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# THE EFFECT OF SEX STEROIDS ON BODY FAT DISTRIBUTION IN TRANSGENDER PERSONS

**MARIEKE TEBBENS**





# **The effect of sex steroids on body fat distribution in transgender persons**

Marieke Tebbens

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PERSONS**

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# CHAPTER 1



## **General introduction**



Adipose tissue, or body fat, consists mostly of adipocytes, which main role is to store lipids that can be mobilized during energy deprivation. However, it has been long known that adipose tissue is a metabolic active endocrine organ as well <sup>1</sup>. The human body contains different types and depots of adipose tissue, with their own phenotype and function. Excessive amounts of adipose tissue can pose a risk to our health, with the effect of the enlarged fat depot depending on the tissue distribution, e.g., excessive amounts of visceral fat are associated with metabolic and cardiovascular risks <sup>2</sup>, while excessive amounts of bone marrow fat are associated with an increased risk of osteoporosis <sup>3</sup>. The regulation of adipose tissue distribution is multifactorial, of which a major group of regulators are sex steroids. Sex steroids, or sex hormones, are a group of steroid hormones that include estrogens, androgens and progestogens. The ratio in concentration between these hormones differs for women and men. Women typically have a high serum concentration of estrogens that fluctuates with the menstrual cycle, and a low serum concentration of androgens. In contrast, men have a stable, low serum concentration of estrogens and a high serum concentration of androgens. In transgender persons receiving gender affirming hormone treatment, the ratio of these sex steroids changes during their transition. This process has a large impact on adipose tissue distribution <sup>4</sup>. The overall objective of this thesis is to investigate the effects of sex steroids on body fat distribution in transgender persons. This general introduction provides background information on different types of adipose tissue, body fat distribution, the health risks associated with different types and distributions of adipose tissue, sex steroids, and gender dysphoria. Further, this general introduction will elaborate on the current knowledge on the effects of sex steroids on fat distribution and the current gaps in that knowledge. Finally, I will outline how this thesis can contribute to closing these gaps.

### **Adipose tissue**

The first published article on adipose tissue dates back to 1837, and warns of the risks of inflammation in this tissue <sup>5</sup>. At that time, it was thought that adipose tissue was a passive reservoir for energy storage. In 1987, the first hormone produced by adipose tissue was discovered in rodents <sup>6</sup> and in 1994, the discovery of leptin confirmed that adipose tissue was metabolically active <sup>7</sup>. We can distinguish different types of adipose tissue in the human body. The two major types of adipose tissue are white and brown adipose tissue. White adipocytes are cells that store energy in order to allow an interval between meals <sup>8</sup>. They contain one large lipid droplet and their nucleus is pressed to the periphery <sup>9</sup>. Brown adipocytes on the other hand, are multilocular adipocytes that burn lipids for heat <sup>10</sup>. White adipose tissue can be found in two main depots; subcutaneous adipose tissue (SAT)

and visceral adipose tissue (VAT). Subcutaneous adipose tissue is the adipose tissue beneath the skin, and visceral adipose tissue is the tissue surrounding the internal organs. Visceral fat in particular, is associated with insulin resistance and metabolic and cardiovascular risks <sup>11,12</sup>, however abdominal subcutaneous adipose tissue is also associated with adverse cardiometabolic risk factors, albeit less than abdominal visceral adipose tissue <sup>13</sup>. Another type of fat that is associated with insulin resistance and metabolic and cardiovascular risks, is liver fat <sup>14</sup>. Liver fat is not a fat depot, such as subcutaneous or visceral adipose tissue, but a type of ectopic fat. Ectopic fat deposition is the storage of triglycerides in non-adipose tissues such as liver, muscles, heart or pancreas <sup>15</sup>. If the accumulation of fat exceeds 5% of the total liver weight, it is called hepatic steatosis <sup>16</sup>. Not only is a fatty liver associated with insulin resistance and metabolic and cardiovascular risks, hepatic steatosis can also progress into the development of cirrhosis and hepatocellular carcinoma <sup>16</sup>. The final fat depot that we focus on in this thesis, is another type of fat that is associated with health risks, namely bone marrow fat. This is a distinct fat depot and differs in phenotype and function from white and brown fat <sup>17</sup>. Bone marrow adipocytes contain one large lipid droplet but are not grouped in lobules as in the other fat depots, but are scattered within the hematopoietic tissue <sup>18</sup>. Bone marrow adipose tissue is associated with low bone mass, osteoporosis and fractures <sup>3,19,20</sup>.

## Sex steroids

Sex steroids, or sex hormones, are a group of steroid hormones that can activate steroid hormone receptors to exert their effects. Sex steroids include estrogens, androgens and progestogens. The sex steroids I will focus on in this thesis are estrogens and androgens, because these hormones have been associated with body fat distribution <sup>21</sup>. The first mentioning of the existence of testosterone was in 1849, by Arnold Berthold. He discussed the transplantation of testes in six roosters <sup>22</sup>. He removed both testes and transplanted one of the testes back in the abdomen to discover that their sexual behavior had maintained intact. Since the nerves were no longer connected, this could only mean that the testes exert their effect through the blood on the body as a whole. Since Berthold was the first to experiment with, and describe an hormonal effect of one organ on distant organs, he has been named the 'Father of Endocrinology'. The name testosterone is coined from the combination of *testis* (Latin for testicle, which is regarded as a special application of *testis* "witness"), *sterol* (abstracted from cholesterol) and *one* (chemical suffix). Estrogens were discovered in 1923, by Edgar Allen and Edward Doisy <sup>23</sup>. These men extracted the hormone out of liquor isolated from hog folliculi and injected it in ovariectomised rats and mice. These rats and mice

showed an increased epithelium of the vagina and were therefore in full estrus. The word estrus derives from the Greek word οἰστρος, which translates to gadfly, but also frenzy/mad impulse. In 1926, Parkes and Bellerby used the term *estrin* to describe this hormone that could induce estrus in animals <sup>24</sup>.

Together, these hormones control reproduction by their involvement in sexual development, brain organization and sexual behavior <sup>25</sup>. However, sex steroids affect numerous other organs, such as the gastrointestinal tract, the immune system, bones, adipose tissue etc. Estrogens and androgens exert their effect by binding to steroid hormone receptors, including the nuclear receptors estrogen receptor  $\alpha$  and  $\beta$  and the androgen receptor <sup>26</sup>. When these hormones bind with their receptor, they form transcription factors, which bind to hormone response elements and either stimulate or inhibit gene expression. Estradiol also signals through the G protein-coupled estrogen receptor, a transmembrane receptor.

The most important androgens are testosterone, dehydroepiandrosterone (DHEA), androstenedione and dihydrotestosterone (DHT). DHEA is synthesized from cholesterol and is the precursor of testosterone and androstenedione. DHT is the most potent androgen and is converted from testosterone in the skin and reproductive tissue. The most common estrogens are estrone ( $E_1$ ), estradiol ( $E_2$ ) and estriol ( $E_3$ ). Estrone is synthesized by the aromatization of androstenedione and can be converted into estradiol. Estradiol is aromatized out of testosterone and can also be converted into estrone. Estriol is synthesized from  $16\alpha$ -hydroxyestrone which is synthesized from estrone and is only present during pregnancy. Estradiol is the most potent and abundant estrogen in premenopausal women. After the menopause estrone is more abundant since it is synthesized from androstenedione in adipose tissue <sup>27</sup>.

Both testosterone and estradiol are produced in men and women, however in a different ratio. In premenopausal women, estradiol is cyclically produced in the ovaries and serum concentrations vary monthly between 30 and 3000 pmol/L <sup>28</sup>. After the menopause this concentrations drops to <26 pmol/L <sup>28</sup>. Testosterone is biosynthesized from cholesterol in both the ovaries and adrenal glands and serum concentrations remain low, at a median of 1.2 nmol/L with an interquartile range (IQR) of 0.80 to 1.76 <sup>29</sup>. In men, testosterone is mainly produced in the testes and in lesser amount in the adrenal glands. Serum concentrations decrease in adult men from median 18.4 nmol/L (IQR 14.7 to 22.3) at age 19 to 39, to 16.5 nmol/L (IQR 12.5 to 20.9) at age 80 to 99 <sup>30</sup>. Estradiol in men is synthesized by conversion from testosterone by the enzyme aromatase. The serum concentration is around

84.0 ± 22.4 (SD) pmol/l and does not change with age <sup>31</sup>. The concentration of sex steroids is mostly linked to one's age and gender as described above, but can differ strongly for persons with gender dysphoria.

