serum capacity was tested thus potentially including also an LDL mediated effect.

We thank the reviewer for her/his comment. We are surprised that the reviewer found the novelty of the present article very limited since in the first revision the comment was "Thus, new investigations on cardiovascular implications of the increasing transgender HT may deliver valuable novel insights on its safety as well as about effects of sex hormones on cardiovascular physiology".
Relative to novelty, to the best of our knowledge, only one study reported the effects of gender affirming hormone therapy on the effect of HDL efflux capacity, the one mentioned by the Reviewer (reference 18 of the present manuscript): this study evaluated CEC in THP-1 derived macrophages in 10 samples from trans men and 10 samples from trans women before and after treatment.
Besides the evaluation of HDL total CEC, a parameter that could be considered not novel, our study evaluated other aspects in transgender individuals: (1) CEC mediated by each individual efflux mechanism that contribute to total efflux, *e.g.*, ABCA1- and ABCG1-mediated effluxes; (2) correlations of CEC with HDL and hormone plasma levels; (3) the impact of hormone therapy on HDL subclass distribution and, (4) the proatherogenic potential of sera from the studied subjects, namely serum capacity to load macrophage with cholesterol (CLC).

In the light of these observations, we are convinced that our study adds more and new insights compared to what is presently available in the literature in reference to the impact of hormone affirming therapy on HDL cholesterol effluxing capacity.

Concerning the hypothesis of the role of LDL in cholesterol efflux, this certainly exceeds the limits of our investigations. The aim of our study was to evaluate the HDL function by measuring their capacity to promote cholesterol efflux, one of the main and known atheroprotective functions of these lipoproteins. Notably, in most published CEC evaluations, apoB-depleted serum (HDL fraction) has been utilized: *Fallah S. et al, J Clin Lipidol. 2021 Jan-Feb;15(1):218-226.e1; Cervellati C, et al. Atherosclerosis; 2019 Jun;285:64-70; Niisuke K. et al, Curr Opin Lipidol. 2018 Aug;29(4):293-298; Gkolfinopoulou C, et al. J Rheumatol. 2015 Sep;42(9):1652-60; Vigna G.B et al. Nutr Metab Cardiovasc Dis. 2014 Jul;24(7):777-83: Minicocci I. et al. Int J Cardiol. 2013 Oct 9;168(4):4375-8; Tedesco S., Adorni M.P., et al. Endocrine. 2019 2019 Nov;66(2):360-369; Adorni MP et al. J Clin Endocrinol Metab. 2019 104(8):3097-3107; Greco D, Kocyigit D et al. Nutr Metab Cardiovasc Dis. 2018 28(8):822-829; Adorni MP et al. Ther Clin Risk Manag. 2017 Dec 11;13:1555-1562; Zimetti F et al. J Lipid Res 2017, 58(10):2051-2060; Yahya R, Favari E et al. Atherosclerosis. 2016 May 11;251:15-18; Pisciotta L et al. J Clin Lipidol. 2015 Nov-Dec;9(6):837-46; Zimetti F, Favari E et al. Atherosclerosis. 2015 Aug 10;242(2):443-449; Pisciotta L et al. Circ Cardiovasc Genet. 2012 Feb 1;5(1):42-50; Calabresi L et al. Atherosclerosis. 2009 Aug; 205(2):506-11.*

In addition, we have previously shown that CEC measured with apoB-depleted serum or with whole serum leads to similar results (*Pisciotta L et al. Circ Cardiovasc Genet. 2012;5:42-50; Pisciotta L et al. J Clin Lipidol. 2015 Nov-Dec;9(6):837-846).*
However, in order to evaluate the contribution of LDL, we also carried out in our samples the evaluation of the serum capacity to load macrophage with cholesterol (CLC), reflecting also LDL function, and found essentially no hormone effects (Fig.2), thus allowing to conclude that the new

interpretation of an LDL related mechanism is unlikely to play a major role in the described efflux changes.

