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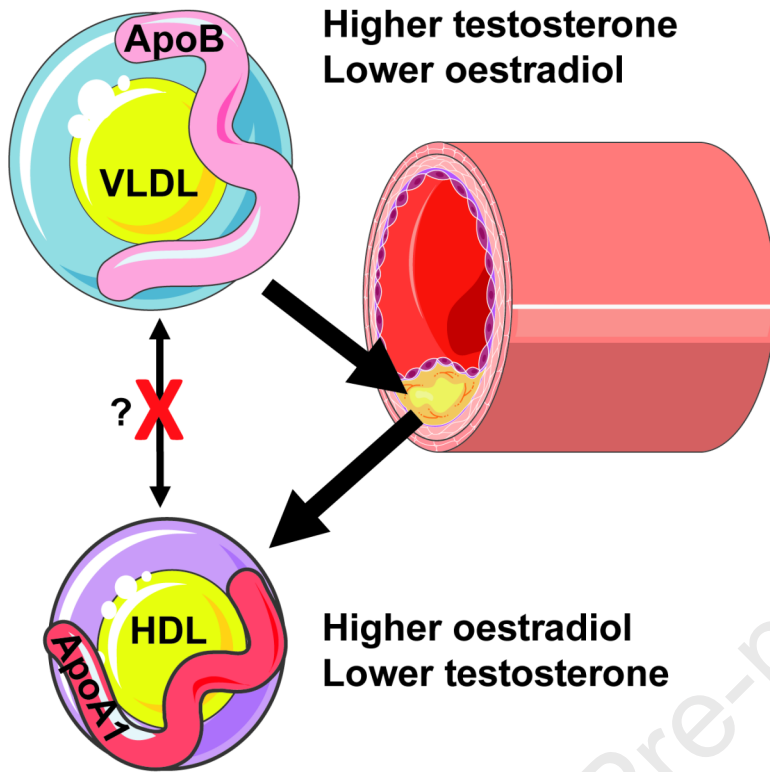
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Sex hormones drive changes in lipoprotein metabolism

George A Robinson^{1,2}, Junjie Peng², Hannah Peckham², Anna Radziszewska², Gary Butler^{3,4},
Ines Pineda-Torra^{5†}, Elizabeth C Jury^{1†}, Coziana Ciurtin^{1,2†*}

¹Centre for Rheumatology Research, Division of Medicine, University College
London, Rayne Building, London WC1E 6JF, U.K.

²Centre for Adolescent Rheumatology Research, Division of Medicine, University College
London, Rayne Building, London WC1E 6JF, U.K.

³Department of Paediatric and Adolescent Endocrinology, UCLH and Great Ormond Street
Institute of Child Health, University College London, London, U.K.

⁴Gender Identity Development Service (GIDS), Tavistock and Portman NHS Foundation Trust,
London, U.K.

⁵Centre for Cardiometabolic and Vascular Science, Department of Medicine, University College
London, London WC1E 6JF, U.K.

†Share senior authorship

*Lead contact

Corresponding authors:

Dr Coziana Ciurtin: c.ciurtin@ucl.ac.uk, +44 (0)20 7380 9230

Professor Liz Jury: e.jury@ucl.ac.uk, +44 (0)20 3108 2161

Professor Ines Pineda-Torra: i.torra@ucl.ac.uk, +44 (0)20 7679 6535

Dr George Robinson: george.robinson.15@ucl.ac.uk, +44 (0)20 3108 2161

Summary

Women have a reduced cardiovascular disease (CVD) risk compared to men which could be partially driven by sex hormones influencing lipid levels post-puberty. The interrelationship between sex hormones and lipids was explored in pre-pubertal children, young post-pubertal cis-men/women, and transgender individuals on cross-sex-hormone treatment (trans-men/women) using serum metabolomics assessing 149 lipids. High-density lipoproteins (HDL, typically atheroprotective) were significantly increased and very-low- and low-density lipoproteins (typically atherogenic) were significantly decreased in post-pubertal cis-women compared to cis-men. These differences were not observed pre-puberty and were induced appropriately by cross-sex-hormone treatment in transgender individuals, supporting that sex hormones regulate lipid metabolism *in vivo*. Only atheroprotective apolipoprotein (Apo)A1 expressing lipoproteins (HDL) were differentially expressed between all hormonally unique comparisons. Thus, oestradiol drives a typically atheroprotective lipid profile through upregulation of HDL/ApoA1 which could contribute to the sexual dimorphism observed in CVD risk post-puberty. Together, this could inform sex-specific therapeutic strategies for CVD management.

Keywords: Sex differences, Metabolomics, Lipids, Lipoproteins, Hormones, Cardiovascular, Transgender, Cross-sex-hormone therapy, HDL, Apolipoprotein-A1

Introduction

Prior to menopause, it is known that women have a lower risk of cardiovascular disease (CVD) and coronary heart disease compared to age matched men; it is reported that women have around half the CVD risk and almost a 10-year delay in first myocardial infarction event compared to men (Lloyd, 2011; Roger *et al.*, 2011; Wilmot *et al.*, 2015). Various traditional risk factors have been shown to confer differential CVD susceptibilities for men compared to women (Yusuf, Hawken and Ounpuu, 2004).

Atherosclerosis, a chronic inflammation of the medium-sized to large arteries, secondary to lipid deposition within the sub-endothelial intimal layer by oxidised apolipoprotein (Apo)B expressing lipoproteins, is a major cause of CVD and mortality. Sex differences in CVD risk could be due to reduced low and very low-density lipoproteins (LDL and VLDL, typically atherogenic) and increased high density lipoproteins (HDL, typically atheroprotective) in women, as shown in the Framingham Offspring Study (Freedman *et al.*, 2004a). Sex hormones have been proposed to drive these differences mechanistically (Arnold *et al.*, 2017) and there is evidence that early versus late menarche may result in differential long term cardiovascular traits, including altered lipoprotein levels, in women (Bell *et al.*, 2018). In support of this observation, following menopause a reduction in circulating oestrogen levels increases susceptibility to developing metabolic diseases including metabolic syndrome, non-alcoholic fatty liver disease, and diabetes in women (Della Torre *et al.*, 2014). In addition, LDL is increased post-menopause in women to the same levels as observed in age-matched men, thus reducing CVD protection; although HDL remains higher in women compared to men at all ages (Matthews *et al.*, 1989; Abbey *et al.*, 1999; Kannel, 1983).

Despite previous studies reporting differences in lipoproteins between men and women, less is known about the sex-biased cardiovascular risk in younger individuals and the specific role of sex hormones in driving these differences post-puberty. It is of particular importance to investigate changes in lipoproteins and CVD risk in individuals with gender dysphoria undergoing cross-sex-

hormone therapy. In this case, young individuals are issued with gonadotrophin-releasing hormone agonists (“puberty blockers”), followed by testosterone (in those born phenotypically female, trans-men), or oestradiol (in those born phenotypically male, trans-women) (Butler *et al.*, 2018). Another group of particular interest are individuals with autoimmune diseases characterised by a significant sex bias such as juvenile systemic lupus erythematosus (JSLE) (with a common onset around puberty and significant female bias), juvenile idiopathic arthritis (JIA) overall (with onset before 16 years of age, with a slight female bias overall, but with clear sex differences in incidence based on the disease phenotype), systemic sclerosis, autoimmune thyroid disease, multiple sclerosis and rheumatoid arthritis (with strong female predominance and onset in adulthood), suggesting a sex hormone influence in disease etiopathogenesis (Watson *et al.*, 2012; Cattalini *et al.*, 2017; Chiaroni-Clarke, Munro and Ellis, 2016), as well as dyslipidaemia and increased CVD risk through accelerated atherosclerosis (Barsalou, Bradley and Silverman, 2013; Schanberg *et al.*, 2009; Coulson *et al.*, 2013). Therefore, both young individuals with gender dysphoria undergoing sex hormone replacement therapy and patients with autoimmunity could be significantly affected by changes in lipoprotein metabolism mediated by sex hormones which could influence long-term cardiovascular health outcomes; this represents an unmet research need.