## Gender dysphoria

Gender identity refers to a person's sense of being male, female, or an alternative gender, and this may or may not correspond to the sex assigned at birth <sup>32</sup>. The term transgender refers to persons whose gender identity does not match their sex assigned at birth. In this thesis I will use the terms trans women: male assigned at birth, identifies as female; and trans man: female assigned at birth, identifies as male. Not all people identify themselves within the binary gender system <sup>33</sup>. Non-binary is an umbrella term for people identifying themselves outside this binary system, examples of non-binary gender identities are 'genderqueer', 'pangender' or 'agender'. Transgender persons might experience gender dysphoria, distress caused by the incongruence between their sex assigned at birth and experienced gender <sup>34</sup>. The prevalence of people with gender dysphoria who sought professional help has increased drastically in recent decades <sup>35</sup>. The prevalence over the years 1975 to 1992, in the Netherlands, was 1:11,900 for assigned males at birth and 1:30,400 for assigned females at birth <sup>36</sup>. The prevalence in 2015 in the Netherlands was 1:3,800 for assigned males at birth and 1:5,200 for assigned females at birth <sup>35</sup>. The median age of adults at presentation has decreased in recent decades from around 35 years in 2005 to 25 years in 2015 in trans women and from 30 year in 2005 to 22 years in 2015 in trans men <sup>35</sup>. People with gender dysphoria might seek medical help. This medical help can consist of psychological diagnosis and treatment, hormone treatment and/or surgery. Hormone treatment in trans women consists of antiandrogens and/or estradiol. The antiandrogens described in the gender clinic of the Amsterdam UMC, location Vrije Universiteit are cyproterone acetate, an antiandrogen with progestagenic properties, and decapeptyl, a gonadotrophin releasing hormone (GnRH) analogue. In our gender clinic, estradiol is given as 17β-estradiol in either tablet, patches or gel form. Hormone treatment in trans men consists of testosterone and is given as either gel or injections. All gender affirming hormone treatment protocols described in this thesis are in line with the Endocrine Society guideline for the treatment of persons with gender dysphoria <sup>37</sup>.

## Effect of sex steroids on body fat distribution

Body fat distribution differs between women and men. In 1947, Vague first mentioned the gynoid fat distribution (hip and thigh) in women and the android fat distribution (upper body and trunk) in men <sup>38</sup>. Women have more body fat than

men. This is already present before puberty, but the difference increases drastically during puberty <sup>39</sup>. Women have more subcutaneous adipose tissue, while men are more susceptible to visceral adipose tissue and liver fat accumulation <sup>40,41</sup>. In premenopausal women and men, the amount of liver fat, visceral fat, and the visceral/subcutaneous fat ratio increase gradually with aging <sup>41,42</sup>. However, around menopause, the amount of liver fat and visceral fat, as well as the visceral/subcutaneous fat ratio increase rapidly, resulting in similar amounts of liver fat, visceral fat and visceral/subcutaneous fat ratio in women compared to men <sup>43-45</sup>. The final fat depot of interest for this thesis, bone marrow fat, shows a similar pattern. Men have more bone marrow fat than premenopausal women, and throughout life, this bone marrow fat depot is gradually increasing <sup>46-48</sup>. However, around menopause, the quantity of bone marrow fat steeply increases in women, resulting in approximately 10% more bone marrow fat than men with a similar age <sup>46,48</sup>. The fact that body fat distribution changes mostly during puberty and menopause suggests a major role for sex steroids in the regulation of body fat distribution. The major role of sex steroids in the regulation of body fat distribution has been studied in different populations with varying ratios of estrogens to androgens. These populations include (but are not limited to) postmenopausal women with or without hormone replacement treatment, hypogonadal men and transgender persons.

As mentioned above, visceral fat, liver fat and bone marrow fat increase during the menopause. When giving estradiol as hormone replacement treatment, visceral fat, liver fat and bone marrow fat decrease <sup>49-51</sup>. Since testosterone concentrations remain low in postmenopausal women, the major sex steroid regulating body fat distribution seems to be estradiol. In men, we see a similar pattern, where visceral fat and liver fat increase if sex steroids are being suppressed <sup>52</sup>. Gonadal suppression in men has not been studied in the context of bone marrow fat, but Mistry et al., did find that lower total estradiol and testosterone concentrations in older men are associated with more bone marrow fat <sup>53</sup>. However, as mentioned above, testosterone is converted into estradiol in adipose tissue by aromatase. Therefore, it is difficult to study the effects of testosterone and estradiol separately in men.

A complicating factor in studying the effect of sex steroids on body fat distribution, is the correlation between genetic factors and sex steroids. Cis women (assigned female at birth, identify as female) mostly have XX sex chromosomes and cis men (assigned male at birth, identify as male) mostly have XY sex chromosomes. The sex-determining region Y (SRY) gene on the Y chromosome initiates testes

differentiation and therefore androgen production, while the absence of the SRY gene initiates ovaries differentiation and therefore estrogen production <sup>54</sup>. XX chromosomes are therefore correlated with high estradiol concentrations (until menopause) and low testosterone concentrations and XY chromosomes are correlated with high testosterone concentrations and medium estradiol concentrations. The metabolic effect of sex chromosomes, independently of sex steroids, has been examined in animals with the four core genotypes mouse model. This mouse model separates the SRY gene from the Y chromosome, making it possible to have XX mice with either male or female gonads, and XY mice with either male or female gonads <sup>55</sup>. This model showed that both mice with male and female gonads with two X chromosomes had higher body weight and more body fat than mice with one X and one Y chromosome <sup>56</sup>. This animal model suggests that genetic factors do affect body fat distribution and therefore, when studying the effect of sex steroids on body fat distribution, we want to separate the effects of genetics and the effects of sex steroids. In humans it is more difficult to study the effects of sex chromosomes separately from sex steroids. Persons with Klinefelter syndrome (XXY) display an increased risk of visceral obesity, however, they also have low concentrations of testosterone <sup>57</sup>.

Separating the effects of sex chromosomes and sex steroids on body fat distribution can be done in transgender persons undergoing hormone treatment who keep their sex chromosomes, but change in sex steroid ratio. Previous studies in transgender persons have shown that trans women using hormone treatment increase in body fat, mainly in the gynoid region and slightly in the android region <sup>58</sup>. Trans men using hormone treatment, decrease in body fat in the gynoid region and remain stable in body fat in the android region. Elbers et al., zoomed in on the different fat depots and assessed visceral and subcutaneous fat in 20 trans women and 17 trans men by MRI before and after one year of hormone treatment <sup>59</sup>. In trans women, they observed an increase in subcutaneous fat and a small increase in visceral fat. In trans men they observed a reduction in subcutaneous fat and a slight increase in visceral fat. In contrast, Klaver et al., estimated visceral fat by dual-energy X-ray absorptiometry (DXA) in a much larger cohort and found no change in visceral fat in trans women and trans men <sup>60</sup>. The difference in imaging method may have contributed to this difference since DXA is not validated in transgender persons starting hormone treatment and not validated for the longitudinal assessment of visceral fat. Both studies followed the standard treatment protocol and could therefore not separate the effects of lowering testosterone and increasing estradiol in trans women and increasing testosterone and lowering estradiol in trans women. Liver fat and bone marrow fat

have not yet been assessed in transgender persons using hormone treatment, but this is important to do because liver fat and bone marrow fat seem to be regulated by sex steroids (among other things) and are associated with major health risk such as cardiovascular disease and osteoporosis, respectively.