Clinical relevance is also low. Author agrees with my comments that in this study "the decreased HDL-CEC in trans women is likely to be attributable to the use of cyproterone acetate; which, aside from the anti-androgen activity also exert progestogen-like effects." Thus, the present results are not addressing general effects of gender affirming hormonal treatment protocols, but rather the effect of this specific antiandrogen. Moreover, Authors agree with my comment about the lack of appropriate power of the study. The Study is underpowered as N=14 is assumed for homogeneous treatment conditions, here HT treatment is not homogeneous. Authors state in their answer to this Reviewer that: "Due to lack of statistical power, it is difficult to appreciate differences among the different treatment modalities". In table 2 it is said that hormonal concentrations were independent of treatment, though we clearly see how the delta estradiol (values -17 - 74) in 4 subjects receiving tablets is very wide.

Although we respect the judgement of this reviewer, we disagree with her/his comment that the clinical relevance of the current study is low. We currently set out to study changes in metrics of HDL function during transgender hormone therapy, as previous studies had shown decreases in HDL-C concentrations in both trans women and trans men. Cyproterone acetate is a commonly prescribed anti-androgen in transgender hormone therapy. Thus, the current study does represent the effects of standard gender affirming hormone therapy for several regions worldwide.

With regard to study power, we assumed no differences in treatment modalities in our initial working hypothesis. The power calculation was therefore based only on the overall study population. Stratification for different treatment modalities (with statistical analyses) were only added at request of this reviewer. We feel the current sample size does not allow for meaningful statistical analysis between treatment modalities, and also comparing by 'eyeballing' should be done with caution. The changes in estradiol levels (IQR -17 to 74), in fact, are based on only four observations. The issue of differences in treatment modalities was raised by this reviewer and therefore would potentially also interest the reader. Therefore, we decided to present the data despite their limitations. The fact that these additional analyses are underpowered has now been also underlined in the limitations section.

Line 362 : ".Despite the described association between body weight and HDL-CEC, in the present study no correlations between BMI/body weight with cholesterol efflux capacity were found".... the effects on cholesterol efflux are independent of weight gained during the 12 months of HT. Are the tablets versus patch estradiol associated to differential BMI gain? Which were the determinants of CEC measurements in this study? assessed by Logistic Regression: Univariate and Multivariate analyses? Change in BMI observed in trans women can induce changes in insulin sensitivity. Interestingly changes in insulin sensitivity have been documented in Men to Female patients. See Clin Endocrinol (Oxf) 2010;72:1-10 .

Changes in BMI were not different for tablets vs patches in the current study (linear regression with an autoregression model: 0.76 (-1.79 – 2.79)), although the same lack of power can be applied here. We know from larger previous studies from our center that weight gain is not differentially affected by different treatment modalities in trans women (*Klaver et al. EJE 201https://doi.org/10.1530/EJE-17-0496*).

Concerning the determinants of CEC measurements, it was assessed by a correlation study by using a univariate logistic regression. This information has been now added in the text in the section of Material and Methods paragraph 2.9 Statistical analysis. It reads as follows "The relationship between parameters was assessed by correlation analysis by using a univariate logistic regression".

As the reviewer correctly suggests, the feminizing hormone therapy may be associated to a worsened insulin resistance, associated to decreased lean and increased fat mass, although the available data are not fully consistent, as highlighted in a recent metanalysis *(Spanos, C. et al. World J Diabetes. 2020 Mar 15;11(3):66-77).* However, considering the slight changes in BMI observed in trans women after hormonal treatment (from 23.3 ± 4.5 vs 24.3±4.5), we decided to not include the measurements of insulin resistance among our evaluations. The impact on insulin resistance is likely to be very negligible.

