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The effect of transdermal gender-affirming hormone therapy on markers of inflammation and hemostasis

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Abstract

Background

Cardiovascular risk is increased in transgender persons using gender-affirming hormone therapy. To gain insight into the mechanism by which sex hormones affect cardiovascular risk in transgender persons, we investigated the effect of hormone therapy on markers of inflammation and hemostasis.

Methods

In this exploratory study, 48 trans women using estradiol patches plus cyproterone acetate (CPA) and 47 trans men using testosterone gel were included. They were between 18 and 50 years old and did not have a history of cardiovascular events. Measurements were performed before and after 3 and 12 months of hormone therapy.

Results

After 12 months, in trans women, systemic and endothelial inflammatory markers decreased (hs-CRP -66%, (95% CI -76; -53), VCAM-1–12%, (95% CI -16; -8)), while platelet activation markers increased (PF-4 +17%, (95% CI 4; 32), β-thromboglobulin +13%, (95% CI 2; 24)). The coagulation marker fibrinogen increased transiently, after 3 months (+15%, (95% CI 1; 32)). In trans men, hs-CRP increased (+71%, (95% CI 19; 145)); platelet activation and coagulation markers were not altered. In both trans women and trans men, leptin and adiponectin changed towards reference values of the experienced gender.

Conclusions

Platelet activation and coagulation marker concentrations increased in trans women using transdermal estradiol plus CPA, but not in trans men using testosterone. Also,

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concentrations of inflammatory markers decreased in trans women, while hs-CRP increased in trans men. Our results indicate that hormone therapy may affect hemostasis in transgender persons, which could be an underlying mechanism explaining the increased cardiovascular risk in this population.

Introduction

Transgender persons experience an incongruence between their sex assigned at birth and their gender identity. This is opposed to cisgender persons, whose sex assigned at birth matches their gender identity. Transgender persons can receive gender-affirming hormone therapy (GAHT) as part of their transition. In trans women (male sex assigned at birth, female gender identity), hormone treatment consists of estrogens, often in combination with antiandrogens (in Europe usually cyproterone acetate, CPA). In trans men (female sex assigned at birth, male gender identity), hormone treatment consists of testosterone [1]. Previous studies have shown that both trans women and trans men receiving hormone therapy have an increased risk of cardiovascular events compared to the general population [2, 3]. Trans women have an increased risk of stroke, myocardial infarction, and venous thromboembolism. Trans men seem to have an increased risk of stroke and myocardial infarction [4, 5].

The pathophysiological mechanism by which hormone therapy affects cardiovascular risk in transgender persons has not been unraveled yet. In an attempt to do so, the effect of GAHT on different cardiometabolic markers has been studied. While results from different studies are inconsistent, testosterone in trans men seems to elevate lipid levels [3, 6] and blood pressure [2], and decrease insulin resistance [7, 8]. Diversely, estrogens (combined with antiandrogens) in trans women seem to have either a positive or no effect on the lipid spectrum [3, 6], and increase insulin resistance [7, 8]. Alterations in blood pressure in trans women most probably depend on factors as administration type, duration of hormone treatment and measurement method, as both increases as decreases are reported [2, 3]. In conclusion, current evidence is not able to define the mechanism by which cardiovascular risk is increased in transgender persons.

Two key players in the development of cardiovascular disease are the processes of atherosclerosis and hemostasis. Endogenous estrogens have beneficial effects on these processes; they promote vasodilatation and endothelial cell-growth and decrease the development of atherosclerosis in cis women [9]. In contrast, oral, but not transdermal, exogenous estrogens increase thrombotic risk in postmenopausal women [10], which may be the result of first-pass hepatic metabolism. Endogenous testosterone has both protecting and deleterious effects on the vasculature [2], and administration of exogenous testosterone does not clearly affect the risk of thrombosis in cis men [11]. As research on this topic in transgender persons is scarce, we aimed to explore the effects of GAHT on inflammation and hemostasis. We selected a broad spectrum of markers associated with systemic, endothelial, or adipose tissue related inflammation. In the context of hemostasis, platelet activation and coagulation markers were investigated.

Materials and methods

Study design

This is a prospective observational study, which is part of the ENIGI (European Network for the Investigation of Gender Incongruence) study. The ENIGI-study is conducted in four

collaborating gender clinics in Amsterdam, Ghent, Oslo and Florence. It is registered at https://clinicaltrials.gov/ct2/show/NCT01072825 and the full study protocol is published elsewhere [12]. The overall study protocol was approved by the ethical review board of Ghent University Hospital, Belgium and local ethical review boards of the other participating centers. Participants of the ENIGI-study are 18 years or older, diagnosed with gender dysphoria according to the revised fourth or fifth edition of the Diagnostic and Statistical Manual of Mental Disorders and receive routine clinical transgender care. Exclusion criteria are previous or current use of hormone treatment. Written informed consent is obtained.

In this study, data from participants included in Amsterdam between June 2012 and July 2019 was analyzed. All subjects were 50 years or younger, used transdermal hormone treatment and had available blood samples at baseline and after three and / or twelve months. Trans women received estradiol patches (100 mcg/24 hours, twice weekly) combined with CPA (50 mg daily) and trans men received testosterone gel (50 mg daily). If necessary, the dosage of the hormone treatment was adjusted to achieve adequate estradiol or testosterone concentrations as suggested by applying guidelines [1]. Exclusion criteria were use of anti-inflammatory medication or medication that affects hemostasis (e.g. platelet inhibitors, anti-coagulation, SSRI's, etc.). Trans men were not allowed to use hormonal medication to suppress medication. None of the participants underwent gender-affirming genital surgery before or during the study-period. Follow-up duration was one year.

Data collection

Venous blood samples were taken and Body Mass Index (BMI) and blood pressure were measured before the start of GAHT (baseline) and after 3 and 12 months of treatment. Estradiol (pmol/L) and testosterone (nmol/L) concentrations were measured at the Laboratory for Endocrinology of the Amsterdam University Medical Centers.

Outcome measures

Our primary outcome measures consisted of several inflammatory markers. We selected a broad spectrum of markers in order to investigate different influences on inflammation, hemostasis and adipose tissue, the latter already known to be altered by hormone therapy in transgender persons [7, 13]. Of the examined systemic inflammatory markers, high-sensitivity C reactive protein (hs-CRP) is directly associated with cardiovascular risk [14] while cytokines may play an intermediate role in the acute phase response (α-1-antitrypsin, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukine (IL)-1b, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-22) and vascular adhesion molecule 1 (VCAM-1) is expressed during endothelial activation. Adipose tissue specific marker leptin is associated with obesity and is a predictor of myocardial infarction [15, 16], while adiponectin has anti-inflammatory effects, like increasing insulin sensitivity [16, 17]. Of the examined coagulation markers, fibrinogen is associated with cardiovascular risk [18], and plasminogen activator inhibitor-1 (PAI-1) is altered by hormone therapy in postmenopausal cis women [19]. Platelet activation markers consisted of platelet-specific proteins platelet factor 4 (PF-4), β-thromboglobulin and p-selectin, which are released by platelets upon activation [20].