## **Aims and outline of this thesis**

The overall aim of this thesis is to investigate the effects of sex steroids on body fat distribution. I aim to assess the effect of sex steroids on bone marrow fat and liver fat, for the first time in transgender persons. Further, I aim to separate the effects of estradiol and testosterone on body fat distribution. In **chapter 2** we start by exploring which estrogens exert their effect on body fat distribution. We separately assess the effect of estradiol ( $E_2$ ) and its metabolite estrone ( $E_1$ ) on body fat distribution. Based on previous *in vitro* studies, we hypothesize that estrone has no effect on body fat distribution. In **chapter 3** we determine the effects of estradiol and testosterone on liver fat content, visceral fat and subcutaneous fat, measured by magnetic resonance imaging (MRI). Trans women were treated with GnRH analogues, followed by GnRH analogues combined with estradiol, while trans men were split into two groups; GnRH analogues, testosterone and an aromatase inhibitor; or GnRH analogues and testosterone. This treatment protocol provides the opportunity to separate the effects of testosterone and estradiol. We hypothesize that estradiol is the main regulating sex steroid of liver fat content, visceral fat and subcutaneous fat, in women and men. In **chapter 4**, we compare the gold standard for visceral fat measurement, MRI, with a more accessible method for visceral fat estimation, DXA. Although previous studies with DXA have been performed in transgender persons, visceral fat estimation by DXA has not been validated in transgender persons and has not been validated for longitudinal assessment of visceral fat. In **chapter 5** we assess the effects of estradiol and testosterone on the amount of bone marrow fat in humans. Previous studies in postmenopausal women have shown that estradiol is the major sex steroid regulating bone marrow fat in women, and in this chapter we examine whether estradiol is the main sex steroid regulating the amount of bone marrow fat in men as well. Since estradiol rapidly decreases the amount of bone marrow fat in women, we hypothesize that estradiol has a direct effect on bone marrow adipocytes. Therefore in **chapter 6** we assess the effect of estradiol on adipocyte number, lipid droplet area and adipokine expression. Finally, we move on to subcutaneous fat distribution in the face. In **chapter 7** we assess the effects of estradiol and testosterone on facial changes in transgender persons. We use 3D imaging to compare the surface of the face of trans women and trans men before and after one year of hormone treatment. **Chapter 8** contains the discussion, in

which I will summarize the results of the studies in this thesis, discuss strengths and limitations, and give recommendations for future research.

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# CHAPTER 2



# **The role of estrone in feminizing hormone treatment**

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## ABSTRACT

**Context:** In trans women, hormone treatment induces feminization, however the degree of feminization varies from person to person. A possible contributing factor could be estrone, a weak estrogen that interferes with the estrogen receptor.

**Objective:** To assess whether estrone is involved in feminization induced by hormone treatment.

**Design:** Prospective cohort study, with one year follow-up.

**Setting:** Gender identity clinic.

**Participants:** 212 adult trans women, starting hormone treatment between July 2017 and December 2019, median age 25 years.

**Intervention:** Gender affirming hormone treatment.

**Main outcome measures:** Change in fat percentage and breast development.

**Results:** After 12 months of hormone treatment, estrone concentration was 187pmol/L (95%CI 153 – 220) in transdermal and 1516pmol/L (95%CI 1284 – 1748) in oral estradiol users. Fat percentage increased by 1.2% (IQR 0.3 – 4.8) in transdermal and 4.6% (IQR 2.5 – 5.9) in oral estradiol users. This was not associated with estrone concentrations in transdermal(+4.4% (95%CI -4.0 – 13) per 100pmol/L increase in estrone concentration) nor in oral estradiol users (-0.7% (95%CI -1.7 – 0.3)). Breast volume increased by 69ml (IQR 58 – 134) in transdermal and 62ml (IQR 32 – 95) in oral estradiol users. This was not associated with estrone concentrations in transdermal (+ 14% (95%CI -49 – 156) per 100pmol/L increase in estrone concentration) nor oral estradiol users (+ 11% (95%CI -14 – 43)).

**Conclusions:** Change in fat percentage and breast development in trans women were not associated with estrone concentrations nor with administration route. Therefore measurement of estrone concentrations does not have a place in the monitoring of feminization in trans women.

## INTRODUCTION

Feminizing hormone treatment is an important part of the transition process of trans women. Combined treatment with estradiol and an antiandrogen induces female secondary sex characteristics. Breast development and gynoid fat deposition are among the most anticipated changes <sup>1</sup>. However, the magnitude of these changes varies among trans persons and in some cases the changes are limited, which might cause severe disappointment <sup>2</sup>. Previous studies have focused on differences in administration route of estradiol, estradiol concentrations, types of antiandrogens and lifestyle. Albeit, none of these factors seems to explain the difference in amount of feminization <sup>3,4</sup>.

Another possible factor in the amount of feminization, is estrone, an estradiol metabolite and estrogen receptor agonist. Both oral and transdermal administered estradiol are partly converted to estrone <sup>5</sup>. However, due to the first-pass effect, oral estradiol causes much higher estrone concentrations than transdermal estradiol, both in the peripheral bloodstream and the portal vein <sup>6-9</sup>. Estrone is a weak estrogen agonist, with a relative binding affinity of 4 – 10% for estrogen receptor (ER)  $\alpha$  and 2 – 3.5% for ER $\beta$ , compared to estradiol <sup>10,11</sup>. Therefore, estrone is mainly considered to be a precursor pool for the more potent estradiol <sup>5,12,13</sup>. On the other hand, a previous in vitro study suggested that a high estrone/estradiol ratio can interfere with the cooperative binding capacity of estradiol and thereby act as an estrogen antagonist <sup>14</sup>. This has led to speculations about a possible antagonistic effect of estrone on internet fora and in the transgender community.

Clinical data about the role of estrone in feminizing hormone treatment is lacking. Therefore, in this study, the main aim is to assess the association between estrone concentrations and feminization in trans women. We will assess feminization by change in body fat and breast development, since these clinical features are known to change under the influence of estradiol as part of feminizing hormone treatment and are among the most anticipated changes <sup>1,4,15</sup>.

## METHODS

### Subjects and study design

We performed a prospective cohort study, which was part of the European Network for the Investigation of Gender Incongruence (ENIGI) <sup>16</sup>. All subjects were 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 planned

to start with hormone treatment. Exclusion criteria were previous or current use of hormone treatment, diversion from the local treatment protocol and inability to understand the patient information and informed consent. A more detailed description of the study protocol is described elsewhere <sup>16</sup>. The ENIGI study was approved by the local ethics committee of Ghent University Hospital, Belgium and the participation of Amsterdam UMC in this study was approved by the local ethics committee of the Amsterdam UMC, location VUmc. The ENIGI partners from the other participating centers have given their approval for publishing this manuscript.

Contrary to the measurement of serum estradiol concentrations, measurement of serum estrone concentrations is not part of the routine follow-up of hormone treatment in transwomen <sup>17</sup>. With the switch to tandem-MS based measurements of estradiol in the Amsterdam UMC, estrone concentrations could be easily measured in the same run together with estradiol <sup>18</sup>. Therefore, in this study, we analyzed all trans women included in the ENIGI cohort in Amsterdam from the introduction of tandem-MS based measurements of estradiol and estrone in our laboratory, in July 2017 until the end of our study in December 2019. Since estrone concentrations were only measured in Amsterdam, in this study, only trans women included in the Amsterdam cohort were analyzed.

The association between estrone concentrations and change in fat percentage or breast development was assessed at 12 months. The association between estrone concentrations and administration route or SHBG concentrations was assessed at 3 months since estrone, estradiol and SHBG concentrations were stable between 3 and 12 months and no changes in administration form or dosage occurred before the 3 months measurement.

### **Treatment protocol**

Trans women were treated with estradiol, either oral (estradiol valerate 2 to 6 mg daily) or transdermal (estradiol patches 50 to 100 mcg/24 hours twice weekly), mostly in combination with an antiandrogen, either cyproterone acetate (10 to 50 mg daily), triptorelin (3,75 mg once every four weeks) or spironolactone (100 to 150 mg daily). The dosage of either estradiol or antiandrogens could be adjusted to achieve adequate serum estradiol (200-600 pmol/L) and testosterone concentrations (<2.5 nmol/L) during follow-up.