Investigating the relationship between sex hormones and lipid metabolism is therefore important for understanding the aetiology of atherosclerosis in different physiological and disease settings. Using an unbiased analysis of lipid metabolomics in a cohort of young post-pubertal cis-gendered-men and women, as well as age matched transgender individuals on cross-sex-hormone treatment, we found a direct association between oestradiol (in young cis-woman and trans-women) and increased typically atheroprotective HDL subsets and ApoA1, the dominant apolipoprotein associated with HDL. Conversely, testosterone (in young cis-men and trans-men) was associated with an increase in typically atherogenic VLDL subsets and ApoB:ApoA1 ratio. Strikingly, these sex differences in lipids were lost or altered between young post-pubertal men

and women with JSLE or JIA, respectively, and were not observed in healthy pre-pubertal children. Together, this indicates that sex hormones are likely to play a crucial role in lipoprotein metabolism, contributing to the sexual dimorphism observed in atherosclerotic and CVD risk post-puberty. This detailed knowledge of sex differences in lipoprotein taxonomy post-puberty could help inform sex-tailored strategies for cardiovascular risk management from a younger age, potentially leading to a decrease in CVD related morbidity and mortality overall.

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Results:**Young post-pubertal men and women have unique serum lipid profiles associated with atherosclerotic risk that are partially reversed by cross-sex-hormone treatment**

The onset of puberty is known to alter lipid profiles (Freedman *et al.*, 2004b; Freedman *et al.*, 2001; Fonseca *et al.*, 2019; Mascarenhas *et al.*, 2015; Eissa *et al.*, 2016). In-depth nuclear magnetic resonance (NMR) spectroscopy analysis of 149 serum lipid metabolites was performed on pre-pubertal children and young post-pubertal cis-men and cis-women (Figure 1 for study plan and detailed explanation of study groups, Table S1 for list of metabolites). This analysis accounts for >20 times the number of lipid measures included in a routine serum lipid profile panel. No sex-associated differences were observed in lipid metabolites from age matched pre-pubertal children (Figure 2A, Table S2 for demographics). By contrast, following false discovery rate (FDR) correction for multiple t-tests, 31 lipid metabolites were significantly increased in young post-pubertal cis-men compared with 22 lipid metabolites increased in age matched post-pubertal cis-women (Figure 2B, Table 1 for demographics, Table SD1 for full metabolite analysis), strongly supporting a role for sex hormones in altering lipid metabolism. Notably, a significant increase in VLDL particle subsets (VLDL particle concentration and size and VLDL lipid composition including cholesterol, cholesterol esters, free cholesterol, phospholipids and triglycerides, TG) and serum TGs was identified in young post-pubertal cis-men. Conversely, in young post-pubertal cis-women a significant increase in HDL particle subsets (HDL particle concentration and size and HDL lipid composition including cholesterol, cholesterol esters and free cholesterol), HDL-associated ApoA1, polyunsaturated fatty acid (PUFA) and docosahexaenoic acid (DHA) was seen post-puberty.

To investigate the extent to which sex hormones can influence lipid metabolism, we explored the serum lipid profile of young transgender individuals on puberty blockers and early cross-sex-hormone treatment: trans-men (on puberty blockers and exogenous testosterone) and trans-

women (on puberty blockers and exogenous oestradiol) (Table 1 for demographics and clinical data). Strikingly, young trans-women had an increase in HDL subsets, ApoA1 and DHA compared to age matched cis-men (Figure 2C, Table SD2 for full metabolite analysis), resembling the profile of young post-pubertal cis-women. In contrast, young trans-men had decreased HDL subsets, ApoA1 and DHA, an increased ApoB:ApoA1 ratio and an up to 4-fold significant increase in VLDL subset expression compared to age matched cis-women (Figure 2D, Table SD3 for full metabolite analysis), resembling the profile of young post-pubertal cis-men. These statistically significant differences were validated by logistic regression analysis (Figure S1). No significant differences in lipid measures were observed between young cis-men and trans-men or between young cis-women and trans-women following FDR correction (Table SD4 for full metabolite analysis). There was no significant difference in BMI between each gender group, however ethnicity did vary between cis- and trans-gender populations – with trans-men and trans-women being predominately white (Table 1 for demographics and clinical data).

HDL-associated metabolites were significantly influenced by all sex hormone changes

A total of 18 metabolites were significantly altered in all comparisons between hormonally unique adolescent groups using FDR corrected multiple t tests (Figure 3A) and validation by logistic regression analysis (Figure S1). Interestingly, these metabolites were all HDL subsets (total, medium, large and extra-large particles) and ApoA1 (Figure 3A-B, Table S3 for full metabolite overlap list, Figure S2 for raw data plots of 18 overlapping metabolites). Furthermore, HDL subsets and ApoA1, but not VLDL subsets, were significantly altered between young trans-women and trans-men (Figure 3C). ApoA1 remained statistically significant in all comparisons when adjusted for ethnicity by logistic regression, despite ethnic differences observed between cohorts (Table 1 for demographics, Table S4 for adjusted logistic regression data). In addition, circulating ApoA1 levels correlated positively with the duration of cross-sex-hormone (oestradiol) treatment and with serum oestradiol concentration in young trans-women (Figure 3D-E).

However, ApoA1 did not correlate with testosterone levels in young trans-men (Figure 3D-E), suggesting that only oestradiol may drive changes in HDL expression irrespective of the background sex chromosomes. In support, ApoA1 was not significantly altered in young post-pubertal individuals with Turner syndrome (females with only one functional X sex chromosome, rather than the usual two) compared to age, ethnicity and puberty matched young healthy controls (Figure 3F, Table S5 for clinical information).

Lipoprotein interaction networks are altered under different *in vivo* hormone conditions

Lipoprotein metabolism is a tightly regulated network, largely co-ordinated by the liver, to control the concentration of circulating lipids and subsequent peripheral tissue lipid uptake and efflux (Zhang, Temel and Martel, 2014). Therefore, we next investigated associative networks between both lipoprotein subsets and other lipid metabolites to explore possible mechanistic metabolite interactions across the hormonally unique groups (Figure 4, Table S6 for key with node numbers matched to metabolites). This could help to explain how sex hormones systemically alter the lipoprotein metabolic network and change metabolites serum concentrations as identified previously. As expected, typically atheroprotective HDL subsets (including the 18 identified as overlapping in Figure 3A, represented as enlarged nodes in Figure 4) and ApoA1, were tightly and separately clustered away from typically atherogenic lipoproteins (LDL, VLDL and intermediate density lipoprotein, IDL) and ApoB in all of the cohort networks (Figure 4A-D). The typically atherogenic metabolite group appeared more tightly clustered and incorporated more lipoprotein subsets and fatty acids in young post-pubertal cis-men compared to cis-women (Figure 4A-B). In fact, the network for cis-women excluded multiple small-HDL subsets, extra-large-VLDL subsets and the majority of high TG-containing lipoproteins. In the young transgender groups on cross-sex-hormone treatment (Figure 4C-D), the typically atherogenic and atheroprotective metabolite clusters were cross-linked together through correlative relationships. This was more

evident in the young trans-women group where many more connections were observed, suggesting that exogenous oestradiol may induce more tightly regulated lipoprotein networks. Saturated and monounsaturated fatty acids (SFA and MUFA) were incorporated into the typically atherogenic lipoprotein cluster, whilst total unsaturated fatty acids, Omega-3 and DHA were excluded from the network entirely in young cis- and trans-men (Figure 4A and C). In contrast, all fatty acids were excluded from the network in young cis-women and only total unsaturated fatty acids were excluded from the network in young trans-women, where other fatty acid subtypes bridged connections between the clusters (Figure 4B and D). Together, this data supports that sex hormones may play a pivotal but mechanistically complex role in modifying serum lipid metabolism in post-pubertal young individuals which may contribute to the sex-biased atherosclerotic risk.

Finally, lipoproteins were assessed in post-pubertal adolescents with JSLE and JIA, autoimmune diseases characterised by sex bias and onset at young age, as well as dyslipidaemia and increased CVD risk through accelerated atherosclerosis linked to chronic inflammation/medication. This makes them of particular interest for exploring the role of sex hormones and lipoproteins on CVD risk in younger populations with autoimmune conditions. Strikingly, all previously identified post-pubertal sex differences in lipids (Figure 2B) were lost between young age matched men and women with JSLE (Figure S3A, Table S7 for demographics and clinical data). In JIA, young women maintained the more typically atheroprotective HDL/ApoA1 profile compared to men, whilst men lost the VLDL profile observed in healthy individuals compared to women (Figure S3B, Table S8 for demographics and clinical data). Despite no statistical significance, HDL subsets were reduced whilst VLDL subsets and TGs were increased in young women with JSLE compared to age matched healthy women (Figure S3C); interestingly, the opposite was observed for young men with JSLE compared to age matched healthy men, suggesting possible differential metabolic mechanisms by pathology and

cardiovascular implications for JSLE patients depending on their sex. Similar directional patterns were observed by sex in JIA for lipoproteins compared to age matched HCs (Figure S3D).