Secondary outcome measures were estradiol and testosterone levels, BMI and blood pressure. BMI and blood pressure are influenced by GAHT [21], and are associated with inflammation [22, 23]. They were included to rule out that observed changes in concentrations of inflammatory markers were actually explained by changes in BMI or blood pressure.

Biomarker assays

The above-mentioned markers were measured at the Netherlands Organization for Applied Scientific Research (Leiden, The Netherlands), using serum (for PF-4 and β-thromboglobulin) and EDTA (for all other markers) plasma samples and miniaturized biomarker assays. The samples from transgender men and women were randomized and evaluated on the same plates. To minimize analytical variability, all measurements of a particular biomarker were carried out on the same day, and longitudinal samples of a subject (0, 3 and 12 months) were analyzed on the same plate (96 wells). More specifically, cytokine concentrations were determined with a CorPlex™ Cytokine Panel on an SP-X™ imaging system (Quanterix, Billerica, MA, USA). All other markers were quantified by enzyme-linked immunosorbent assay using the following antibody sets: hs-CRP (DY1707); VCAM-1 (DY809); adiponectin (DY1065); PF4 (DY795); β-thromboglobulin (DY393); p-selectin (DY137); PAI-1 (DY9387); all R&D Systems (Abingdon, UK); α-1-antitrypsin (NBP2-60541) and fibrinogen (NBP2-60465) from Novus Biologicals (Wiesbaden, Germany); leptin (10-1199-01) from Mercodia (Uppsala, Sweden). For each biomarker, the linear range and optimal dilution factor was optimized prior to the measurements using commercially available reference plasma from female (n = 20) and male blood donors (n = 20) (TCS Bioscience Ltd, Buckingham, UK). The linear range, limit of quantification and dilution factor of the inflammatory markers are provided in S1 Table.

Statistical analysis

Statistical analyses were performed using STATA®, version 15.1. Baseline characteristics and hormone concentrations are presented as median with interquartile range (IQR). Concentrations of inflammatory markers were log transformed for analysis and back-transformed for presentation. Normality of the residuals was verified via visual inspection of the histogram. Linear mixed models [24] with a random intercept for each subject were used, with the inflammatory marker as the dependent variable and duration of treatment (0, 3 and 12 months) as a categorical covariate. We adjusted for potential confounders (change in BMI or blood pressure) and stratification factors (age, hormone level during treatment, smoking status). The obtained regression coefficient was back-transformed to the ratio and converted to percentage change. No adjustment for multiple comparisons was performed.

Results

In this study, 48 trans women and 47 trans men were included. The study flowchart is shown in Fig 1. Table 1 shows the baseline characteristics of the study population. Hormone concentrations, body mass index and blood pressure at baseline and after 3 and 12 months of hormone therapy are shown in Table 2. Absolute values of the inflammatory markers are shown in Tables 3 and 4; percentage change is shown in Fig 2.

Below, Adjusting the analyses for change in BMI or blood pressure did not affect the results. Also, changes in markers were not different for different age ranges (in trans women only; in trans men no stratification was performed because of the small age range). Lastly, higher hormone concentrations during treatment or different smoking status at baseline did not affect the results either. Therefore, non-adjusted results are reported. We only describe the percentage change after 12 months of hormone therapy, unless the direction of the 3- and 12-month effect was different.

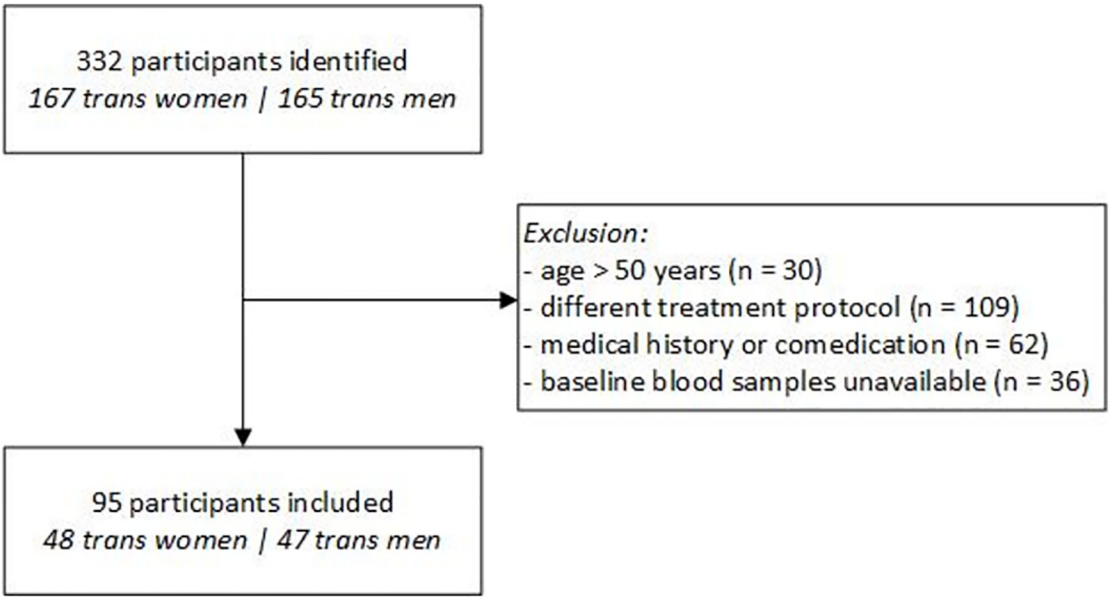

Fig 1. Study flowchart.

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Trans women

Inflammation. Systemic inflammatory markers IL-1b, IL4, IL5, IL12p70 and IFN-γ were undetectable in the majority of samples. Estimates of concentration changes of these markers are not considered reliable and are therefore not reported.

After 12 months of transdermal estradiol plus CPA, systemic and endothelial inflammatory marker concentrations decreased (hs-CRP -66%, (95% CI -76; -53), IL-6–28%, (95% CI -7; -44), IL-8–15%, (95% CI -2; -17), IL-22–34%, (95% CI -7; -44), VCAM-1–12%, (95% CI -16;

Table 1. Baseline characteristics of the study population.

	Trans women (n = 48)	Trans men (n = 47)
Age (years)	30 (24–39)	23 (20–26)
BMI (kg/m^2)	23 (21–26)	23 (21–30)
Ever smoked (%)	16 (33%)	20 (43%)
Blood pressure (mmHg)		
Systolic	129 (121–137)	122 (115–128)
Diastolic	80 (76–87)	77 (72–82)
MAP	96 (91–101)	92 (87–97)
Glucose (mmol/L)	5.5 (5.1–5.7)	5.1 (4.9–5.4)
Total cholesterol (mmol/L)	4.3 (3.7–5.1)	4.3 (3.9–4.9)
Triglycerides (mmol/L)	0.9 (0.7–1.3)	0.7 (0.5–1.0)

Data are presented as median (IQR) for continuous data, and number and % for categorical data. BMI, body mass index. MAP, mean arterial pressure. Number of participants with an underlying disease: diabetes mellitus (1), human immunodeficiency virus (2), pulmonal hypertension (1), ulcerative colitis (1).