Line 271:" In transmen no significant changes were found for LH and estradiol". Indeed estradiol is 158 (103-214) pmol/l and thus almost identical to transwomen. Thus in transmen there is an undelying very high estradiol background that is in my view remarkable. Authors agree that" estrogens in trans men (either due to aromatization or ovarian production) may be of some influence on the associations between testosterone and HDL functional changes. With the current study design, however, we are unable to separate the effects of testosterone and of residual estrogens in transmen." An appropriate power of the study would be advisable to separate the effects of testosterone and of residual estrogens in transmen, this aspect would have raised the incremental novelty of the article.

The main research question in this study was to evaluate changes in functional metrics of HDL during transgender hormone therapy, after observing decreases in HDL-C concentration in a previous report. The estradiol background in masculinizing hormone therapy is a normal occurrence, as discussed by our colleagues in an earlier report (*Defreyne et al. LGBT Health Feb 2020, doi: 10.1089/lgbt.2019.0260*). Thus, the current hormonal environment reported in our study reflects the population of interest. With the main research question in mind, we feel that the results are not limited by this observation. With regard to more mechanistic explanations, it is indeed difficult to separate effects of testosterone from estradiol. However, we feel that by increasing the study sample size, we would not fully be able to dilute specific testosterone effects in trans men with a high estradiol background. A specific group with aromatase inhibitor would have to be added to the study to fully address this point – but is not feasible at this point.

Why Authors did not used ultracentrifugation isolated HDL? this approach would have improved the quality of findings compared to previosly published evidence? Why Murine J774 macrophages and not human THP1 cells, this is also a methodological weakness. Indeed it has been reported tha there are remarkable Sex-related differences in the regulation of macrophage cholesterol metabolism (see: Curr Opin Lipidol. 2001 Oct;12(5):505-10, and Mol Cell Biochem. 2002 Nov;240(1-2):67-73. doi: 10.1023/a:1020604610873).

In order to isolate the HDL fraction of the serum we precipitated the apoB-containing lipoproteins by using polyethylene glycol (PEG). This easy, reproducible procedure, allows to obtain biological samples in which the only lipoprotein present is HDL, and has been extensively used by us and several other authors in studies evaluating cholesterol efflux capacity in different clinical settings *(Fallah S. et al, J Clin Lipidol. 2021 Jan-Feb;15(1):218-226.e1; Cervellati C, et al. Atherosclerosis; 2019 Jun;285:64-70; Niisuke K. et al, Curr Opin Lipidol. 2018 Aug;29(4):293-298; Gkolfinopoulou C, et al. J Rheumatol. 2015 Sep;42(9):1652-60; Vigna G.B et al. Nutr Metab Cardiovasc Dis. 2014 Jul;24(7):777-83: Minicocci I. et al. Int J Cardiol. 2013 Oct 9;168(4):4375-8; Tedesco S., Adorni M.P., et al. Endocrine. 2019 2019 Nov;66(2):360-369; Adorni MP et al. J Clin Endocrinol Metab.*

2019 104(8):3097-3107; Greco D, Kocyigit D et al. Nutr Metab Cardiovasc Dis. 2018 28(8):822-829; Adorni MP et al. Ther Clin Risk Manag. 2017 Dec 11;13:1555-1562; Zimetti F et al. J Lipid Res 2017, 58(10):2051-2060; Yahya R, Favari E et al. Atherosclerosis. 2016 May 11;251:15-18; Pisciotta L et al. J Clin Lipidol. 2015 Nov-Dec;9(6):837-46; Zimetti F, Favari E et al. Atherosclerosis. 2015 Aug 10;242(2):443-449; Pisciotta L et al. Circ Cardiovasc Genet. 2012 Feb 1;5(1):42-50; Calabresi L et al. Atherosclerosis. 2009 Aug; 205(2):506-11.).