These results suggest that sex hormone driven changes in lipid metabolism as well as impact of low-grade chronic inflammation/stable medication associated with disease could influence the atherosclerotic risk associated with sex-biased chronic autoimmune inflammatory conditions such as JSLE and JIA, even when the autoimmune disease is well controlled (e.g. the majority of patients with low disease activity and none of the JIA patients were treated with steroids or biologics, while JSLE patients have been treated only with the equivalent of less than 10 mg Prednisolone daily).

Discussion:

In this study we identified for the first time that cross-sex-hormone treatment induced early sex-specific lipoprotein and apolipoprotein changes in a dose dependent manner, supporting a role for sex hormones in lipid metabolism; our data suggests that this may not be significantly affected by sex chromosome dosage and could explain the observation that, following puberty, young men develop a more typically atherogenic lipoprotein profile compared to women, who develop a more typically atheroprotective profile. We also identified that lipoprotein metabolic interaction networks may differ depending on the circulating sex hormone environment and that sex differences in lipid profiles were lost or altered in patients with JSLE or JIA, respectively, suggesting that sex hormone-induced lipoprotein metabolism may be disrupted in autoimmunity. The information reported here may help us to understand why men and women are differentially susceptible to CVD from a young age (Lloyd, 2011; Roger *et al.*, 2011; Wilmot *et al.*, 2015).

It is established that clinical measures of lipoproteins differ between healthy men and pre-menopausal women, where men are at an increased risk of developing CVD (Matthews *et al.*, 1989; Abbey *et al.*, 1999; Kannel, 1983). Despite this, the role of sex hormones in driving these differences in humans has not been explored using detailed metabolomic analysis. In this study, assessing in depth lipid fraction characterisation, far greater than available in a clinical setting, we found that VLDL subsets and TGs were significantly increased in young post-pubertal cis-men, whilst HDL subsets, ApoA1 and DHA were increased in cis-women; no differences were observed pre-puberty. This provides evidence that, from the onset of puberty, young cis-men and cis-women are already segregated by unique metabolic signatures that could contribute to a differential CVD risk. In support, a large cohort study of cardiometabolic outcomes post-puberty, including lipoprotein measures, showed trends towards increasing CVD traits, including increased VLDL and reduced HDL, in women with delayed menarche (Bell *et al.*, 2018); the study, however, speculated that pre-pubertal BMI and adiposity were significant confounders. Measurement of

adiposity was beyond the scope of this study; however, we found no difference in the mean BMI between the cohorts. The INTERHEART study, a standardised case-control study of acute myocardial infarction in 52 countries, showed that abnormal lipids had the highest population attributable risk to CVD in both men (49.5%) and women (47.1%), highlighting the importance of lipids when studying CVD risk factors (Yusuf, Hawken and Ounpuu, 2004).

Our study suggests that post-pubertal cis-men may need to consider specific dietary interventions from a younger age to counteract their more typically atherogenic lipid profile. A diet intervention trial where healthy individuals followed a diet rich in PUFAs from childhood to adulthood resulted in a decrease in circulating levels of saturated fat and LDL in both men and women, whereas monounsaturated fat levels decreased in men only (Lehtovirta *et al.*, 2018). We identified lower levels of circulating DHA (a PUFA) in young cis- and trans-men compared to cis-women as well as altered sex-specific network interactions between fatty acids and lipoproteins. This supports a sex-specific differential role for dietary PUFAs in normalising serum lipid profile; sex differences in DHA have also been reported in a systematic review of 51 publications, thus supporting these findings (Lohner *et al.*, 2013). The Cardiovascular Risk in Young Finns Study, a prospective study following the dietary intake of children through to adulthood, showed that young women had a more favourable diet than young men in terms of cardiovascular health, while the men diet containing relatively more total, saturated and monounsaturated fat (Mikkila *et al.*, 2004). The study also showed that the children's diets were a significant determinant of their adult diet, even after the age of 21 years, supporting that improved sex-specific dietary public health advice given to young individuals could improve long-term cardiovascular outcomes, particularly for young cis- and trans-men who have more typically atherogenic lipid profiles as shown by our study.

Speculation that sex hormones may drive changes in lipid metabolism and CVD risk is not new. Studies have shown that the combined oral contraceptive pill (COCP) (combination of

oestradiol and progesterone) increased circulating TGs and HDL-cholesterol, whereas the progestin only oral contraception had little effect on the serum lipid profiles (Wang *et al.*, 2016). In the same study, COCP also decreased PUFAs, suggesting a differential role of sex hormones in regulating lipid metabolism. These observations support the association between exogenous oestradiol administration and an atheroprotective lipid serum profile. We found that HDL subsets were increased in young trans-women treated with oestradiol (on puberty blockers) and were lost in young trans-men treated with testosterone (on puberty blockers) where an increase in VLDL and LDL was detected. It is well reported that TG-rich VLDL particles are responsible for residual cardiovascular risk (Duran and Pradhan, 2021). Interestingly, no difference in VLDL or LDL subsets were seen in young trans-women, suggesting HDL levels and cellular lipid efflux to HDL may be more sensitive to oestradiol fluctuations. Negative feedback interactions between HDL and the levels of typically atherogenic lipoproteins may explain why an increase in VLDL was observed following blockade of physiological oestradiol production in young trans-men with a gonadotropin-releasing hormone agonist, as shown by our network analysis. Clinical studies of lipids in large transgender cohorts are rare, however, a few small studies have shown contradictory observations regarding routine serum lipid measurements. A study showed that adolescent and adult trans-men develop increased TGs, total cholesterol and LDL-cholesterol as well as decreased HDL-cholesterol following puberty blockers and cross-sex-hormone treatment with testosterone. Adolescent and adult trans-women in the same study showed decreased total cholesterol and LDL-cholesterol (Fisher, 2016). Another report also identified a decrease in HDL levels in young trans-men but found no difference in the lipid profile of young trans-women compared to matched cis-gendered individuals (Jarín *et al.*, 2017). Discrepancies between studies may be due to differences in circulating exogenous sex hormone concentrations following cross-sex-hormone treatment, length of time on therapy, and conventional and other CVD risk factors, such as BMI, hypertension, smoking and associated medical conditions (e.g. diabetes or chronic inflammatory conditions). Longitudinal studies will help us to identify the long-term cardio-

pathogenic effects of these sex hormone driven lipid changes in individuals with gender dysphoria (Martinez *et al.*, 2020). Together, these observations could explain why cis-women lose their cardio-protective advantage over cis-men following the cessation of ovarian functions post-menopause (Della Torre *et al.*, 2014); in addition, the atheroprotective benefits of oestradiol are not age-dependent, as there is also evidence that oral oestradiol therapy can increase HDL post-menopause (Walsh *et al.*, 1999; Shufelt and Manson, 2021).

While the role of sex hormones in modifying lipid metabolism is more difficult to explore in humans, studies of circulating oestradiol in mice have begun to identify potential mechanisms. Della-Torre *et al.* identified that the reproductive cycle in mice determines the size and efficiency of HDL particles produced by the liver (Della Torre *et al.*). Smaller, more lipid efficient HDL are produced during high oestradiol phases of the menstrual cycle and this results in greater cholesterol efflux from cells. In our study, we observed an increase in medium-to-large-sized HDL subsets in the presence of oestradiol as well as an exclusion of small-HDL subsets and high TG-containing HDL subsets from the lipid network in young women, suggesting complex mechanistic differences between human and mouse lipoprotein metabolism. It has been shown recently that cholesterol in larger vs smaller HDL particles infer a respective higher vs lower CVD protection humans, whilst TGs in all HDL particle sizes infer a greater CVD risk (Holmes *et al.*, 2018); this supports an increased CVD protection in cis- and trans-women with respect to our analysis and lipid networks. This effect on HDL efflux in mice was due to increased DNA binding of oestrogen receptor alpha when oestradiol levels were high, speculated to promote the binding and transcriptional activity of Liver-X-Receptors (LXRs), master regulators of cholesterol metabolism and HDL efflux (Della Torre *et al.*). This suggests a key mechanism of action for oestradiol and HDL metabolism. High plasma levels of oestrogen in murine models have also shown to increase *de novo* lipid clearance of VLDL and LDL, increase synthesis of HDL and alter the expression of lipoprotein modifiers (Villa *et al.*, 2012; Della Torre *et al.*). In contrast a study in macrophages