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Table 2. Hormone concentrations, body mass index and blood pressure at baseline and after 3 and 12 months of hormone therapy.

	Trans women			Trans men		
	Visit (months)					
	0	3	12	0	3	12
Estradiol (pmol/L)	101 (79–126)	246 (120–342)	258 (178–496)	273 (168–442)	184 (147–250)	158 (116–204)
Testosterone (nmol/L)	18 (14–23)	0.6 (0.5–0.8)	0.7 (0.5–0.8)	1.3 (1.0–1.7)	27 (14–42)	20 (15–30)
BMI (kg/m^2)	23 (21–26)	23 (21–26)	24 (22–27)	23 (21–30)	23 (22–29)	23 (22–28)
BP (mmHg)						
Systolic	129 (123–138)	123 (117–134)	127 (117–135)	122 (115–126)	123 (117–130)	122 (116–129)
Diastolic	80 (73–88)	78 (73–83)	78 (73–84)	77 (72–81)	75 (70–82)	76 (72–82)
MAP	96 (91–101)	93 (88–98)	94 (89–100)	92 (87–97)	90 (86–98)	92 (87–96)

Data are presented as median (IQR). BP, blood pressure. BMI, body mass index. MAP, mean arterial pressure.

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-8)). Concentrations of both adipokines increased (leptin +202%, (95% CI 139; 282), adiponectin +7% (95% CI -2; 16)). For percentage change after 3 and 12 months, see Fig 2.

Hemostasis. Concentrations of platelet activation markers increased after 12 months of treatment (PF-4 +17%, (95% CI 4; 32), β-thromboglobulin +13% (95% CI 2; 24)). Coagulation

Table 3. Inflammation and hemostasis markers at 0, 3 and 12 months in trans women (absolute values).

	Visit (months)				
	0	3		12	
Inflammation					
Systemic inflammatory markers					
hs-CRP (μg/ml)	0.8 (0.6, 1.1)	0.5 (0.3, 1.0)	p = 0.04	0.3 (0.2, 0.4)	p < 0.001
α-1-antitrypsin (μg/ml)	186 (173, 199)	198 (177, 222)	p = 0.62	178 (165, 192)	p = 0.24
TNF-α (pg/ml)	2.9 (2.6, 3.3)	2.5 (2.2, 2.9)	p = 0.06	2.6 (2.3, 2.9)	p = 0.02
IL-6 (pg/ml)	1.1 (0.9, 1.4)	1.0 (0.7, 1.4)	p = 0.48	0.8 (0.6, 1.0)	p = 0.01
IL-8 (pg/ml)	6.3 (5.6, 7.0)	5.9 (5.1, 6.9)	p = 0.52	5.3 (4.6, 6.1)	p = 0.02
IL-10 (pg/ml)	0.6 (0.5, 0.8)	0.5 (0.4, 0.6)	p = 0.33	0.5 (0.4, 0.6)	p = 0.06
IL-22 (pg/ml)	0.8 (0.6, 0.9)	0.5 (0.4, 0.7)	p = 0.33	0.5 (0.4, 0.7)	p = 0.06
Endothelial inflammatory markers					
VCAM-1 (ng/ml)	382 (358, 407)	328 (299, 360)	p < 0.001	337 (315, 360)	p < 0.001
Adipose tissue markers					
Leptin (ng/ml)	3.5 (2.4, 5.1)	7.6 (5.1, 11.4)	p < 0.001	9.8 (7.5, 12.8)	p < 0.001
Adiponectin (μg/ml)	2.3 (2.1, 2.6)	2.6 (2.1, 3.2)	p = 0.015	2.5 (2.1, 2.8)	p = 0.13
Hemostasis					
Platelet activation markers					
Platelet factor 4 (PF-4) (μg/ml)	2.0 (1.8, 2.3)	1.9 (1.4, 2.4)	p = 0.28	2.4 (2.2, 2.6)	p = 0.01
β-thromboglobulin (μg/ml)	5.6 (5.1, 6.1)	5.3 (4.2, 6.5)	p = 0.35	6.4 (5.8, 6.9)	p = 0.02
P-selectin (ng/ml)	36 (32, 40)	39 (35, 43)	p = 0.29	34 (30, 38)	p = 0.37
Markers of coagulation					
Fibrinogen (mg/ml)	3.7 (3.3, 4.1)	4.3 (3.5, 5.2)	p = 0.04	3.8 (3.4, 4.1)	p = 0.78
PAI-1 (ng/ml)	23 (20, 26)	24 (20, 30)	p = 0.46	21 (18, 25)	p = 0.45

Predicted average marker levels (geometric mean) with 95% confidence intervals and p-values, not adjusted for multiple comparisons. Measures were log-transformed for analysis and back-transformed for presentation.

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Table 4. Inflammation and hemostasis markers at 0, 3 and 12 months in trans men (absolute values).

	Visit (months)				
	0	3		12	
Inflammation					
Systemic inflammatory markers					
hs-CRP (μg/ml)	0.3 (0.2, 0.6)	0.5 (0.3, 0.8)	p = 0.23	0.5 (0.3, 0.9)	p = 0.005
α-1-antitrypsin (μg/ml)	176 (165, 188)	166 (150, 184)	p = 0.10	166 (150, 184)	p = 0.10
TNF-α (pg/ml)	2.7 (2.4, 3.0)	2.8 (2.5, 3.1)	p = 0.82	2.8 (2.5, 3.1)	p = 0.85
IL-6 (pg/ml)	1.1 (0.9, 1.4)	1.1 (0.8, 1.6)	p = 0.76	1.0 (0.8, 1.4)	p = 0.53
IL-8 (pg/ml)	5.6 (4.8, 6.4)	6.1 (5.4, 6.9)	p = 0.15	6.7 (5.7, 7.8)	p = 0.10
IL-10 (pg/ml)	0.5 (0.4, 0.5)	0.5 (0.4, 0.6)	p = 0.64	0.5 (0.4, 0.6)	p = 0.40
IL-22 (pg/ml)	0.8 (0.6, 1.0)	0.7 (0.5, 1.0)	p = 0.64	0.8 (0.6, 1.1)	p = 0.91
Endothelial inflammatory markers					
VCAM-1 (ng/ml)	386 (364, 410)	407 (371, 446)	p = 0.13	390 (361, 421)	p = 0.37
Adipose tissue markers					
Leptin (ng/ml)	14.0 (10.8, 18.0)	9.9 (7.1, 13.7)	p < 0.001	6.6 (4.9, 9.1)	p < 0.001
Adiponectin (μg/ml)	3.1 (2.6, 3.6)	2.4 (2.1, 2.9)	p < 0.001	2.5 (2.1, 2.9)	p < 0.001
Hemostasis					
Platelet activation markers					
Platelet factor 4 (PF-4) (μg/ml)	2.1 (1.9, 2.3)	1.7 (1.3, 2.2)	p = 0.04	2.1 (1.8, 2.4)	p = 0.85
β-thromboglobulin (μg/ml)	5.4 (5.1, 5.9)	4.4 (3.4, 5.8)	p = 0.03	5.5 (5.0, 6.1)	p = 0.99
P-selectin (ng/ml)	33 (30, 37)	36 (32, 40)	p = 0.56	34 (30, 38)	p = 0.72
Markers of coagulation					
Fibrinogen (mg/ml)	3.5 (3.2, 3.9)	3.0 (2.3, 3.9)	p = 0.10	3.2 (2.8, 3.6)	p = 0.36
PAI-1 (ng/ml)	23 (20, 25)	26 (21, 32)	p = 0.40	21 (17, 25)	p = 0.35

Predicted average marker levels (geometric mean) with 95% confidence intervals and p-values, not adjusted for multiple comparisons. Measures were log-transformed for analysis and back-transformed for presentation.