### **Data collection**

Bioelectrical impedance analysis (BIA) measurements was performed using a Tanita MC-780 MA (Tanita Europe B.V., Amsterdam, The Netherlands) at baseline,

after 3 months and after 12 months of hormone treatment. This device measures whole body and segmental bioelectrical impedance using eight electrodes. Participants were measured in underwear and barefoot. The machine output of fat percentages was used. A more detailed description of the BIA protocol is described elsewhere <sup>3</sup>.

Breast volume was measured with 3D imaging, using the VECTRA XT scanner (Canfield Inc.). For this study, measurements at baseline and 12 months were included for analyses. Breast volume was measured by manual placement of landmarks and the assessment software (version 5.7.1.) of the VECTRA XT. A more detailed description of this protocol can be found elsewhere <sup>19</sup>.

### Hormone analyses

Blood samples were collected at baseline, after 3 months and after 12 months of hormone treatment. Estradiol and estrone concentrations were measured in serum using an in house developed LC-MS/MS method in our endocrine laboratory <sup>18</sup>. In short, stable, isotopically labelled internal standards (13C3-labelled estradiol; obtained from IsoSciences, King of Prussia, PA, USA and 13C3-labelled estrone; obtained from Sigma Aldrich, Saint Louis, MO, USA) were added to every specimen (sample, control, calibrator). Estrogens were extracted from 150 µL of sample using a 4:1 (volume/volume) mixture of hexane and ether. The supernatant was subsequently dried under a stream of nitrogen and reconstituted in 75 µL of a 1:1 (volume/volume) mixture of methanol and water. Analysis of the samples was performed using tandem mass spectrometry with a 2D Xevo TQ-S mass spectrometer (Waters Corp., Milford, MA) with electrospray in negative mode using an injection volume of 50 µL. Separation was achieved on two analytical columns, a C4 Acquity UPLC column (Waters Corp), 2.1 x 50 mm, 1.7 µm particle size, and a Phenomenex Kinetex Biphenyl, 2.1 x 50 mm – 1.7 µm column (Torrance, CA, USA), with a methanol:water gradient elution at a flow rate of 0.6 and 0.4 mL/minute (water containing 100 µM NH<sub>4</sub>F). Total analysis time was 8.2 minutes. The monitored transitions were m/z 271 to 145 and 183 and m/z 274 to 148 and 186 for estradiol and its internal standard and m/z 269 to 143 and 145 and m/z 272 to 146 and 148 for estrone and its internal standard, respectively.

The LC-MS/MS method has a lower limit of quantification of 10 pmol/L for estradiol and 10 pmol/L for estrone. For estradiol, the intra assay variation was <5% between 20 and 1700 pmol/L and the inter assay variation was 9% at 21 pmol/L and <7% at 179 and 760 pmol/L. For estrone, the intra assay variation was <10% over the whole concentration range and the inter assay variation was 10% at 30

pmol/L and <6% at 220 and 640 pmol/L. All samples were run in duplicate. SHBG was measured using an automated immunoassay (Architect, Abbott Diagnostics, Chicago) with an inter assay CV of <8% over the whole concentration range.

### **Statistical analysis**

Baseline characteristics are presented as mean ( $\pm$  standard deviation), or as median (interquartile range) when not normally distributed. The use of tobacco and type of hormone treatment at 3 or 12 months are presented as number and percentage of users. All outcome variables were log-transformed before analyses, back-transformed to ratios, and converted to percentage change. The independent variables estrone and estradiol concentrations are presented as percentage change per 100 pmol/L increase in estrone or estradiol. This was calculated by multiplying the regression coefficients and 95% CIs by 100 before back-transformation.

Linear regressions were performed to analyze the associations between estrone concentrations and change in fat percentage or breast development at 12 months. Mean estrone and estradiol concentrations were calculated by taking the mean of the serum concentrations at 3 and 12 months. If only one measurement was performed, the only available concentration was used in the analyses (13 for estrone and 15 for estradiol). Other linear regressions were performed to analyze the associations between estrone concentrations and SHBG concentrations at 3 months. Finally, linear regressions were performed to analyze the effect of either transdermal or oral estradiol on the estrone/estradiol ratio, SHBG concentrations, change in fat percentage and breast development.

The regression models for change in fat percentage, breast development and SHBG were stratified by route of estradiol administration at the time of the measurement. Possible confounders were added to the model and considered a confounder if the regression coefficient increased or decreased by 10% or more.

STATA Statistical Software, version 15.1 (Statacorp, College Station, Texas, USA) was used to perform analyses and create graphs.

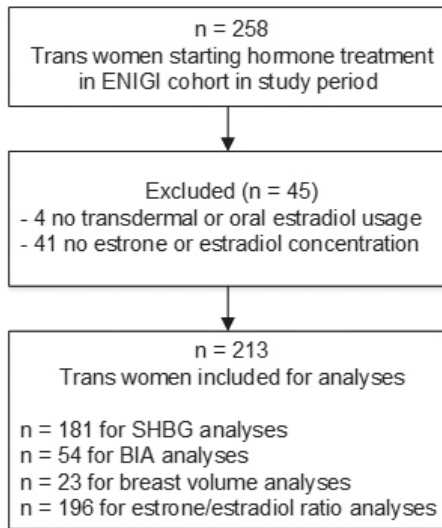
## RESULTS

Between July 2017 and December 2019, 258 trans women were included in the ENIGI study. After applying our inclusion criteria, 212 trans women were eligible for analyses (Fig. 1). The baseline characteristics are presented separately for trans women who were prescribed transdermal estradiol and oral estradiol (Table 1). The median age at baseline was higher for transdermal estradiol users than for oral estradiol users. The mean estradiol and estrone concentrations were similar at 3 and 12 months of hormone treatment. In transdermal estradiol users, the estradiol concentration was 298 pmol/L (95% CI 240 to 355) at 3 months and 360 pmol/L (95% CI 276 to 443) after 12 months. The estrone concentration was 200 pmol/L (95% CI 159 to 240) after 3 months and 187 pmol/L (95% CI 153 to 220) after 12 months. In oral estradiol users, the estradiol concentration was 239 pmol/L (95% CI 215 to 263) at 3 months and 241 pmol/L (95% CI 210 to 271) after 12 months. The estrone concentration was 1443 pmol/L (95% CI 1274 to 1612) after 3 months and 1516 pmol/L (95% CI 1284 to 1748) after 12 months. This led to a mean estrone/estradiol ratio of  $0.96 \pm 1.06$  in transdermal estradiol users and of  $6.04 \pm 1.84$  in oral estradiol users (see Fig. 2).

**Table 1.** Baseline characteristics

|                           | Transdermal (n=88) | Oral (n=124)   |
|---------------------------|--------------------|----------------|
| Age (years)               | 43 [27 to 54]      | 22 [20 to 26]  |
| BMI (kg/m <sup>2</sup> )  | 24.3 $\pm$ 3.6     | 22.9 $\pm$ 4.7 |
| Current smoker, n (%) yes | 19 (22)            | 25 (21)        |
| Estrone (pmol/L)          | 119 $\pm$ 52       | 106 $\pm$ 32   |
| Estradiol (pmol/L)        | 82 $\pm$ 28        | 77 $\pm$ 22    |
| Estrone/estradiol ratio   | 1.5 $\pm$ 0.4      | 1.4 $\pm$ 0.3  |
| Testosterone (nmol/L)     | 19 $\pm$ 7         | 18 $\pm$ 7     |
| Antiandrogens             |                    |                |
| CPA, n (%)                | 72 (83)            | 113 (92)       |
| GnRH analogues, n (%)     | -                  | 1 (1)          |
| Spironolactone, n (%)     | -                  | 1 (1)          |
| None, n (%)               | 15 (17)            | 8 (6)          |

Baseline characteristics, displayed per prescribed estradiol administration route at baseline. Abbreviations: BMI = body mass index, CPA = cyproterone acetate. Data are presented as mean  $\pm$  SD, median [inter quartile range], or number (percentage).