taken from trans-women undergoing sex hormone therapy showed that total HDL efflux decreased by 10.8% and ATP-binding cassette transporter A1 (ABCA1) mediated HDL efflux by 23.8%; this hormone driven effect may therefore be tissue specific (van Velzen *et al.*, 2021). In addition, a study has reported that male patients with primary and secondary hypogonadism (low testosterone) presented with lower ABCA1/G1, suggesting an association with reduced cholesterol efflux (Adorni *et al.*, 2019). The liver may therefore be a sexually dimorphic organ providing tight control of systemic lipid metabolism. As for mouse models (Della Torre and Maggi, 2017; Della Torre *et al.*, 2018; Palmisano, Zhu and Stafford, 2017), investigating the mechanisms by which sex hormones regulate the hepatic transcriptome and function will be a key focus for research going forward in humans. Oestradiol levels may also contribute to decreased CVD risk through direct effects on non-hepatic cell types including anti-inflammatory, apoptotic and oxidative implications (Nofer, 2012). As supported by our study, the sex bias observed in JSLE and JIA and the increased autoimmune-associated CVD risk of JSLE and JIA patients could therefore be due to a breakdown in oestradiol signalling, influencing both atherogenic lipid metabolism and inflammation (Klein and Flanagan, 2016). With this respect, it has been shown in different species and cell culture systems that low levels of oestradiol promote Th1-type and cell-mediated immunity, whereas high levels of oestradiol promote Th2-type and humoral immune responses, thus adding to the complexity of sex-hormone signalling and inflammation (Straub, 2007). In addition, castration of male vs female mouse models increases vs decreases the respective incidence of autoimmune diseases (Voskuhl, 2011). Whilst chronic inflammation represents a CVD risk factor alone, changes in lipoprotein metabolism in autoimmunity may also be driven by inflammation of the liver, a master regulator of systemic lipid metabolism, where altered liver function is common in SLE (Gibson and Myers, 1975; Matsumoto *et al.*, 2000; Runyon, Labrecque and Anuras, 1980), as well as influenced by medications (such as corticosteroids or other targeted disease modifying therapies). It is also plausible that testosterone drives atherogenic lipoprotein production and/or inhibits HDL production in men; this remains to

be explored mechanistically, although the effects of testosterone replacement therapy on serum lipoproteins has been investigated by several studies with no clear conclusions (Gencer *et al.*, 2021). One study showed that testosterone administration in men increased the activity of hepatic lipase and decreased the levels of HDL and size of LDL (although VLDL was not measured). This could explain the increase in VLDL seen in young trans-men through increased hydrolysis of TGs in chylomicrons to produce VLDL (Herbst *et al.*, 2003). Despite this, treatment with testosterone has also been associated with improved CVD symptoms such as angina and claudication (Gencer *et al.*, 2021). Alternatively, a study has shown that young trans-women on puberty blockers alone (prior to oestradiol treatment) experienced a statistically significant decrease in lean mass over 12 months, whereas no difference was seen for young trans-men on puberty blockers alone (Ghelani *et al.*, 2020). This suggests that testosterone may play a greater role in the glycolytic control of muscle development over lipid metabolism.

Limitations of study:

In our study, we had the unique opportunity to investigate the *in vivo* effects of sex hormones in young donors; this study, however, had some limitations. The cross-sectional design and low sample size may limit the statistical power of some of the metabolite analyses. Thus, it would be important to validate our findings in external cohorts; this will also account for genetic, demographic and lifestyle differences between populations. In our analysis, a statistically significant difference in ethnicity were identified between cohorts. However, we observed a sustained statistically significant difference in ApoA1 concentration, the dominant apolipoprotein associated with HDL, when ethnicity was adjusted for in the logistic regression analysis. Furthermore, longitudinal data was not available, and analysis was performed at one timepoint per individual. This was specifically an early timepoint following initiation of cross-sex-hormone treatment to investigate the initial influences of sex hormones on lipids; this meant that hormone levels rarely reached physiological levels, however, this is common in young individuals where

low doses are administered initially and tapered gradually to avoid adverse effects (Meyer, Boczek and Bojunga, 2020). In addition, stage of menstrual cycle was not recorded for young cis-women and it was beyond the scope of the study to quantify hormone levels in the cis-gendered cohorts. Based on our observations in transgender individuals, differing circulating hormone concentrations per individual may have influenced the lipid levels to some extent, something that is difficult to control in human studies. Heterogeneity in cross-hormone treatments was also difficult to control for as transgender individuals were treated differently based on individual factors, such as tolerability and absorption efficiency of different sex hormone formulations and route of administration, BMI and rate of development of secondary sex characteristics upon initiation of cross-sex hormone therapy. Here we opted to perform metabolite analysis compared to circulating levels of hormones to account for differences in dose and absorption (and therefore bioavailability). It was beyond the scope of this study to karyotype the cis/transgender individuals; however, both of these cohorts had no clinical features suggestive of genetic disorders. In support, a recent study evaluating the same transgender cohort, reported that all 44 transgender individuals evaluated had 'normal' karyotypes for their birth genders (Carmichael *et al.*, 2021). It is typical that individuals with Turner's syndrome are treated with hormone therapy due to the reduced/dysfunctional X chromosome; this confounder was therefore difficult to control for in our analysis. Prospective studies for the quantification of the risks and benefits of hormonal treatment are of high demand, particularly regarding the stability of metabolic changes and cardiovascular outcomes. Finally, the data reported here are mostly associative; further functional studies are required to investigate the mechanism of sex hormone regulation of lipid metabolism post-puberty.

In conclusion, we report unique changes in lipoprotein metabolism induced by sex hormones following puberty in young healthy donors and in an age matched cohort of young transgender individuals in the initial stages of cross-sex-hormone treatment. The presence of oestradiol (and

puberty blockers) was associated with a typically atheroprotective lipoprotein profile, whereas the presence of testosterone (and puberty blockers) was associated with a typically atherogenic lipoprotein profile. Together, this highlights the importance of considering sex determinants in all biological research studies and interventional clinical trials and the results reported here will help to inform future sex-specific therapeutic considerations for CVD.

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Figure 1: Study design and analysis flow diagram

Terminology: **Cis-woman**: a person who identifies as female and was also assigned female at birth. This may have been based on genitals and/or having two X chromosomes (XX); **Cis-man**: a person who identifies as male and was also assigned male at birth. This may have been based on genitals and/or having one X and one Y chromosome (XY); **Trans-woman**: a person who identifies as female but was assigned male at birth, on the basis of their genitals and/or having one X and one Y chromosome (XY). Sometimes known as MTF; Male-to-female; **Trans-man**: a person who identifies as male but was assigned female at birth, on the basis of their genitals and/or having two X chromosomes (XX). Sometimes known as FTM; Female-to-male; **Puberty blocker**: medication that blocks the production of the hormones oestrogen and testosterone, which cause female or male puberty, respectively. These are given to young people before they begin taking cross-sex hormones to begin their transition. In this case, GnRHa (gonadotrophin-releasing hormone agonists).

Figure 2: Young post-pubertal men and women have an altered serum lipid profile driven by hormones

Metabolites were measured in serum by Nightingale Metabolomics (Table S1). **(A-D)** Volcano plots displaying fold change of all metabolites and Log₁₀ p values from multiple unpaired t-tests comparing **(A)** pre-pubertal boys and girls (n=10 and 10), **(B)** young post-pubertal cis-men and cis-women (n=15 and 17) (Table SD1), **(C)** young transgender individuals undergoing cross-sex-hormone treatment with oestradiol, trans-woman (n=25) and young post-pubertal cis-men (assigned male at birth, n=15) (Table SD2) **(D)** young transgender individuals undergoing cross-sex-hormone treatment with testosterone, trans-men (n=26) (Table SD3), and post-pubertal cis-women (assigned female at birth, n=17). Bottom y-axis line, p=0.05; top y-axis line, adjusted p value threshold following 6% false discovery rate (FDR) adjustment for multiple comparisons

(Benjamini, Krieger and Yekutieli approach). Coloured dots represent different metabolite groups.

Abbreviations: VL/I/L/HDL (very low/intermediate/low/high density lipoproteins), Apo (apolipoprotein).