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marker fibrinogen transiently increased after 3 months (+15%, (95% CI 1; 32)) and normalized after 12 months (+1%, (95% CI -8; 12)); PAI-1 was not altered. For percentage change after 3 and 12 months, see Fig 2.

Trans men

Inflammation. After 12 months of testosterone treatment, concentrations of systemic inflammatory markers did not change, except for hs-CRP (+71%, (95% CI 19; 145)). Endothelial marker VCAM-1 was not clearly affected. Adipokine concentrations decreased (leptin -49% (95% CI -59; -37), adiponectin -20% (95% CI -27; -14)). For percentage change after 3 and 12 months, see Fig 2.

Hemostasis. Concentrations of platelet activation markers and coagulation markers did not clearly change after 12 months of treatment (PF-4 +0%, (95% CI -18; 23), β-thromboglobulin +2% (95% CI -16; 23), fibrinogen -8% (95% CI -23; 9)). For percentage change after 3 and 12 months, see Fig 2.

Discussion

In this study, we assessed the association between hormone treatment and markers of inflammation and hemostasis in transgender persons. We observed that hormone treatment in trans

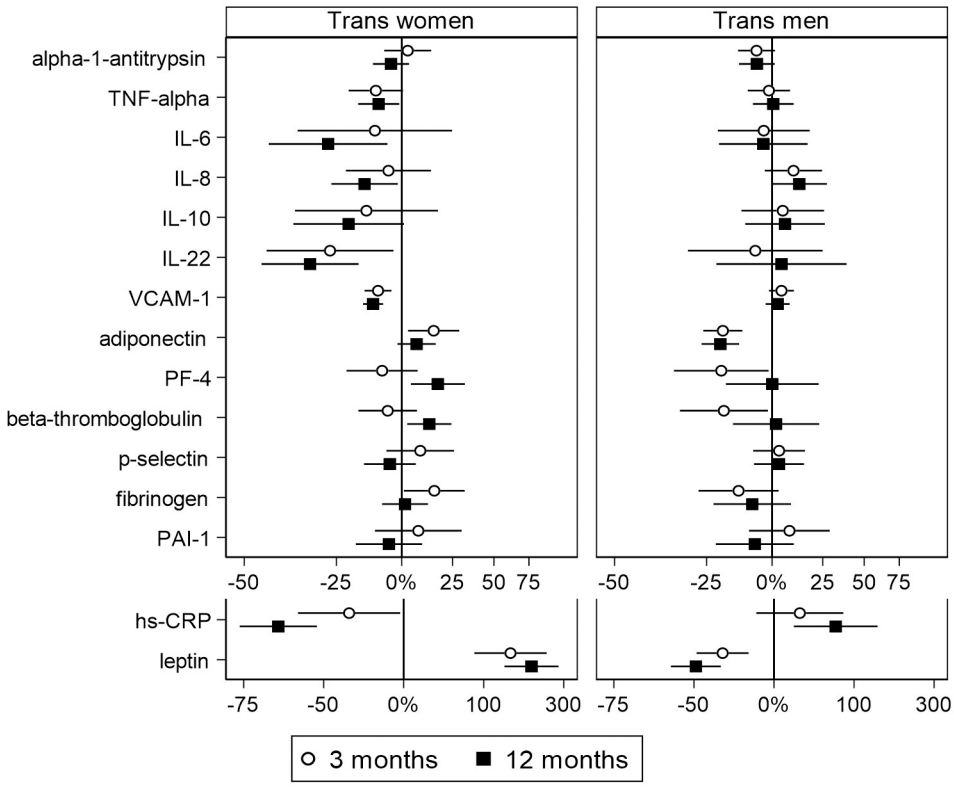

Fig 2. %-change after 3 and 12 months of hormone therapy in trans women and trans men. Values are presented as geometric mean with 95% confidence interval. Measures were log-transformed for analysis and back-transformed for presentation. Scales are logarithmic. For hs-CRP and leptin, a larger scale is used.

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women was associated with a decrease in inflammatory marker concentrations and an increase in hemostasis marker concentrations. In contrast, in trans men, hormone treatment was associated with no change in inflammatory and hemostasis marker concentrations, except for an increase in hs-CRP.

Trans women

Inflammation. Transdermal estradiol combined with CPA was associated with a decrease in concentrations of systemic and endothelial inflammatory markers in trans women. This is not in line with previous studies in postmenopausal and trans women, which have found that transdermal estradiol was not associated with a change in inflammation markers and oral estrogens were associated with an increase in inflammation marker concentrations [25–27]. However, both the postmenopausal and trans women included in these studies, were older than our participants. The potential beneficial effect of hormone therapy on inflammation decreases with age, as estradiol may not inhibit the development of atherosclerosis once this is present [28]. Further, the increase in concentrations of inflammatory markers associated with oral but not with transdermal estrogens implies that the hepatic first pass effect may be responsible.

In contrast with the other inflammatory markers, hormone treatment was associated with increased adipokine concentrations. These results are in line with previous studies in transgender persons using hormone treatment, which showed that concentrations of adipokines changed towards reference values of the experienced gender [7, 13]. The increased leptin

concentration may, at least partly, be explained by a change in fat distribution from visceral depots to the subcutaneous depot [29]. Subsequently, the change in adipokine concentrations can be interpreted as a consequence of metabolic adjustments in energy balance and body fat distribution.

Hemostasis. In trans women, transdermal estradiol plus CPA was associated with an increase in levels of platelet activation markers. This is in line with a previous study in cis women, which has shown that platelets express estrogen receptors and platelet activation differs per phase of the menstrual cycle [30]. However, studies in postmenopausal women are inconclusive, with some studies reporting an increase and other reporting a decrease in platelet activity after hormone treatment [31, 32].