**Figure 1.** Study flowchart.

The mean SHBG concentrations after 3 months were 37 nmol/L (95% CI 33 to 40) in transdermal estradiol users and 44.67 nmol/L (95% CI 40 to 49) in oral estradiol users. After adjustment for age, BMI at baseline and estradiol concentrations, SHBG concentrations at 3 months were 45% (95% CI 23 to 71) higher in trans women using oral estradiol compared to transdermal estradiol. In oral estradiol users, SHBG concentrations increased by 2.7% (95% CI 1.9 to 3.6) for every 100 pmol/L increase in estrone concentration (Table 2). After adjustment for estradiol concentrations and BMI at baseline this increase was 1.9% (95% CI 0.5 to 3.2) for every 100 pmol/L increase in estrone concentration. In transdermal estradiol users, SHBG concentrations at 3 months were not associated with estrone concentrations.

**Table 2.** Association between estrone or estradiol and SHBG concentrations at 3 months of hormone treatment

|                                        | Transdermal estradiol users | Oral estradiol users    |
|----------------------------------------|-----------------------------|-------------------------|
| SHBG concentration                     |                             |                         |
| Estrone (per 100 pmol/L)               | 2.45% (-3.99 to 9.32)       | 2.74% (1.89 to 3.59)    |
| Estradiol (per 100 pmol/L)             | 4.54% (-0.01 to 9.30)       | 18.46% (11.36 to 26.00) |
| Estrone adjusted for estradiol         | -1.65% (-8.95 to 6.25)      | 2.33% (0.84 to 3.82)    |
| Estrone adjusted for estradiol and BMI | -1.37 (-8.35 to 6.14)       | 1.87% (0.51 to 3.24)    |

Regression analyses, crude analyses for estrone and estradiol and adjusted analyses. Data in percentage change with (95% CI). Estrone and estradiol in 100 pmol/L, SHBG in nmol/L and BMI in kg/m<sup>2</sup>.

### Estrone and change in fat percentage

After 12 months, the increase in fat percentage was 1.2% (IQR 0.3 – 4.8) in trans women using transdermal estradiol and 4.6% (IQR 2.5 – 5.9) in trans women using oral estradiol. This change in fat percentage over 12 months in trans women was not associated with estrone concentrations in transdermal (+4.4% (95% CI -4.0 to 13) for every 100 pmol/L increase in average estrone concentration) and oral estradiol users (-0.7% (95% CI -1.7 to 0.3)). This did not change after adjustment for estradiol concentrations and other possible confounders, -9.4% (95% CI -22 to 4.8) and -0.8% (95% CI -2.2 to 0.7), for transdermal and oral estradiol users, respectively (Table 3). Change in fat percentage, adjusted for age and BMI at baseline, did not differ between oral and transdermal estradiol users, 1.1% (95% CI -13 to 18).

**Table 3.** Association between estrone concentrations and change in fat percentage and breast growth after 12 months of hormone treatment

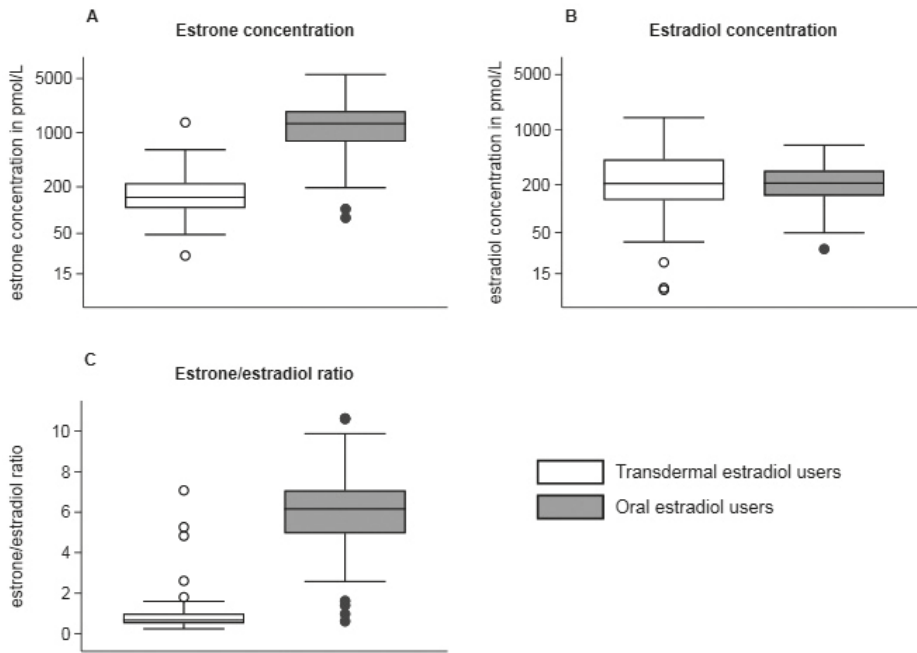
|                                  | Transdermal estradiol users | Oral estradiol users       |
|----------------------------------|-----------------------------|----------------------------|
| Change in fat percentage         |                             |                            |
| Estrone (100 pmol/L)             | 4.38% (-3.96 to 13.44)      | -0.69% (-1.70 to 0.33)     |
| Estradiol (100 pmol/L)           | 3.00% (-0.38 to 6.50)       | -3.74% (-11.34 to 4.50)    |
| Estrone adjusted for estradiol   | -4.55% (-17.21 to 10.05)    | -0.71% (-2.16 to 0.77)     |
| Estrone adjusted for confounders | -9.40% (-21.69 to 4.82)     | -0.80% (-2.22 to 0.65)     |
| Breast growth                    |                             |                            |
| Estrone (pmol/L)                 | 14.47% (-48.73 to 155.57)   | 10.92% (-14.00 to 43.03)   |
| Estradiol (pmol/L)               | 14.10% (-20.06 to 62.85)    | -16.67% (-85.11 to 366.38) |
| Estrone adjusted for estradiol   | 8.50% (-54.80 to 160.41)    | 62.51% (5.45 to 150.44)    |
| Estrone adjusted confounders     | -3.74% (-79.69 to 356.21)   | 49.3% (-7.27 to 140.41)    |

Regression analyses, crude analyses for estrone and estradiol and adjusted analyses. Data in percentage change with (95% CI). Estrone and estradiol in 100 pmol/L, age in years and BMI in kg/m<sup>2</sup>. Change in fat percentage and breast growth were measured over 12 months of hormone treatment, estrone and estradiol concentrations are average concentrations over 3 and 12 months. Confounders change in fat percentage: estradiol concentration, testosterone concentration, age, BMI at baseline. Confounders breast growth: estradiol concentration, age, BMI at baseline.

### Estrone and breast development

After 12 months, the breast development in trans women was 69 ml (IQR 58 – 134) in trans women using transdermal estradiol and 62 ml (IQR 32 – 95) in trans women using oral estradiol. Breast development in trans women was not associated with estrone concentrations in trans women using transdermal (+ 14% (95% CI -49 to 156) for every 100 pmol/L increase in average estrone concentration) or oral estradiol (+ 11% (95% CI -14 to 43)). Adjustment for estradiol,

age and BMI did not change this (Table 3.). Breast development, adjusted for age, BMI at baseline, estradiol and testosterone concentrations, did not differ between oral and transdermal estradiol users, -33% (95% CI -85 to 209).



**Figure 2.** The serum (A) estrone and (B) estradiol concentrations and (C) estrone/estradiol ratio in trans women after 3 months of hormone therapy. Panel A and B are in logarithmic scale.

## DISCUSSION

The main result of this study is that we found no relationship between estrone concentrations and change in body fat or breast development after 12 months of hormone treatment. Further, we found no association between administration route and these feminization markers, despite the fact that oral estradiol was clearly associated with much higher estrone levels compared with transdermal estradiol.