Figure 3: HDL metabolites are associated with the presence of circulating oestradiol

(A) Venn diagram (<http://www.biovenn.nl/>) displaying the proportional overlap of statistically significantly altered metabolites (Table S3) that overlap between different gender group comparisons from Figure 2. Abbreviations: M (men), W (women), HDL (high density lipoproteins), Apo (apolipoprotein), C (cholesterol), CE (cholesterol ester), FC (free cholesterol), L (total lipids), P (particles), PL (phospholipids). **(B)** Violin plot comparing levels of ApoA1 in young post-pubertal cis-men and cis-women (n=15 and 17) and in young transgender individuals undergoing cross-sex-hormone treatment with oestradiol, trans-women (n=25), or testosterone, trans-men (n=26). One-way ANOVA, *= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$, ****= $P < 0.0001$. **(C)** Volcano plot displaying fold change of all lipid metabolites (Table S1) and Log₁₀ p values from multiple unpaired t-tests comparing young trans-women (n=25) with age matched trans-men (n=26). Bottom y-axis line, $p = 0.05$; top y-axis line, adjusted p value threshold following 6% false discovery rate adjustment for multiple comparisons (Benjamini, Krieger and Yekutieli approach). Coloured dots represent different metabolite groups. **(D-E)** Pearson's correlation between serum ApoA1 levels and **(D)** number of months on cross-sex-hormone treatment or **(E)** circulating serum levels of hormones following cross-sex-hormone treatment matched to the time of metabolomic analysis in either young trans-women (n=25, oestradiol treatment) or trans-men (n=26, testosterone treatment). **(F)** Violin plot comparing the level of ApoA1 between young post-pubertal individuals with Turner syndrome (females with only one functional X sex chromosome, rather than the usual two, n=8) compared to age, ethnicity and puberty matched young healthy controls (n=8). One-way ANOVA. Abbreviations: VL/I/L/HDL (very low/intermediate/low/high density lipoproteins), Apo

(apolipoprotein), C-M (cis-men), T-M (trans-men), C-W (cis-woman), T-W (trans-women), HC (healthy control), TS (Turner syndrome).

Figure 4: Lipoproteins have altered interaction networks depending on hormone presence

Network analysis displaying correlative relationships between lipid metabolites in **(A)** young post-pubertal cis-men (n=15), **(B)** young post-pubertal cis-women (n=17), **(C)** young trans-men (n=26) undergoing cross-sex-hormone treatment with testosterone, or **(D)** young trans-women (n=25) undergoing cross-sex-hormone treatment with oestradiol. The high-dimensional undirected graphs were produced using the R package 'high dimensional undirected graph estimation' (HUGE). Each of the coloured nodes represents a single lipid metabolite variable: a key showing metabolite names corresponding to numbers on the nodes is in Table S6. Edges between nodes show likely correlations between two lipid metabolites, where a dense cluster of nodes implies a stronger relationship between the metabolites than the sparser clustered nodes. Nodes (or small groups of nodes) with no linking edge implies that they are conditionally independent compared to the other metabolites. Enlarged nodes represent lipid metabolites that overlap between all hormone comparisons identified from Figure 3A. Relevant fatty acid metabolites and ApoA1 are labelled with arrows. Abbreviations: VL/I/L/HDL (very low/intermediate/low/high density lipoproteins), Unsat (unsaturated), SFA (saturated fatty acids), MUFA (monounsaturated fatty acids), ApoA1 (Apolipoprotein-A1).

Table 1: Demographic and clinical comparisons between young post-pubertal cis-men and cis-women and transgender cohorts

Demographic:	Cis-Men	Cis-Women	Trans-Men	Trans-Woman	P value*
Number	15	17	26	25	-
Age, mean (SD)	19 (2.73)	18.94 (3.07)	18.38 (0.57)	18.44 (0.87)	0.6316 ⁺
BMI, mean (SD)	23.99 (2.18)	22.28 (3.59)	24.75 (4.38)	24.03 (4.92)	0.4757 ⁺
Ethnicity, number (%):					
White	7 (47)	8 (47)	23 (88)	22 (88)	0.0008*
Asian	5 (33)	3 (18)	0 (0)	1 (4)	0.0046*
Black	1 (7)	1 (6)	1 (4)	0 (0)	0.6608*
Other	2 (13)	5 (29)	2 (8)	2 (8)	0.1604*
Tanner Stage at time of sample, n (%):					
Tanner Stage 2-3	0 (0)	0 (0)	-	-	>0.9999*
Tanner Stage 4-5	15 (100)	17 (100)	-	-	>0.9999*
Tanner Stage at time of puberty block, n (%):					
Tanner Stage 2-3	-	-	1 (4)	11 (44)	0.0008*
Tanner Stage 4-5	-	-	25 (96)	14 (56)	0.0008*
Cross-sex-hormone treatment:					
Time on treatment (months), mean (SD)	-	-	11.83 (7.40)	12.04 (8.34)	0.9234 ⁺
Estradiol valerate (2-6mg oral/day), n (%)	-	-	-	19 (76)	-
Estradiol/Evorel patch (1.6-6.4 mg/patch), n(%)	-	-	-	6 (24)	-
Oestrogen serum level (pmol/L), median (IQR)	-	-	75.50 (45.5-111.5)	126.0 (44.0-256.0)	0.0470 ⁺
Sustanon (100-250mg IM/4 weeks), n (%)	-	-	23 (88)	-	-
Nebido (1000mg IM/12 weeks), n (%)	-	-	2 (8)	-	-
Testogel (25mg/day), n (%)	-	-	1 (4)	-	-
Testosterone serum level (nmol/L), median (IQR)	-	-	15.5 (6.1-25.8)	0.4 (0.4-1.1)	<0.0001 ⁺
Other medication, number (%):					
Contraception (combined pill)	-	2 (12)	-	-	-
Anti-depressants	0 (0)	0 (0)	4 (15)	1 (4)	0.0862*

Table 1: Demographic and clinical comparisons between young post-pubertal cis-men and cis-women and transgender cohorts. *Fisher's exact (2 groups) or chi-square (4 groups) test, ⁺one-way ANOVA, or ⁺unpaired t-test was used. For transgender individuals, the Tanner stage was their most recent Tanner stage prior to puberty blocking therapy. IM: Intramuscular.

STAR★METHODS**Resource availability****Key resources table**

REAGENTS or RESOURCE	SOURCE	IDENTIFIER
Peripheral blood (serum) from healthy individuals	University College London Hospital and University College London	N/A
Peripheral blood (serum) from trans-gender individuals with gender dysphoria	University College London Hospital	N/A
Peripheral blood (serum) from patients with Turner syndrome	University College London Hospital	N/A
Peripheral blood (serum) from patients with JSLE	University College London Hospital or Great Ormond Street Hospital (GOSH)	N/A
Peripheral blood (serum) from patients with JIA	University College London Hospital or Great Ormond Street Hospital (GOSH)	N/A
Data		
Metabolomics data from healthy post-pubertal cis-men/women and individuals with gender dysphoria (trans-men/women).	Nightingale Health	Robinson, George; Pineda-Torra, Ines; Jury, Elizabeth; Ciurtin, Coziana (2021), "Metabolomics data on serum from cis-gender and trans-gender individuals", Mendeley Data, doi: 10.17632/3nxny453ch.1
Software and code		
Prism v9	GraphPad	https://www.graphpad.com/
RStudio	R	https://www.R-project.org/
Venn analysis	BioVenn	https://www.biovenn.nl/
High dimensional undirected graph estimation' (HUGE)	R	https://rdrr.io/cran/huge/
Graphical lasso (glasso) with the stability approach to regularization selection (StARs)	R	(Liu, Roeder and Wasserman, 2010)

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr Coziana Ciurtin (c.ciurtin@ucl.ac.uk).

Materials availability

This study did not generate unique reagents.

Data and code availability

- Metabolomic data can be found at Mendeley Data (<https://data.mendeley.com/>), with source and identifier detailed in the 'Key resources table'.
- This paper does not report original code. The source and identifier of analysis code used in the paper can be found in the 'Key resources table' and/or 'Quantification and statistical analysis' section.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

Experimental model and subject details

Terminology describing human cohorts included in the study:

Cis-woman: a person who identifies as female and was **also** assigned female at birth. This may have been based on genitals and/or having two X chromosomes (XX).

Cis-man: a person who identifies as male and was **also** assigned male at birth. This may have been based on genitals and/or having one X and one Y chromosome (XY).

Trans-woman: a person who identifies as female but was assigned male at birth, on the basis of their genitals and/or having one X and one Y chromosome (XY). Sometimes known as *MTF*; *Male-to-female*.

Trans-man: a person who identifies as male but was assigned female at birth, on the basis of their genitals and/or having two X chromosomes (XX). Sometimes known as *FTM*; *Female-to-male*.