The concentration of the coagulation marker fibrinogen transiently increased after 3 months of transdermal estradiol combined with CPA. Similarly, a recent study on the effect of GAHT on coagulation parameters observed more procoagulant profiles in trans women [33]. Our observed increase in fibrinogen concentration after 3 months of hormone therapy may indicate temporarily increased pro-coagulant activity. The transient increase in fibrinogen concentration could be caused by CPA. Oral contraceptives containing CPA are associated with a higher thrombotic risk than contraceptives containing levonorgestrel [34]. Also, the dosage of CPA that has antiandrogenic effects is higher than the dosage used in contraception (50 vs 2 mg), which may even be more prothrombotic. While we know that trans women using hormone treatment have an increased thrombotic risk, we do not know if those using CPA have a higher thrombotic risk than those using other (for example GnRH-analogues or spironolactone) or no antiandrogens.

Trans men

Inflammation. In trans men, testosterone was associated with an increase in hs-CRP concentration, but not with a change in other systemic inflammatory markers. This is in contrast with previous studies in hypogonadal cis men, which found no effect or even a decrease in levels of inflammation markers [35, 36]. This difference may be explained by the effect of testosterone administration on estrogen concentrations. In hypogonadal cis men, testosterone is converted into estradiol by aromatase, increasing the concentration of estradiol. However, trans men start with a high concentration of estradiol, which is decreased by testosterone Since our results in trans women suggest that estrogens are associated with a decrease in inflammatory markers the increase of hs-CRP in trans men may be associated with the decline in estrogen concentrations. This however does not explain why we observed no change in concentrations of the other inflammatory markers.

Further, we found that testosterone decreased adipokine levels towards reference values for men, which is in line with our results in trans women and the results of previous studies in transgender persons [7, 13]. This is probably partially explained by a change in fat distribution from subcutaneous towards visceral fat depots [29].

Hemostasis. While estradiol plus CPA increased hemostatic marker concentrations in trans women, testosterone did not affect these markers in trans men. After 12 months of hormone treatment, platelet activation marker concentrations did not change. Unfortunately, previous studies on this topic only include animal and ex vivo studies. Some of these studies have suggested that testosterone induces platelet aggregation by influencing platelet receptor expression [37, 38], while another indicated that testosterone induces platelet inhibition [39]. Therefore, the effect of testosterone on platelet activity needs to be further examined.

Administration of testosterone also did not affect coagulation markers in trans men. This is in line with a recent study on coagulation parameters in trans men, which observed no

apparent changes [33]. Also, there is absence of evidence for an increased risk of venous thromboembolic disease in trans men [2–5]. Similarly, in hypogonadal cis men, testosterone replacement therapy does not increase concentrations of coagulation markers [40], nor the occurrence of venous thromboembolism [11]. While our results indicate that the hemostatic system may play a role in the association between hormone treatment and cardiovascular risk in trans women, this is less apparent in trans men.

Strengths and limitations

As far as we know, this is the first study exploring the effect of gender-affirming hormone therapy on inflammatory and hemostasis markers in both trans women and trans men. The explorative aim of our study required investigating multiple markers and multiple testing. Therefore, our results are hypothesis-generating, and not hypothesis-confirming. A strength of our study is the relatively large homogenous population and the specific hormone regimes and administration types. As it is unethical to include a control group who are withheld from desired hormone therapy, we used a prospective design in which each participant serves as their own control. Our study had some limitations as well. First, our participants were young and healthy and follow-up duration was relatively short, while the occurrence of atherosclerosis and cardiovascular disease increases with age. Second, we were unable to differentiate between the effect of transdermal estradiol and the effect of CPA in trans women. Third, to avoid the first-pass effect of the liver, we investigated the effect of only transdermal estradiol, while the occurrence of thrombosis is especially associated with the use of oral estrogens. Future, larger studies in older transgender persons, with a longer follow-up duration, different hormone regimes and administration types, preferably in comparison to cisgender controls, are necessary to expand our knowledge on this subject. While especially relevant for the trans population, gaining insight into the mechanism by which sex hormones affect cardiovascular risk may help to understand sex differences in cardiovascular disease in the cis population as well.

Supporting information

S1 Table. Linear range, limit of quantification and dilution factor of inflammatory markers.
(DOCX)

S2 Table. Number of analyzed blood samples for trans women and trans men at 0, 3 and 12 months.
(DOCX)

S1 Appendix. Minimal dataset.
(DTA)

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Supporting information

S1 Table. Linear range, limit of quantification and dilution factor of inflammatory markers

	Linear Range	Limit of Quantification	Dilution Factor
hs-CRP	15.625 to 1000 pg/mL	15.625 pg/mL	6000x
α-1-antitrypsin	1.125 to 36 ng/mL	1.125 ng/mL	20000x
TNF-α	0.391 to 400 pg/mL	1.5625 pg/mL	4x
IFN-γ	0.195 to 50 pg/mL	0.1953 pg/mL	4x
IL-1b	0.391 to 100 pg/mL	25 pg/mL	4x
IL-4	3.125 to 200 pg/mL	0.7813 pg/mL	4x
IL-6	0.293 to 300 pg/mL	4.6875 pg/mL	4x
IL-8	1.562 to 400 pg/mL	6.25 pg/mL	4x
IL-10	0.391 to 100 pg/mL	0.3906 pg/mL	4x
IL-12p70	0.293 to 300 pg/mL	1.1719 pg/mL	4x
IL-22	0.098 to 100 pg/mL	0.3906 pg/mL	4x
VCAM-1	15.625 to 1000 pg/mL	15.625 pg/mL	2600x
leptin	0.092 to 4.78 ng/mL	0.092 ng/mL	13x
adiponectin	62.5 to 1000 pg/mL	62.5 pg/mL	5400x
PF-4	15.625 to 500 pg/mL	15.625 pg/mL	16000x
β-thromboglobulin	15.625 to 250 pg/mL	15.625 pg/mL	38000x
p-selectin	125 to 2000 pg/mL	125 pg/mL	40x
fibrinogen	0.165 to 4.444 ug/mL	0.165 ug/mL	1000x
PAI-1	7.8125 to 250 pg/mL	7.8125 pg/mL	270x

Supporting information

S2 Table. Number of analyzed blood samples for trans women and trans men at 0, 3 and 12 months

	Visit (months)					
	0	3	12	0	3	12
Blood samples						
EDTA plasma*	48	21	43	47	31	36
Serum plasma**	46	20	42	45	31	35

*Marker concentrations measured in EDTA plasma: hs-CRP, α-1-antitrypsin, TNF-α, IL-6, IL-8, IL-10, IL-22, VCAM-1, leptin, adiponectin, p-selectin, fibrinogen, PAI-1
**Marker concentrations measured in serum plasma: PF-4, β-thromboglobulin

Response to reviewers

We would like to thank both reviewers for the valuable suggestions on our manuscript. Below, we outline how these suggestions helped us to improve the manuscript.

Reviewer #1: Schutte et al looked at the effect of transdermal GAHT on markers of inflammation. The study is nicely done but the authors need to recognize the limitation of their study. The sample size is very small and no control subjects but 15 markers have been tested. This is a big limitation for the study, the authors should mention that this is an exploratory study and no way is a confirmatory study.

We agree that our study is exploratory and emphasized this by adding this to the abstract (p. 2):

"In this exploratory study, 48 trans women using estradiol patches plus cyproterone acetate (CPA) and 47 trans men using testosterone gel were included."