Notably, the same method has been applied in studies highlighting an inverse relationship between cholesterol efflux capacity and prevalence of cardiovascular (CV) disease, as well as the incidence of CV events, indicating HDL-CEC as a better predictor of CV risk compared to plasma HDL-cholesterol levels *(Soria-Florido MT. et al. Circulation. 2020 Feb 11;141(6):444-453; Khera A.V. et al, Circulation. 2017 Jun 20;135(25):2494-2504; Rohatgi A. et al. N Engl J Med. 2014 Dec 18;371(25):2383-93; Saleheen, D. et al. Lancet Diabetes Endocrinol. 2015 Jul;3(7):507-13. Mody, P. et al. J Am Coll Cardiol. 2016 May 31;67(21):2480-7; Khera A.V. et al. N Engl J Med. 2011 Jan 13;364(2):127-35).* Recently, a head to head comparison between HDL isolated by centrifugation or obtained by PEG precipitation has been performed and the two procedures gave comparable results in CEC studies *(Horiuchi, Y el al. Biosci Rep. 2019 Apr 30; 39(4): reference #27 of the original manuscript). We have added a brief description of this report to the manuscript (paragraph 2.4 Methods section).*

At the same manner, although the use of murine cells as the J774 macrophages can apparently be seen as a methodological weakness, the majority of studies correlating CEC and CV risk have been conducted in this cellular model after incubation with cAMP *(Ritsch A. et al. Biomedicines. 2020 Nov 21;8(11):524; Soria-Florido MT. et al. Circulation. 2020 Feb 11;141(6):444-453; Khera A.V. et al, Circulation. 2017 Jun 20;135(25):2494-2504; Rohatgi A. et al. N Engl J Med. 2014 Dec 18;371(25):2383-93; Saleheen, D. et al. Lancet Diabetes Endocrinol. 2015 Jul;3(7):507-13. Mody, P. et al. J Am Coll Cardiol. 2016 May 31;67(21):2480-7; Khera A.V. et al. N Engl J Med. 2011 Jan 13;364(2):127-35).* We thus felt more confident in adopting this widely used cell model, in order to draw reliable conclusions on the potential CV impact of sex hormones based on CEC results..

We absolutely agree with this reviewer on the existence of sex-related differences in terms of macrophage cholesterol metabolism *(Curr Opin Lipidol. 2001 Oct;12(5):505-10).* However, the investigation of macrophage cholesterol metabolism in transgender individuals is not the objective of the present study, focused instead on the characterization of the HDL from these subjects treated with sex hormones in terms of cholesterol efflux capacity, and not on the evaluation of their impact on the capacity of cells to release cholesterol. It would be interesting in future studies to use human monocyte-derived macrophages isolated from blood of these individuals to study potential changes in cholesterol metabolism after hormonal treatments. It is also worth mentioning that in our study the influence of hormonal treatment on cell expression of cholesterol transporters promoting cholesterol efflux may be excluded. In our experimental settings evaluating HDL- CEC, we exposed cells to very low concentrations of apoB-depleted serum (1 or 2% in medium volume). It is thus unlikely that the hormones present in such low amounts may reach an adequate concentration to modulate the expression of cell cholesterol transporters, as it has been reported for estradiol on ABCA1 *(Mol Cell Biochem. 2002 Nov; 240(1-2):67-73).*

Reviewer #2: In this revised manuscript the authors have included new data and text to answer most of the questions raised.
Minor point: I agree that the relative rate of efflux to SR-BI does not have to be measured if the role for SR-BI in the author's in vitro model system does not contribute to cholesterol efflux. However, many in vivo studies in mice have shown that SR-BI does play an important role in the

efflux of cholesterol from macrophages and the associated protection against atherosclerosis [see for instance Van Eck M et al. Am. J. Pathol (2004)]. As such, the authors should at least mention in the text that changes in SR-BI functionality could possibly be involved in the effects of hormone treatment on macrophage CEC in vivo (i.e. in the limitation paragraph of the discussion section).
According to the Reviewer's suggestion, we have included a comment on the SR-BI mediated CEC in the limitation paragraph of the discussion section (paragraph 4.2).
It reads as follow: "Secondly, the contribution of SR-BI on HDL CEC was not evaluated. SR-BI-promoting cholesterol efflux from macrophages is generally associated to atheroprotection 49. Considering that the main activity of SR-BI is to facilitate the AD efflux 50, the impact of hormone therapy on SR-BI CEC is expected to be quite similar to that observed in the case of AD CEC".