Peripheral blood was collected from young healthy volunteers either children pre-puberty, recruited if blood was being taken for an unrelated clinical purpose (e.g. surgery for routine, non-inflammatory procedures, Table S2 for demographics and clinical information), or young post-puberty (Tanner stage 4-5), recruited from the local community at science outreach events (Table 1 for demographics and clinical information). Blood was also collected from age matched young transgender individuals undergoing cross-sex-hormone treatment (testosterone in those born phenotypically female; trans-men, or oestradiol in those born phenotypically male; trans-women), recruited from UCLH gender dysphoria endocrine clinics (Table 1 for demographics, clinical and treatment information, Figure 1 for study summary). Under current guidelines for NHS treatment of under 18s with gender incongruence, transgender individuals were issued with gonadotrophin-releasing hormone agonists (GnRHa, "puberty blockers", either Gonapeptyl or Decapeptyl) for a minimum of 12 months prior to commencement of cross sex hormone treatment. These medications block the production of hormones oestrogen and testosterone, which induce female or male puberty, respectively. These are given to young people *before* they begin taking cross-sex hormones to begin their transition. All transgender individuals had completed or were nearing completion of physiological puberty of their birth assigned sex prior to starting blocker treatment. All individuals in receipt of a puberty blocker and gender-affirming hormones are encouraged to take calcium and vitamin D supplementation. From the same endocrine clinics, 8 age-matched young post-pubertal women with Turner syndrome (only one functional X sex chromosome, rather than the usual two; all displayed the characteristic phenotype associated with a single X chromosome, although they had varying genotypes) were also recruited for blood collection (Table S5 for treatment and genotype information). Finally, peripheral blood was collected from 35 age matched young juvenile systemic lupus erythematosus (JSLE) patients (fulfilling The American College of Rheumatology (ACR) classification criteria for lupus (1997) (Hochberg, 1997) or the Systemic Lupus International Collaborating Clinics (SLICC) criteria (2012) (Petri *et al.*, 2012) and 121 young juvenile idiopathic arthritis (JIA) patients (fulfilling the

International League of Associations for Rheumatology criteria (Petty *et al.*, 2004)) (puberty Tanner stage 4-5) attending a young adult or adolescent rheumatology clinic at University College London Hospital (UCLH) or Great Ormond Street Hospital (GOSH) respectively (Table S7 and 8 for demographics and clinical data). Patients on biologic therapy were not included in the cohorts due to their known effects on lipids (Hoffman *et al.*, 2018; Daien *et al.*, 2012; Fernandez-Nebro *et al.*, 2014). Informed written consent was acquired from all donors under the ethical approval reference: REC11/LO/0330. Questionnaires provided the Tanner puberty stage of all donors as well as their current use of contraception and any other relevant medication. All information was stored as anonymised data. Detailed demographic and any clinical characteristics, including hormone levels and treatment details, were recorded from NHS databases and questionnaires.

Method details

Metabolomics

Measures of over 140 serum lipid biomarkers were acquired with an established NMR-spectroscopy platform (Nightingale Health) (Robinson *et al.*, 2019) in the serum of study participants. These included both absolute concentrations (mmol/L) and ratios of lipids and apolipoproteins (g/L). Serum lipids measured included fatty acids and very low, low, intermediate density lipoprotein (VLDL, LDL, IDL), and high density lipoprotein (HDL) particles of different sizes ranging from extremely large (XXL), very large, large (L), medium (M), small (S), and very small (XS). Lipids within each lipoprotein subclass included total lipid (L), phospholipids (PL), total cholesterol (C), cholesterol esters (CE), free cholesterol (FC), and triglycerides (TGs) (Table S1 for list of biomarkers). BioVenn (<http://www.biovenn.nl/>) was used to produce proportional Venn diagrams of overlapping metabolites between comparisons.

Quantification and statistical analysis

Statistical analysis was performed using GraphPad Prism-9. Data was tested for normal distribution using Kolmogorov-Smirnov test and parametric/non-parametric tests were used accordingly. Unpaired t-tests and One-way ANOVA (Turkey's post-hoc test) were used as appropriate. In some figures, P values are represented by *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001. Multiple testing was accounted for using a false discovery rate (FDR) approach (Benjamini, Krieger and Yekutieli) to p-values and volcano plots were plotted accordingly. Linear regression was performed using a 95% confidence interval to calculate significance (Pearson correlation). Logistic regression was performed in RStudio (R Core Team. R: A language and environment for statistical computing. Available online at <https://www.R-project.org/>, 2018) on individual metabolomic biomarkers. For metabolomic data networks, high-dimensional undirected graphs were produced using the R package 'high dimensional undirected graph estimation' (HUGE) (Jiang H, 2019). Data pre-processing, neighbourhood screening, graph estimation, and model selection techniques were implanted in the 'huge' package pipeline. In the graph estimation stage, graphical lasso (glasso) was used to estimate the sparse inverse covariance matrix, with the stability approach to regularization selection (StARs) (Liu, Roeder and Wasserman, 2010).