### Effect of administration route on estrone

In line with previous research in trans and postmenopausal women, we found a higher estrone/estradiol ratio in trans women using oral estradiol compared to transdermal estradiol<sup>6-8,20,21</sup>. Orally administered estradiol is already partly metabolized into estrone in the intestines. The ileum produces more estrone

after incubation with estradiol than any other tissue except for the placenta <sup>22</sup>. In contrast, transdermal administered estradiol bypasses the gastrointestinal mucosa and the hepatic system and is slowly metabolized into estrone <sup>7</sup>.

## SHBG

In addition to the clinical outcomes breast development and body fat, we assessed the relation between estrone and SHBG, a binding globulin that is produced in the liver and found to be the most sensitive estrogenic marker in postmenopausal women <sup>23,24</sup>. We found a positive association between estrone and SHBG concentrations in oral estradiol users, in line with a previous in vivo study in postmenopausal women <sup>25</sup>. Further, the positive association between estradiol and SHBG concentrations is also in line with previous in vivo studies in postmenopausal women <sup>20,25-28</sup>. This finding is further supported by previous in vitro studies confirming that estrogens increase the production of SHBG in hepatocytes <sup>29,30</sup>. However, no association was found between estrone and SHBG concentrations in transdermal estradiol users. This could suggest that both the increase in estrone and SHBG concentrations are a result of the oral administration route. Orally administered estradiol leads to high estradiol concentrations in the portal vein and consequently the hepatic microvasculature which stimulates both SHBG production and conversion of estradiol to estrone in the liver <sup>26,31,32</sup>. In addition, around 90% of estrone excreted in the bile is reabsorbed in the intestines, stimulating protein synthesis in the liver even more <sup>33</sup>. Finally, cyproterone acetate is known to decrease the secretion of SHBG in vitro <sup>30</sup>, however in our study, adjustment for cyproterone acetate did not change the association between estrone and SHBG concentrations and our SHBG concentrations match those of postmenopausal women using only estradiol and not cyproterone acetate in other studies <sup>25,26</sup>.

## Change in body fat and breast development

We found no association between estrone concentrations and change in fat percentage or breast development after 12 months of hormone treatment. Estradiol concentrations were also not associated with these outcomes. This is in line with previous studies in trans women, which found no association between estradiol concentrations or administration route and change in fat percentage or breast development <sup>3,19,34</sup>. Our results are in contrast with other studies suggesting that high estrone levels can act as an estrogen antagonist <sup>14,35-37</sup>. An in vitro study found that a high excess of estrone interferes with the positive cooperative binding of estradiol and its receptor, by inducing a change in the estradiol-receptor complex that favors the non-active confirmation of this receptor <sup>14</sup>. This non-active

conformation results in lower affinity for estradiol and DNA <sup>38</sup>. This in vitro study is reinforced by two clinical studies, showing that lowering the upper estrone limit strengthened the association between estradiol and clinical outcome; increase in mammographic density <sup>36</sup> and collagen turnover in urogenital tissue <sup>37</sup>. In our study, lowering the upper limit of estrone did not influence the association between estradiol concentrations and our outcomes (data not shown). This study demonstrates that higher estrone concentrations or higher estrone/estradiol ratios are not associated with antagonistic effect on feminization in trans women. Therefore, estrone has no role in monitoring feminization in trans women using hormone treatment.

### **Estrone and long-term effects**

In this study, we found no association between estrone concentrations and feminization markers in trans women. However, in previous studies in postmenopausal women, estrone concentrations have been associated with an increased risk of diseases such as thrombosis and breast cancer <sup>9,39,40</sup>. Bagot et al. not only found significantly increased thrombin generation in women using oral estradiol, but furthermore, that estrone concentrations in these women were correlated with peak thrombin generation <sup>9</sup>. Other previous studies in postmenopausal women, found that high estrone concentrations were associated with a higher risk of ER positive breast cancer <sup>39,40</sup>. Therefore, estrone concentrations might be associated with increased risks in trans women and future research is needed to assess this.

### **Strengths and limitations**

As far as we know, this is the first study to assess the role of estrone concentrations in feminization of the body in trans women. Due to collaborations within the ENIGI cohort, we were able to assess two markers of feminization, making our conclusions stronger. This study has some limitations as well. Due to our local protocol, almost all women were treated with cyproterone acetate, which makes it impossible to exclude effects of this antiandrogen on our primary outcome parameters. In addition, in accordance with our local protocol, we advised trans women above the age of 40 years to choose transdermal estradiol, while younger transwomen were able to choose either transdermal or oral estradiol. This has led to a significant difference in age between users of both administrations forms. Although we have adjusted our models for age, this might still have an effect on the outcomes.

## **Conclusion**

Estrone concentrations are not associated with breast development or change in body fat in trans women receiving either oral or transdermal estradiol. Moreover, no difference in clinical outcomes was observed between oral and transdermal administration of estradiol, despite the fact that oral estradiol was clearly associated with much higher estrone levels compared with transdermal estradiol. This suggests that estrone has neither an agonistic nor an antagonistic effect on feminization in trans women. Therefore, this study shows that estrone has no role in the monitoring of feminization in trans women.

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## Chapter 2

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# CHAPTER 4



**ABSTRACT**

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## INTRODUCTION

It has been long known that excess visceral fat is associated with hypertension,

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once a day, n=3). The treatment targets were 200-600 pmol/L for serum estradiol