Reviewer #3: The authors addressed all my comments. However, new data involving changes in the HDL function and hormone levels were added to the manuscript as Table 2. These data require statistical analysis. If there was no difference between the treatment modalities, after vs before treatment changes can still be significant for some of them, potentially indicating differences in the potency. This should be evaluated and commented on.
Statistical analyses have been added to table 2 to address this comment. Additionally, these results are also evaluated in the discussion. However, we also wish to stress that these additional analyses are underpowered and should be interpreted with caution. A sentence has been added to the limitation section to address this point. This reads as follows "While the study was sufficiently powered to support the overall changes in total HDL-CEC, additional analyses comparing treatment modalities were underpowered and should be interpreted with caution".

Submission Declaration statement

 The article is not under consideration for publication elsewhere.

 Publication of the article is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out.

 If the article is accepted, it will not be published elsewhere by the authors, including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

To the Editor-in-Chief of Atherosclerosis

Dear Prof. von Eckardstein,

please find enclosed copy of the revised version of the paper “The effect of transgender hormonal treatment on high density lipoprotein function “, by van Velzen D. et al, to be considered for publication on ATHEROSCLEROSIS, together with a detailed list of answers to the comments of the reviewers.

We trust the presently modified version of this paper will be found of interest for the readers of ATHEROSCLEROSIS and we are most grateful for your kind attention to this matter.