We also clarified this in the introduction (p. 4):

"As research on this topic in transgender persons is scarce, we aimed to explore the effects of GAHT on inflammation and hemostasis."

We also mentioned this in the discussion section (p. 18):

"The explorative aim of our study required investigating multiple markers and multiple testing. Therefore, our results are hypothesis-generating, and not hypothesis-confirming."

The English used in the text is also hard to follow and needs rewriting.

We carefully checked the language in our entire manuscript and as a consequence, we made several improvements, especially in the Discussion section.

The abbreviation needs to be spelled out first time used in the body of the manuscript, for example "PAI-1" never been spelled out.

We thank the reviewer for noticing this, and we have now corrected this (p. 6):

"Of the examined coagulation markers, fibrinogen is associated with cardiovascular risk (16), and plasminogen activator inhibitor-1 (PAI-1) is altered by hormone therapy in postmenopausal cis women."

Authors report that "Concentrations of inflammatory markers were log transformed for analysis and back transformed for presentation". While this is probably correct, authors should verify that the residual errors from the mixed linear model have a normal distribution on the log scale by computing a normal goodness of fit statistic (for example Shapiro-Wilk test) and report that normality was verified.

We verified the normality of the residuals via visual inspection of the histogram and added the following to the Statistical analysis section (p. 8):

"Normality of the residuals was verified via visual inspection of the histogram."

If the authors are reporting the antilog of the log scale means, this is called the geometric mean and should be labelled as such.

We thank the reviewer for pointing this out and now added the term 'geometric mean' to the captions of table 3 and figure 2a and b (p. 11 and 13).

Data (IL-1b, IL-4,IL12p70) failing to have a normal distribution ("heavily right skewed"-line 234) is not a good reason for omitting results ("they were not further analyzed"). Non parametric methods can be used. Medians and interquartile ranges can be reported. They can probably ignore these markers if they think they are not important markers otherwise their reason for not analyzing is not acceptable.

We agree with the reviewer that a heavily right skewed distribution is not a good reason for not analyzing markers. However, in the majority of samples, IL-1b, IL-4, IL-5, IL12p70 and IFN-γ were undetectable. We performed analyses in which we replaced undetectable concentrations by the lower limit of detection divided by two. The residuals were normally distributed. As these analyses, performed on raw estimates of concentrations, provide unreliable results with very broad confidence intervals, we do not report them in our manuscript as we believe they do not add value to our study. They are provided below:

*"***Analyses of inflammatory markers IL-1b, IL-4, IL-5 IL12p70 and IFN-γ**

If marker concentrations were undetectable, values were replaced by the lower limit of detection divided by two.

Trans women

After 12 months, IL1-b changed with +199% (95% CI 8 ; 726), IL-4 changed with +37% (95% CI -30 ; 169), IL-5 changed with -14%, (95% CI -44 ; 32), IL-12p70 changed with +20% (95% CI -38 ; 134) and IFN- γ changed with -24% (95% CI -57 ; 33).

Trans men

After 12 months, IL-1b changed with -1% (95% CI -55 ; 117), IL-4 changed with -25% (95% CI -60 ; 40), IL5 changed with -17% (95% CI -41 ; 17), IL-12p70 changed with +12% (95% CI -21 ; 60) and IFN-γ changed with -6% (95% CI -39 ; 47)."

Authors should report the sample size at each time if it is not constant (48 trans females or 47 in trans males) across time.

We have now added this information to the Supporting information (p. 23):

Table 1. Number of analyzed blood samples for trans women and trans men at 0, 3 and 12 months

	Trans women			Trans men		
	Visit (months)					
	0	3	12	0	3	12
Blood samples						
EDTA plasma*	48	21	43	47	31	36
Serum plasma**	46	20	42	45	31	35

*Marker concentrations measured in EDTA plasma: hs-CRP, α-1-antitrypsin, TNF-α, IL-6, IL-8, IL-10, IL-22, VCAM-1, leptin, adiponectin, p-selectin, fibrinogen, PAI-1
**Marker concentrations measured in serum plasma: PF-4, β-thromboglobulin

There is possible selection bias if those with no baseline data were excluded. Authors should provide the flow diagram showing how many were excluded from the initial pool and why they were excluded.

We have now added the flow diagram showing the total number of excluded subjects, including the reasons for exclusion (p. 9):

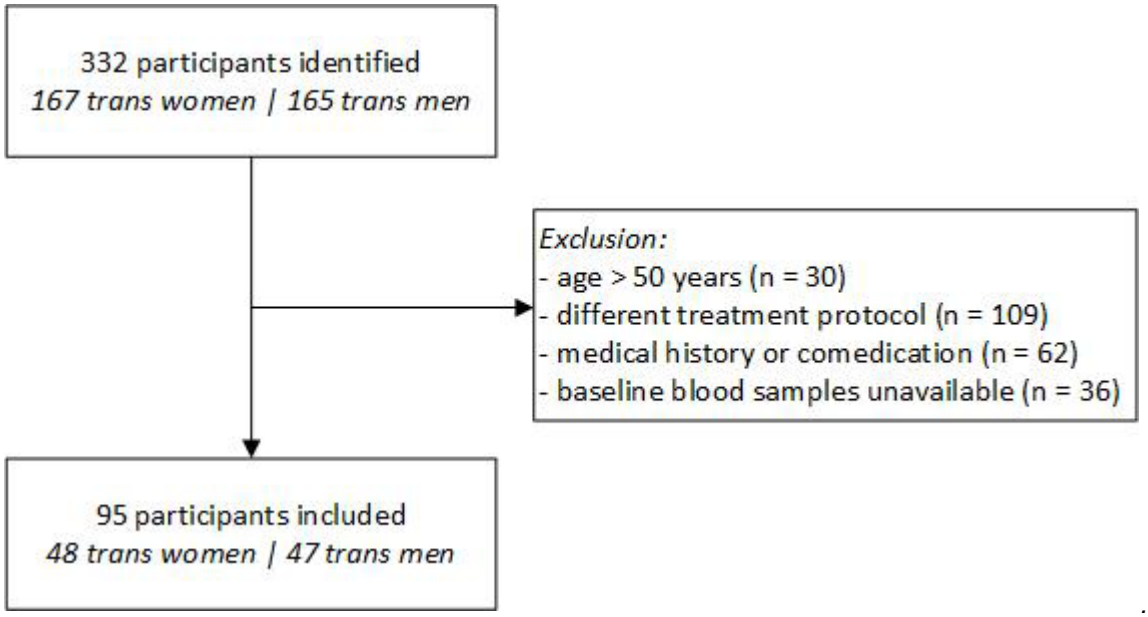

Figure 1. Study flowchart

While the authors did not report BMI or blood pressure change adjusted results, it is not clear why this should even be considered. It would seem that change in BMI and/or blood pressure are not confounders but are another result of the hormone intervention. This needs to be clarified (especially for BP).