Additional resources

N/A

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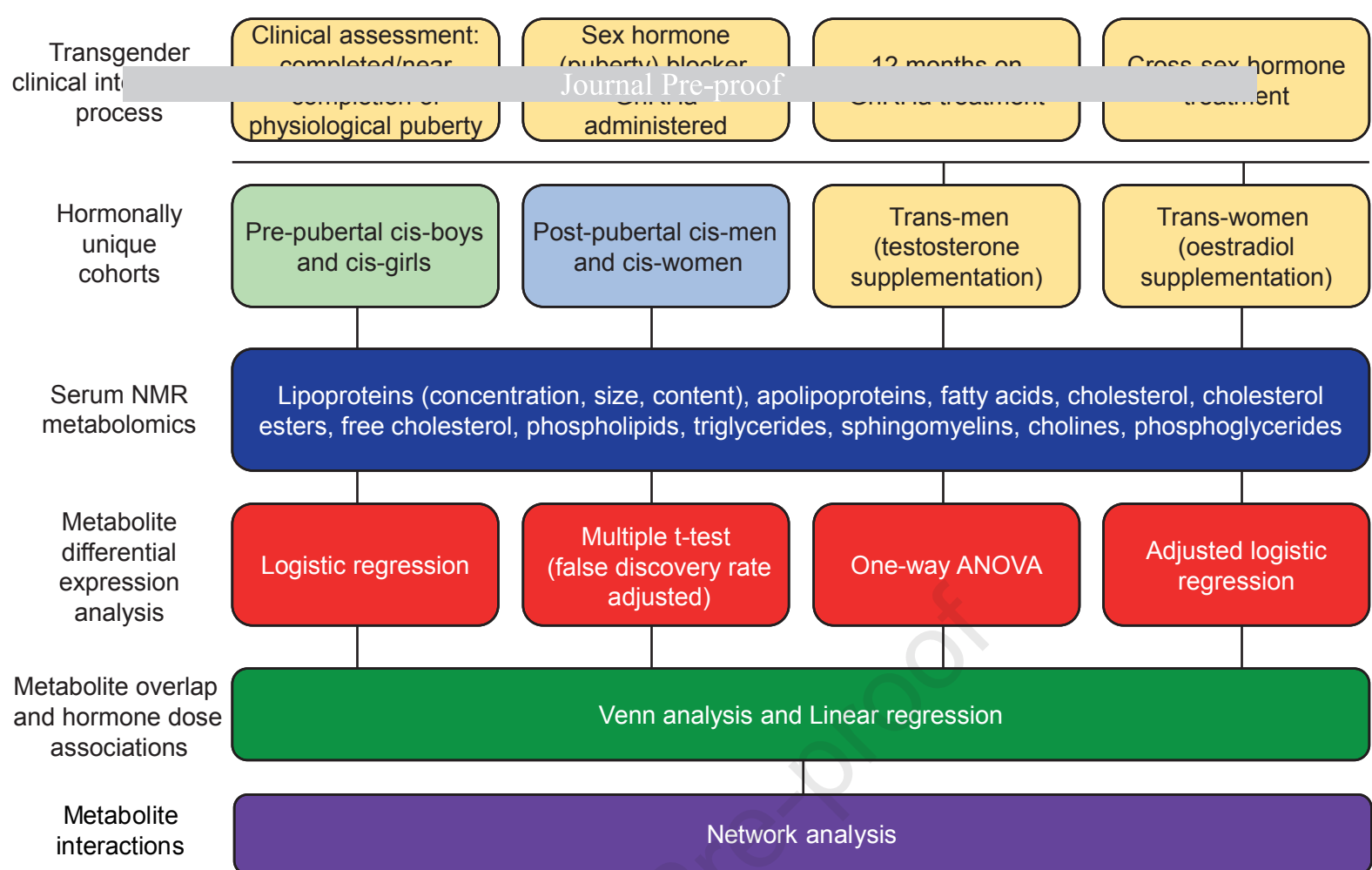
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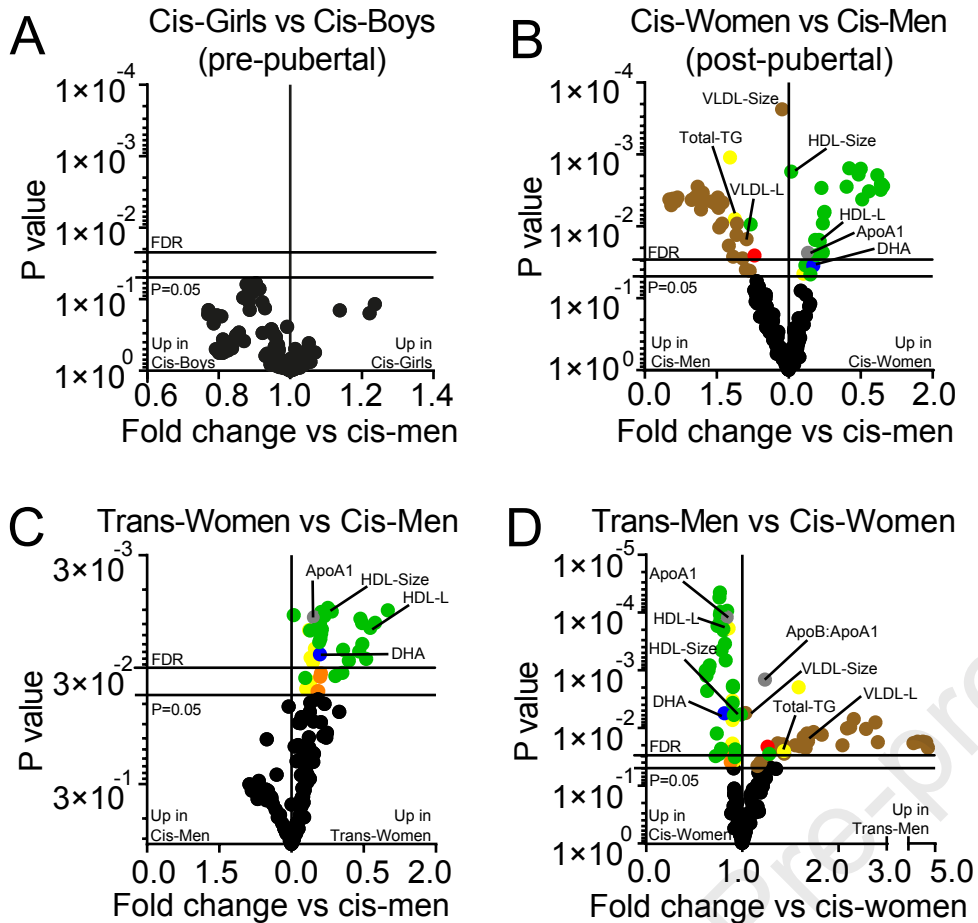
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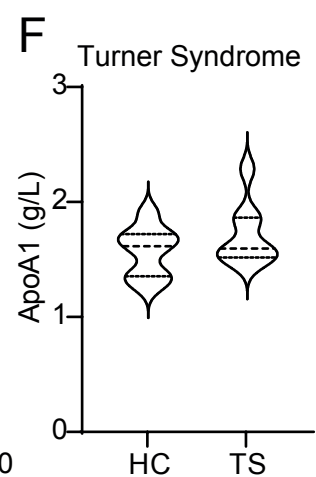
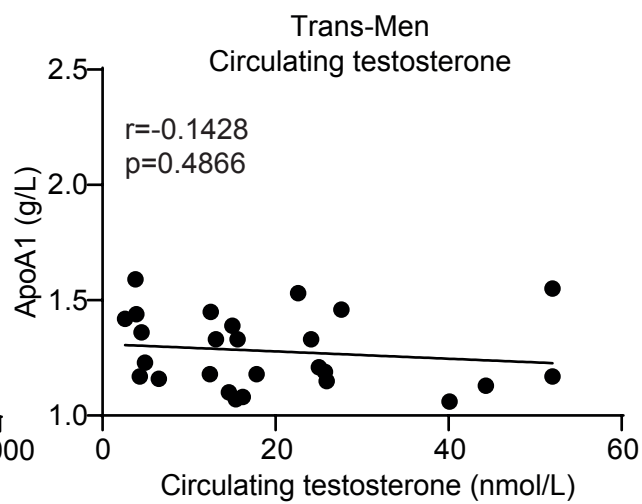
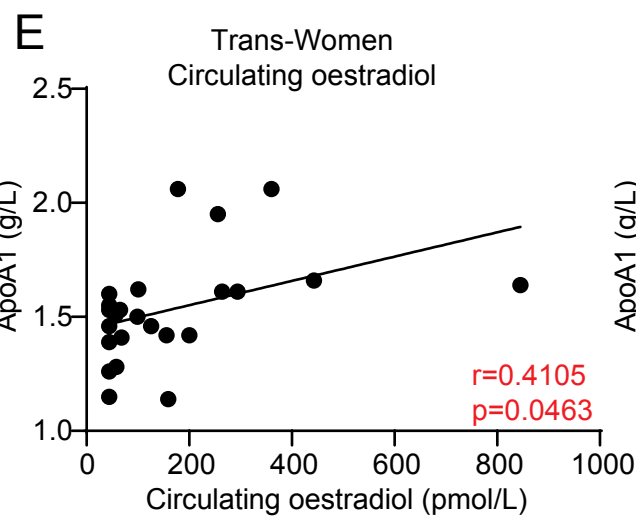
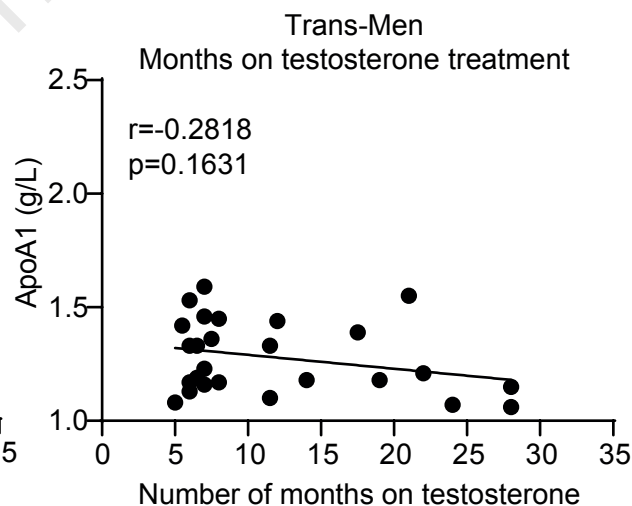
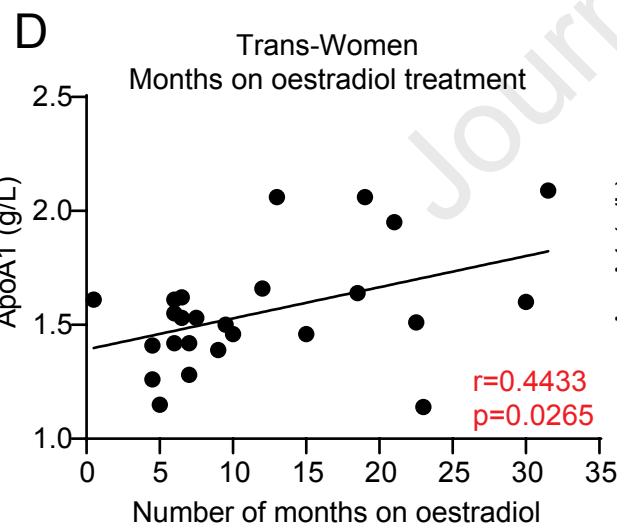
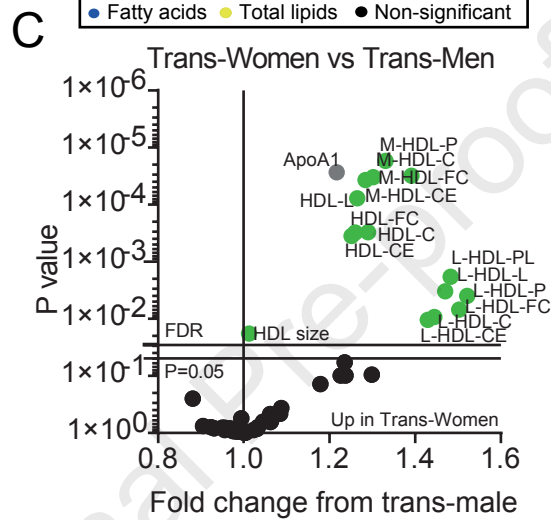
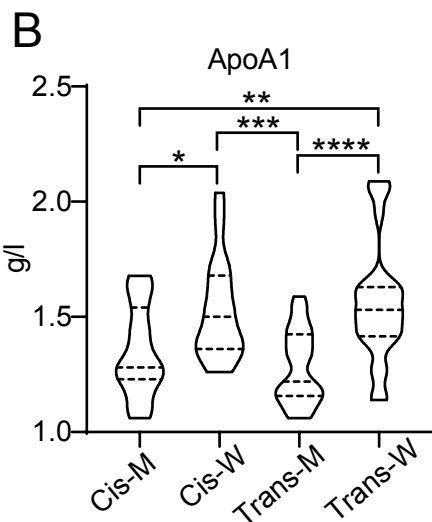
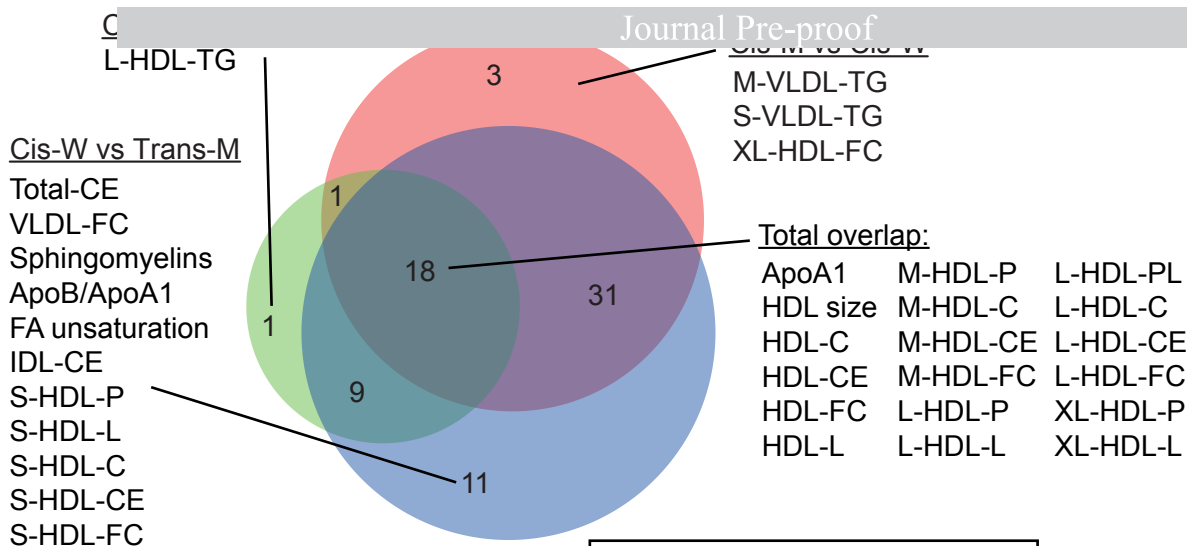
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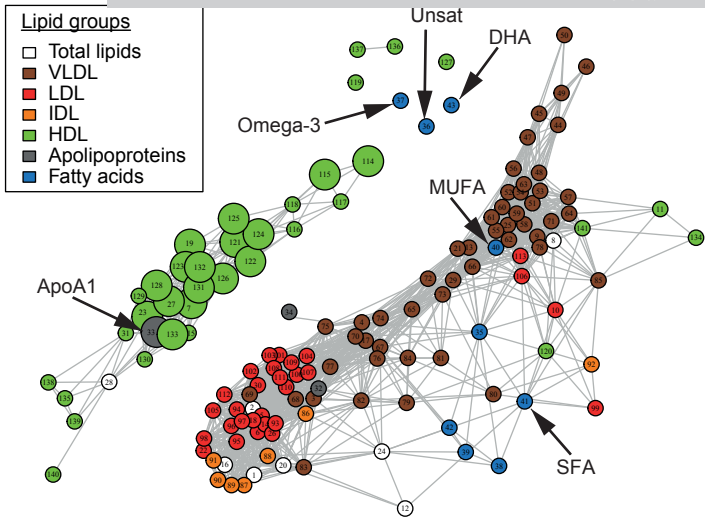
A