Sincerely yours,

Cesare Sirtori

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure 1

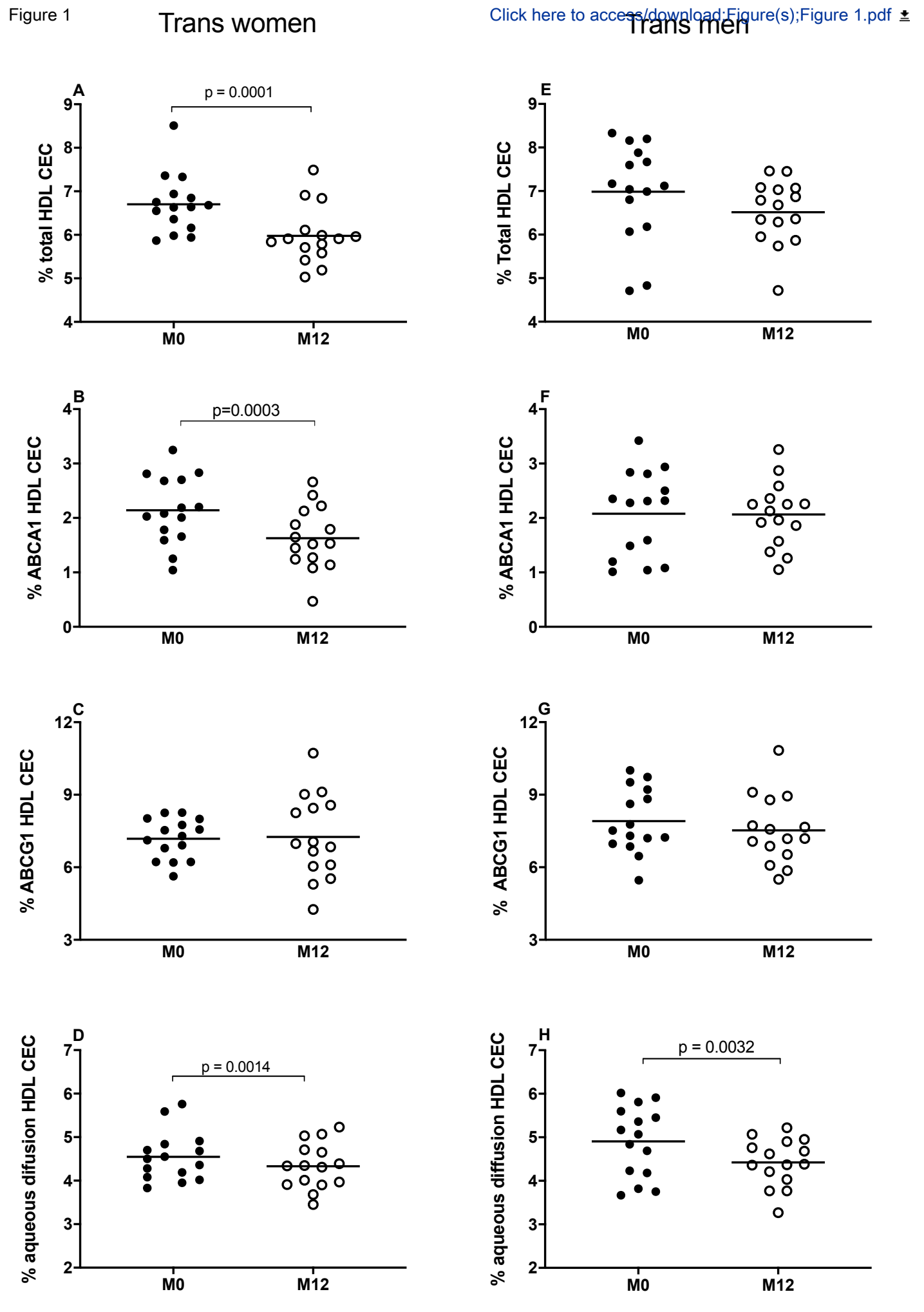

Figure 2

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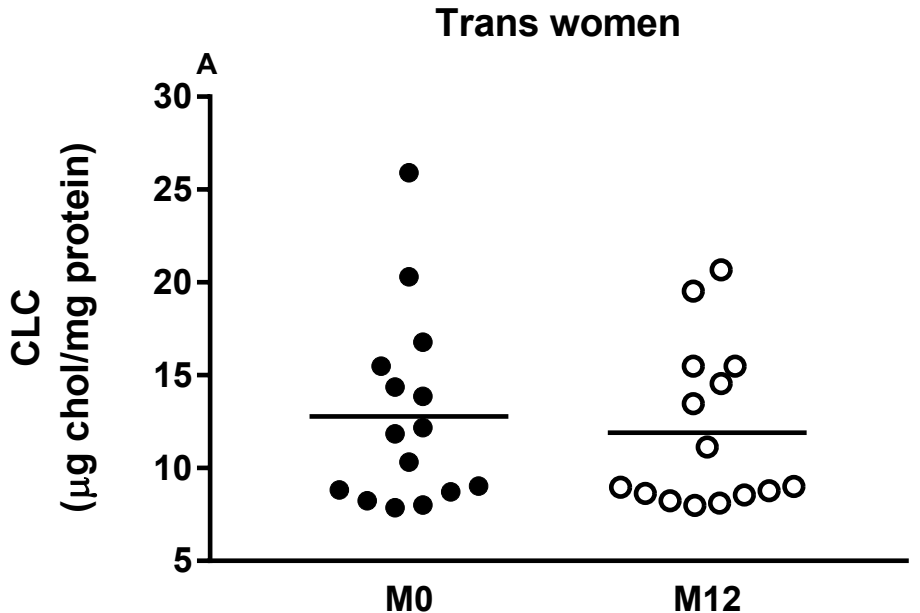