BMI and blood pressure are related to inflammation, and because we did not want to ignore potential confounders, we considered these variables for our analyses. To clarify, we added the following information to the Methods section (p. 6 and 7):

"Secondary outcome measures were estradiol and testosterone levels, BMI and blood pressure. BMI and blood pressure are influenced by GAHT (21), and are associated with inflammation (22, 23). They were included to rule out that observed changes in concentrations of inflammatory markers were actually explained by changes in BMI or blood pressure."

The authors looked at 15 outcomes (Table 3). They should report nominal p values for the change from time 0 and state whether they are adjusting for multiple outcomes and multiple comparisons. The authors are commended for reporting confidence bounds but should clarify that the 95% confidence level is not adjusted for multiple outcomes.

We now explicitly stated that we did not control for multiple comparisons in the Statistical analysis section (p. 8):

"No adjustment for multiple comparisons was performed."

We also added this information and the requested p-values to Tables 3 and 4 (p. 11 and 12):

Table 3. Inflammation and hemostasis markers at 0, 3 and 12 months in trans women (absolute values)

	Visit (months)				
	0	3		12	
Inflammation					
Systemic inflammatory markers					
hs-CRP (µg/ml)	0.8 (0.6, 1.1)	0.5 (0.3, 1.0)	p = 0.036	0.3 (0.2, 0.4)	p = 0.001
α-1-antitrypsin (µg/ml)	186 (173, 199)	198 (177, 222)	p = 0.62	178 (165, 192)	p = 0.24
TNF-α (pg/ml)	2.9 (2.6, 3.3)	2.5 (2.2, 2.9)	p = 0.057	2.6 (2.3, 2.9)	p = 0.021
IL-6 (pg/ml)	1.1 (0.9, 1.4)	1.0 (0.7, 1.4)	p = 0.48	0.8 (0.6, 1.0)	p = 0.012
IL-8 (pg/ml)	6.3 (5.6, 7.0)	5.9 (5.1, 6.9)	p = 0.52	5.3 (4.6, 6.1)	p = 0.024
IL-10 (pg/ml)	0.6 (0.5, 0.8)	0.5 (0.4, 0.6)	p = 0.33	0.5 (0.4, 0.6)	p = 0.056
IL-22 (pg/ml)	0.8 (0.6, 0.9)	0.5 (0.4, 0.7)	p = 0.33	0.5 (0.4, 0.7)	p = 0.056
Endothelial inflammatory markers					
VCAM-1 (ng/ml)	382 (358, 407)	328 (299, 360)	p = 0.001	337 (315, 360)	p = 0.001
Adipose tissue markers					
Leptin (ng/ml)	3.5 (2.4, 5.1)	7.6 (5.1, 11.4)	p = 0.001	9.8 (7.5, 12.8)	p = 0.001
Adiponectin (µg/ml)	2.3 (2.1, 2.6)	2.6 (2.1, 3.2)	p = 0.015	2.5 (2.1, 2.8)	p = 0.13
Hemostasis					
Platelet activation markers					
Platelet factor 4 (PF-4) (µg/ml)	2.0 (1.8, 2.3)	1.9 (1.4, 2.4)	p = 0.28	2.4 (2.2, 2.6)	p = 0.009
β-thromboglobulin (µg/ml)	5.6 (5.1, 6.1)	5.3 (4.2, 6.5)	p = 0.35	6.4 (5.8, 6.9)	p = 0.015
P-selectin (ng/ml)	36 (32, 40)	39 (35, 43)	p = 0.29	34 (30, 38)	p = 0.37
Markers of coagulation					
Fibrinogen (mg/ml)	3.7 (3.3, 4.1)	4.3 (3.5, 5.2)	p = 0.036	3.8 (3.4, 4.1)	p = 0.78
PAI-1 (ng/ml)	23 (20, 26)	24 (20, 30)	p = 0.46	21 (18, 25)	p = 0.45

Predicted average marker levels (geometric mean) with 95% confidence intervals and p-values, not adjusted for multiple comparisons.
Measures were log-transformed for analysis and back-transformed for presentation.

Table 4. Inflammation and hemostasis markers at 0, 3 and 12 months in trans men (absolute values)

	Visit (months)				
	0	3		12	
Inflammation					
Systemic inflammatory markers					
hs-CRP (µg/ml)	0.3 (0.2, 0.6)	0.5 (0.3, 0.8)	p = 0.23	0.5 (0.3, 0.9)	p = 0.005
α-1-antitrypsin (µg/ml)	176 (165, 188)	166 (150, 184)	p = 0.099	166 (150, 184)	p = 0.099
TNF-α (pg/ml)	2.7 (2.4, 3.0)	2.8 (2.5, 3.1)	p = 0.82	2.8 (2.5, 3.1)	p = 0.85
IL-6 (pg/ml)	1.1 (0.9, 1.4)	1.1 (0.8, 1.6)	p = 0.76	1.0 (0.8, 1.4)	p = 0.53
IL-8 (pg/ml)	5.6 (4.8, 6.4)	6.1 (5.4, 6.9)	p = 0.15	6.7 (5.7, 7.8)	p = 0.099
IL-10 (pg/ml)	0.5 (0.4, 0.5)	0.5 (0.4, 0.6)	p = 0.64	0.5 (0.4, 0.6)	p = 0.40
IL-22 (pg/ml)	0.8 (0.6, 1.0)	0.7 (0.5, 1.0)	p = 0.64	0.8 (0.6, 1.1)	p = 0.91
Endothelial inflammatory markers					
VCAM-1 (ng/ml)	386 (364, 410)	407 (371, 446)	p = 0.13	390 (361, 421)	p = 0.37
Adipose tissue markers					
Leptin (ng/ml)	14.0 (10.8, 18.0)	9.9 (7.1, 13.7)	p = 0.001	6.6 (4.9, 9.1)	p = 0.001
Adiponectin (µg/ml)	3.1 (2.6, 3.6)	2.4 (2.1, 2.9)	p = 0.001	2.5 (2.1, 2.9)	p = 0.001
Hemostasis					
Platelet activation markers					
Platelet factor 4 (PF-4) (µg/ml)	2.1 (1.9, 2.3)	1.7 (1.3, 2.2)	p = 0.036	2.1 (1.8, 2.4)	p = 0.85
β-thromboglobulin (µg/ml)	5.4 (5.1, 5.9)	4.4 (3.4, 5.8)	p = 0.032	5.5 (5.0, 6.1)	p = 0.99
P-selectin (ng/ml)	33 (30, 37)	36 (32, 40)	p = 0.56	34 (30, 38)	p = 0.72
Markers of coagulation					
Fibrinogen (mg/ml)	3.5 (3.2, 3.9)	3.0 (2.3, 3.9)	p = 0.095	3.2 (2.8, 3.6)	p = 0.36
PAI-1 (ng/ml)	23 (20, 25)	26 (21, 32)	p = 0.40	21 (17, 25)	p = 0.35

Predicted average marker levels (geometric mean) with 95% confidence intervals and p-values, not adjusted to multiple comparisons. Measures were log-transformed for analysis and back-transformed for presentation.