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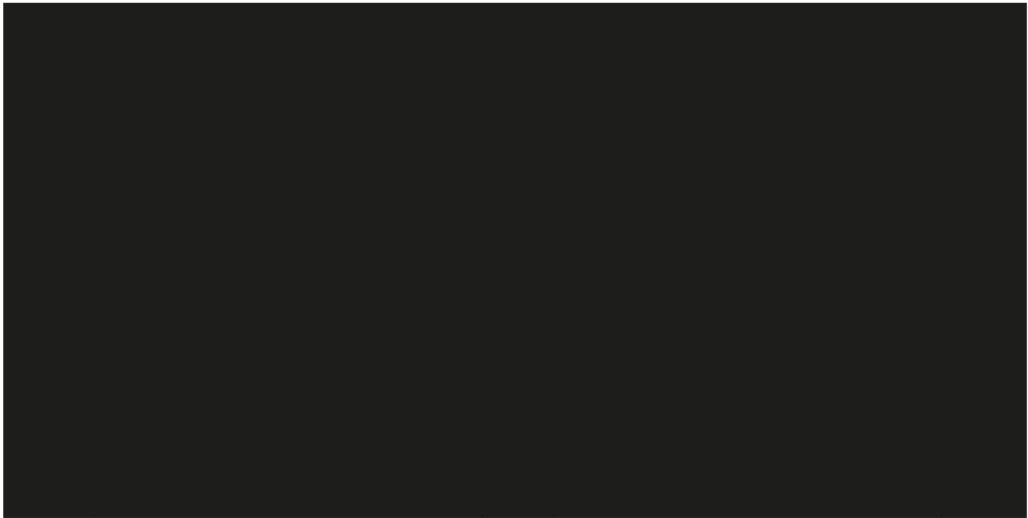
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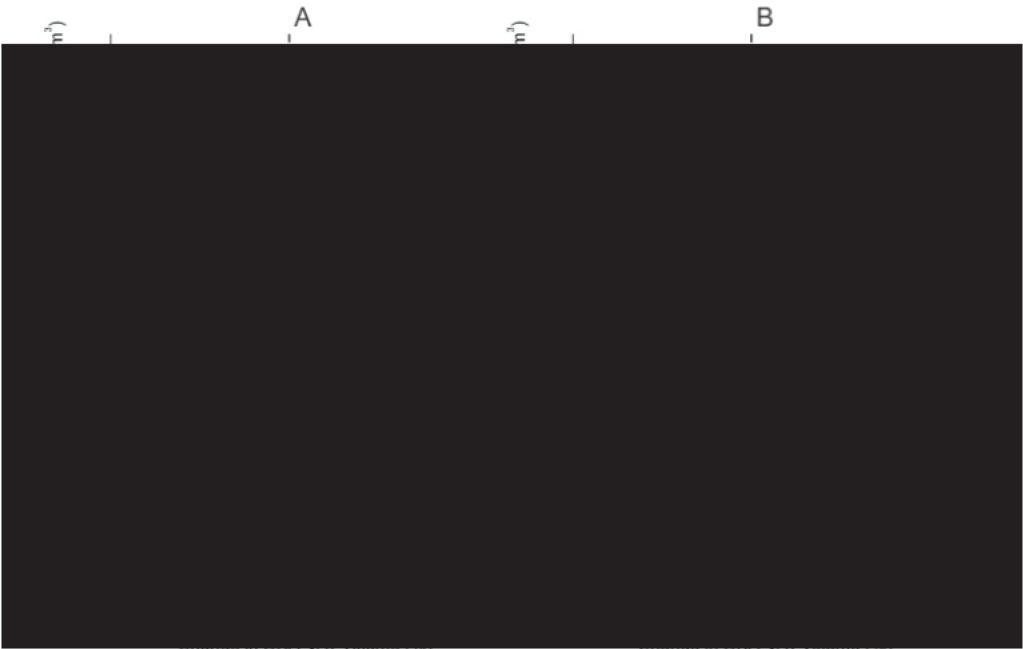
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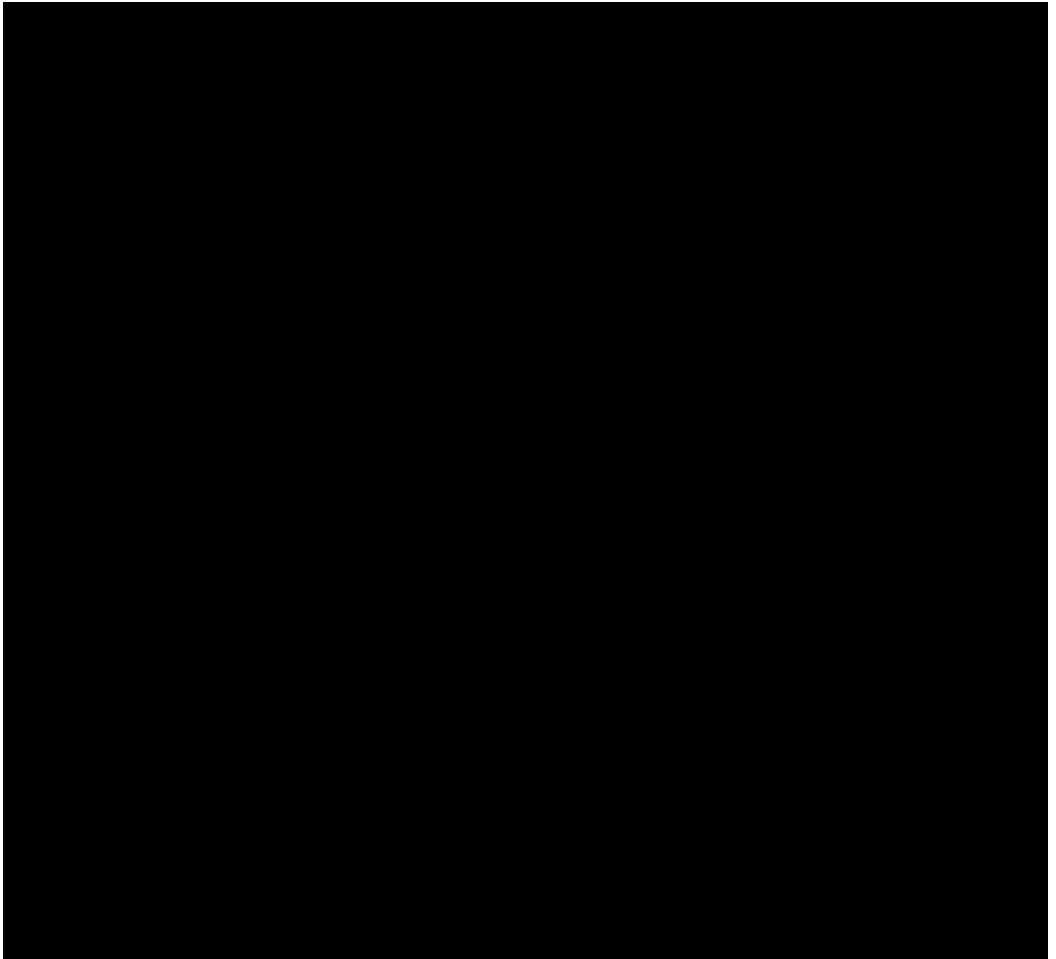