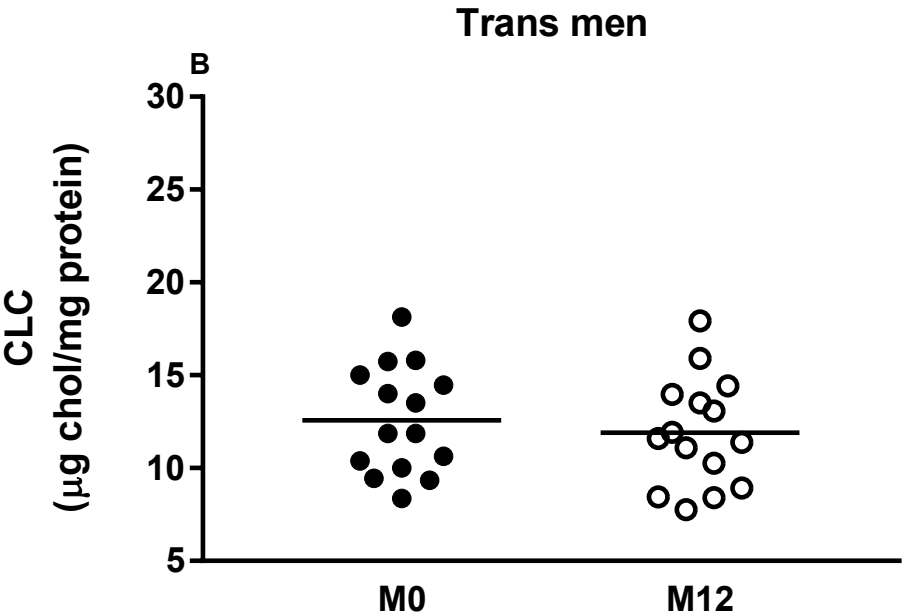


Figure 3

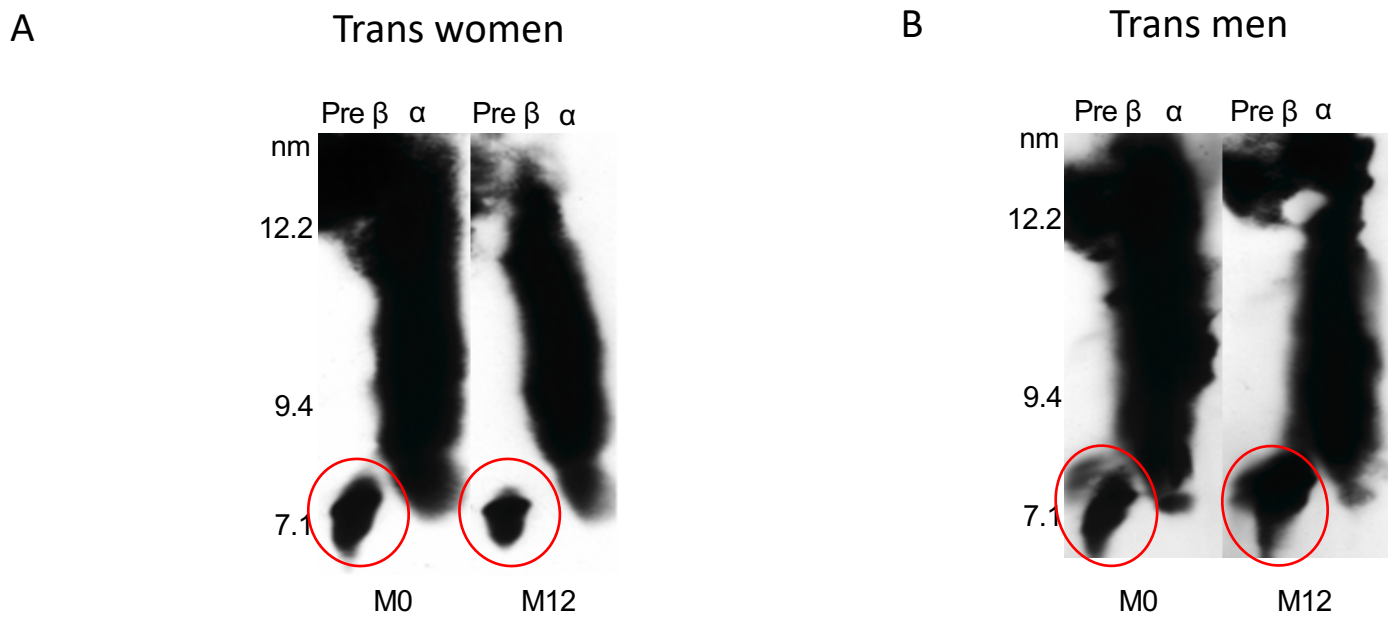
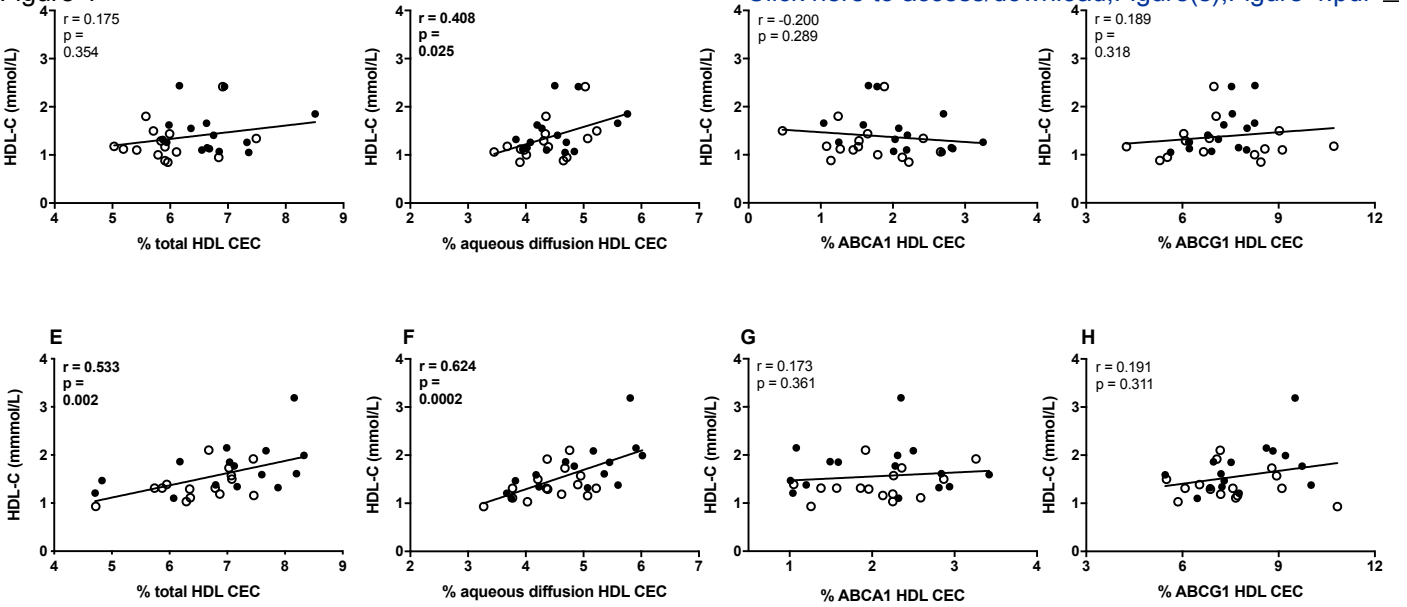


Figure 4



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Conceptualization, D.M.v.V., C.R.S., M.d.H. and M.R.; methodology, D.M.v.V, M.P.A., F.Z., S.S. and M.R.; validation, D.M.v.V., M.P.A., M.d.H. and M.R.; formal analysis, D.M.v.V., M.P.A. F.Z. and A.S. investigation, D.M.v.V., M.P.A., F.Z. and M.R.; resources, D.M.v.V, S.S. and M.d.H; data curation, D.M.v.V, M.P.A., F.Z. and M.R.; writing—original draft preparation, D.M.v.V., M.P.A., F.Z., M.R.. writing—review and editing, D.M.v.V., M.P.A., F.Z., M.d.H., A.S. and M.R.; supervision, C.R.S. and M.d.H. All authors have read and agreed to the submitted version of the manuscript.