Obviously, a stronger study would compare these changes to changes in a cisgender control group but it is acknowledged that this may not be feasible and is beyond the scope of the current study.It would also have been interesting if the change in the outcome had been correlated with the change in hormone but this may be beyond the scope of the current analysis.

We agree with the reviewer that comparison with a cisgender control group would be a great study design. We added this as a suggestion for future research to our Discussion section (p. 19):

"Future, larger studies in older transgender persons with a longer follow-up duration, different hormone regimes (i.e., other or no use of antiandrogens) and different administration types, preferably in comparison to cisgender controls, are necessary to expand our knowledge on this subject."

We also agree with the second recommendation of the reviewer, and therefore we had performed analyses with hormone concentration as a covariate, however there was no correlation between the height of the hormone concentration and the change in outcome (p. 13):

"Lastly, higher hormone concentrations during treatment or different smoking status (at baseline) did not affect the results either."

Reviewer #2: This is an interesting report about the effect of hormone therapy on markers of inflammation and hemostasis in transgender persons. The findings were clear-cut; however, the manuscript has some problems.

We thank the reviewer for the time and effort reviewing our manuscript, and for the useful suggestions that helped us to improve our manuscript.

The keyword cardiovascular events should be revised.

This keyword was changed to cardiovascular risk.

Abstract. The result about the increment in platelet factor 4 (PF-4) in trans women is not mentioned.

This was added to the abstract (p. 2):

"After 12 months, in trans women, systemic and endothelial inflammatory markers decreased (hs-CRP -66%, (95% CI -76 ; -53), VCAM-1 -12%, (95% CI -16 ; -8)), while platelet activation markers increased (PF-4 +17%, (95% CI 4 ; 32), β-thromboglobulin +13%, (95% CI 2 ; 24))."

Introduction. It is not clear why the authors measure leptin and adiponectin, they should explain in a little more detail why they chose these adipokines as adipose tissue markers.

We thank the reviewer for this comment. We clarified our interest in adipose tissue markers in the Introduction section (p. 4):

"We selected a broad spectrum of markers associated with systemic, endothelial, or adipose tissue related inflammation".

We specifically selected leptin and adiponectin because they are known to be influenced by sex hormones (Auer et al., 2018, Elbers et al. 1997), and because they are associated with inflammation. We explained this more clearly in the following sentence (p. 6):

"Adipose tissue specific marker leptin is associated with obesity and is a predictor of myocardial infarction (15, 16), while adiponectin has anti-inflammatory effects, like increasing insulin sensitivity (16, 17)."

Materials and methods. The sentence in line 154 “Using EDTA or serum plasma samples” should be revised.

We now specified this sentence to enhance clarity (p. 7):

“The above-mentioned markers were measured at the Netherlands Organization for Applied Scientific Research (Leiden, The Netherlands), using serum (for PF-4 and β-thromboglobulin) and EDTA (for all other markers) plasma samples and miniaturized biomarker assays.”

Authors did not mention whether the dosage of the hormone therapy was adapted in some participants to reach adequate estradiol or testosterone concentrations as suggested by guidelines. The authors omit important characteristics of the study population as: blood pressure, weight, BMI, glucose levels, and lipid profile at baseline and after 3 and 12 months of hormone therapy.

We thank the reviewer for the valuable suggestions. We added blood pressure and BMI at 3 and 12 months to table 2 (p. 10):

Table 2. Hormone concentrations, body mass index and blood pressure at baseline, after 3 and 12 months of hormone therapy

	Trans women			Trans men		
	Visit (months)					
	0	3	12	0	3	12
Estradiol level (pmol/L)	101 (79-126)	246 (120-342)	258 (178-496)	273 (168-442)	184 (147-250)	158 (116-204)
Testosterone level (nmol/L)	18 (14-23)	0.6 (0.5-0.8)	0.7 (0.5-0.8)	1.3 (1.0-1.7)	27 (14-42)	20 (15-30)
BMI (kg/m^2)	23 (21-26)	23 (21-26)	24 (22-27)	23 (21-30)	23 (22-29)	23 (22-28)
BP (mmHg)						
Systolic	129 (123-138)	123 (117-134)	127 (117-135)	122 (115-126)	123 (117-130)	122 (116-129)
Diastolic	80 (73-88)	78 (73-83)	78 (73-84)	77 (72-81)	75 (70-82)	76 (72-82)
MAP	96 (91-101)	93 (88-98)	94 (89-100)	92 (87-97)	90 (86-98)	92 (87-96)

Data are presented as median (IQR). BMI, body mass index. BP, blood pressure. MAP, mean arterial pressure.

Also, we added glucose, total cholesterol and triglycerides to table 1 (p. 9):

Table 1. Baseline characteristics of the study population

	Trans women (n = 48)	Trans men (n = 47)
Age (years)	30 (24-39)	23 (20-26)
BMI (kg/m^2)	23 (21-26)	23 (21-30)
Ever smoked (%)	16 (33%)	20 (43%)
Blood pressure (mmHg)		
Systolic	129 (121-137)	122 (115-128)
Diastolic	80 (76-87)	77 (72-82)
MAP	96 (91-101)	92 (87-97)
Glucose (mmol/L)	5.5 (5.1-5.7)	5.1 (4.9-5.4)
Total cholesterol (mmol/L)	4.3 (3.7-5.1)	4.3 (3.9-4.9)
Triglycerides (mmol/L)	0.9 (0.7-1.3)	0.7 (0.5-1.0)

Data are presented as median (IQR) for continuous data, and number and % for categorical data. BMI, body mass index. MAP, mean arterial pressure. Participants with an underlying disease: diabetes mellitus (1), human immunodeficiency virus (2), pulmonal hypertension (1), ulcerative colitis (1).

There are no data concerning the liver and renal function for the safety evaluation.

We thank the reviewer for this suggestion. While we acknowledge that safety of the hormone therapy is important, evaluating the safety of hormone therapy was not the aim of this study. Further, also note that we included patients that received routine clinical transgender care in the Netherlands, i.e. there was no experimental manipulation induced by our study. To clarify this issue, we have added this to our Study design section (p. 5):

"Participants of the ENIGI-study are 18 years or older, diagnosed with gender dysphoria according to the revised fourth or fifth edition of the Diagnostic and Statistical Manual of Mental Disorders and receive routine clinical transgender care."

It is not specified if some participants had an underlying disease.

We now added this information to the caption of table 1, see above (p. 9).

In table 3 the authors should provide the p-values of all the comparisons.

P-values of all comparisons were added, see above (p. 11 and 12).

Figure 1 displays a reduction in IL-6, IL-8 and IL-22 in trans women at 12 months; these results are not mentioned in results section and are not discussed.

We now added the reduction in IL-6, IL-8 and IL-22 to the results (p. 13):

“After 12 months of transdermal estradiol plus CPA, systemic and endothelial inflammatory marker concentrations decreased (hs-CRP -66%, (95% CI -76 ; -53), IL-6 -28%, (95% CI -7 ; -44), IL-8 -15%, (95% CI -2 ; -17), IL-22 -34%, (95% CI -7 ; -44), VCAM-1 -12%, (95% CI -16 ; -8)).”