Overlap of significant metabolites



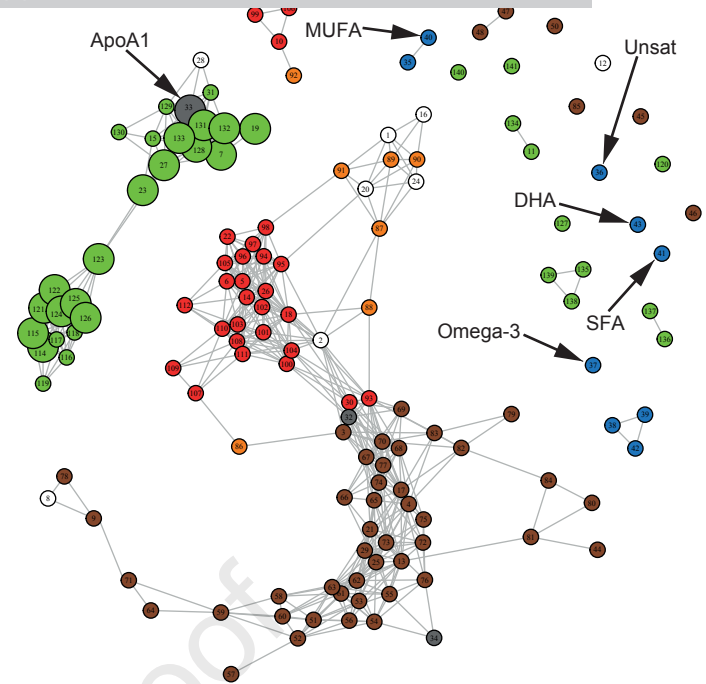
A

Cis-Men

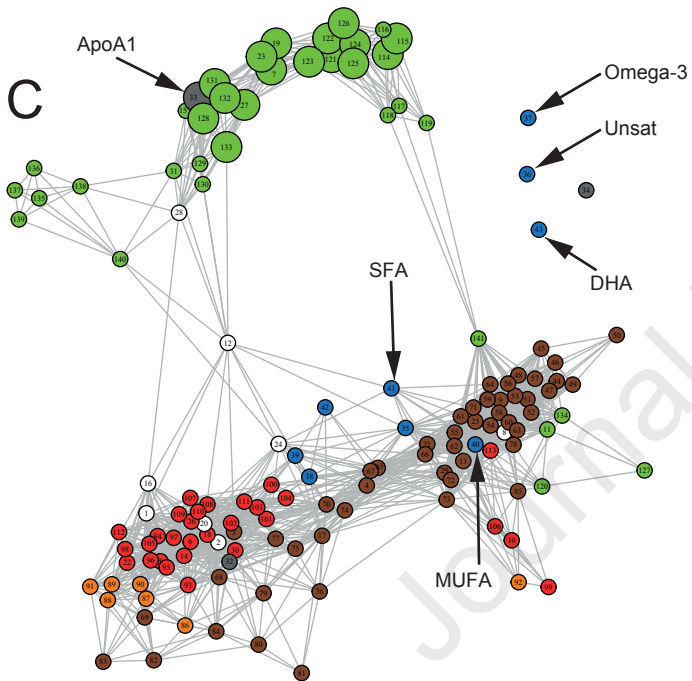


B

Cis-Women

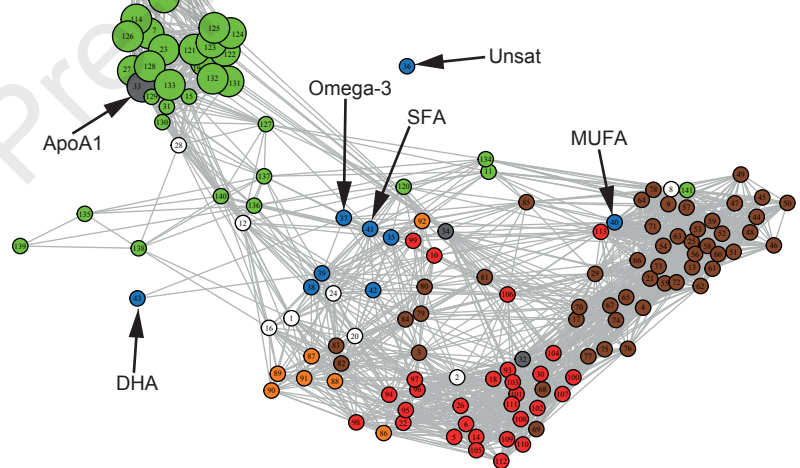


Trans-Men



D

Trans-Women



Highlights:

- Oestradiol increases typically atheroprotective HDL/ApoA1 in cis- and trans-women
- Differences in HDL/ApoA1 are induced in a dose dependent manner by oestradiol
- Sex differences are not identified pre-puberty and are disrupted in autoimmunity
- Serum lipid metabolites could inform sex-tailored strategies for CVD risk management

Journal Pre-proof