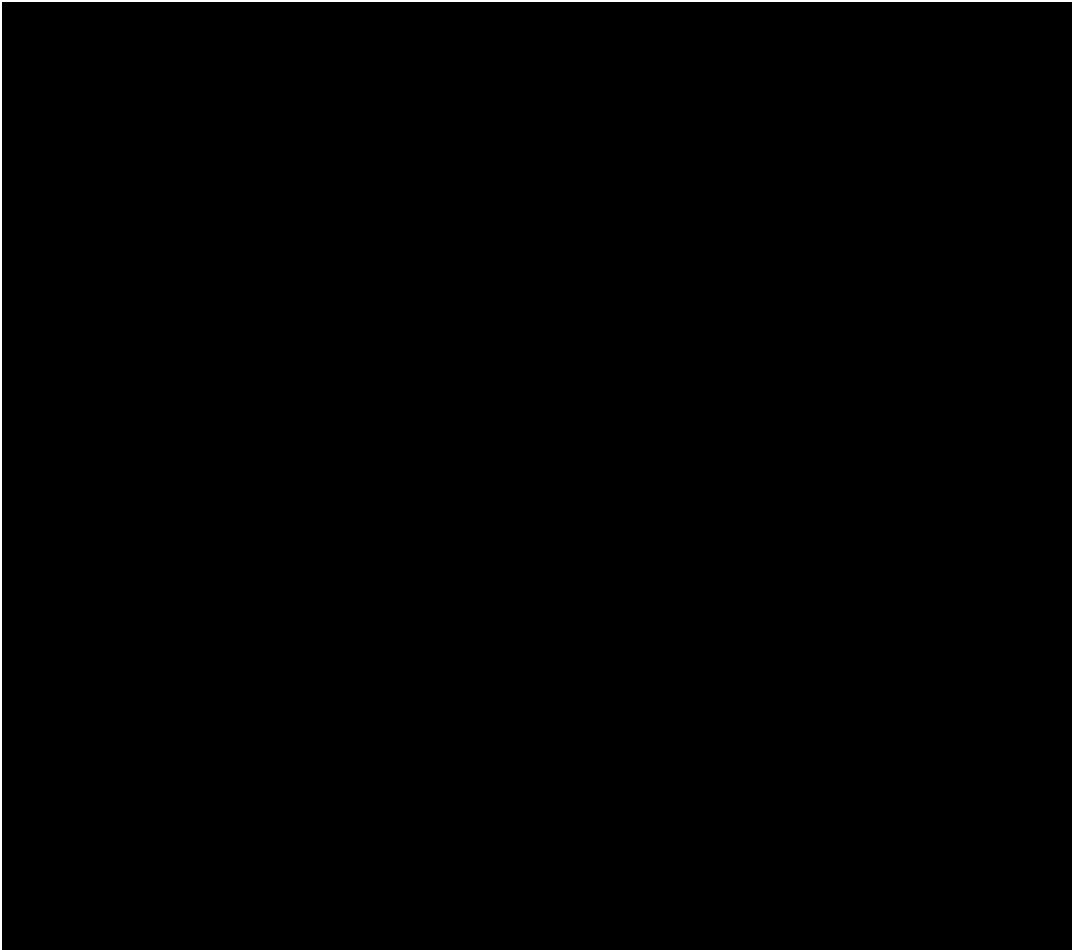
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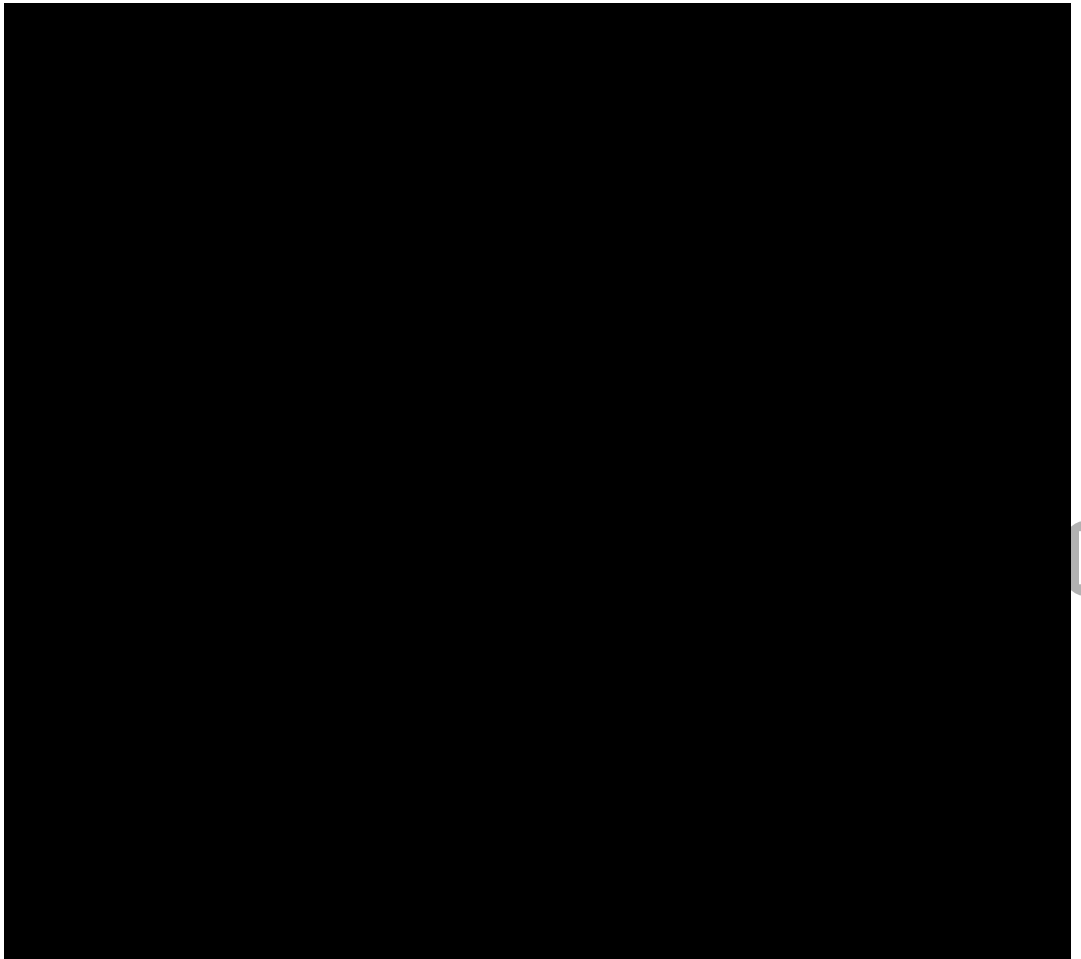
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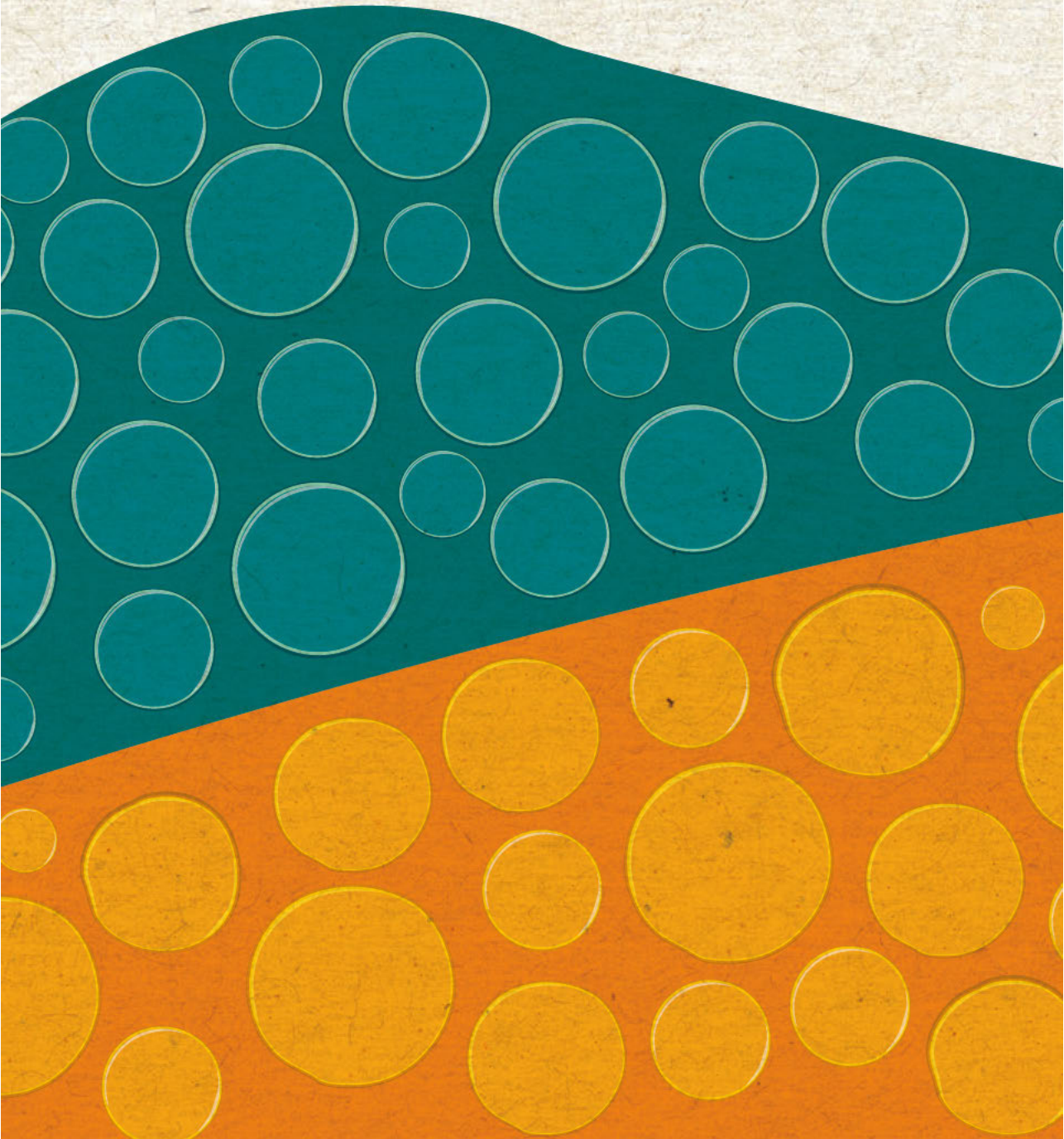








# CHAPTER 5



# **Sex steroids regulate bone marrow fat in men and women: an intervention study in transgender persons**

Marieke Tebbens, Moya Schutte, Marian Troelstra, Aart J. Nederveen, M. den  
Heijer, Peter H. Bisschop



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| United States   | 1.2%         | United States   | 1.2%         |
| Germany         | 0.8%         | Germany         | 0.8%         |
| France          | 0.7%         | France          | 0.7%         |
| Italy           | 0.6%         | Italy           | 0.6%         |
| Spain           | 0.5%         | Spain           | 0.5%         |
| United Kingdom  | 0.4%         | United Kingdom  | 0.4%         |
| Japan           | 0.3%         | Japan           | 0.3%         |
| China           | 0.2%         | China           | 0.2%         |
| India           | 0.1%         | India           | 0.1%         |
| South Korea     | 0.1%         | South Korea     | 0.1%         |
| Canada          | 0.1%         | Canada          | 0.1%         |
| Brazil          | 0.1%         | Brazil          | 0.1%         |
| Russia          | 0.1%         | Russia          | 0.1%         |
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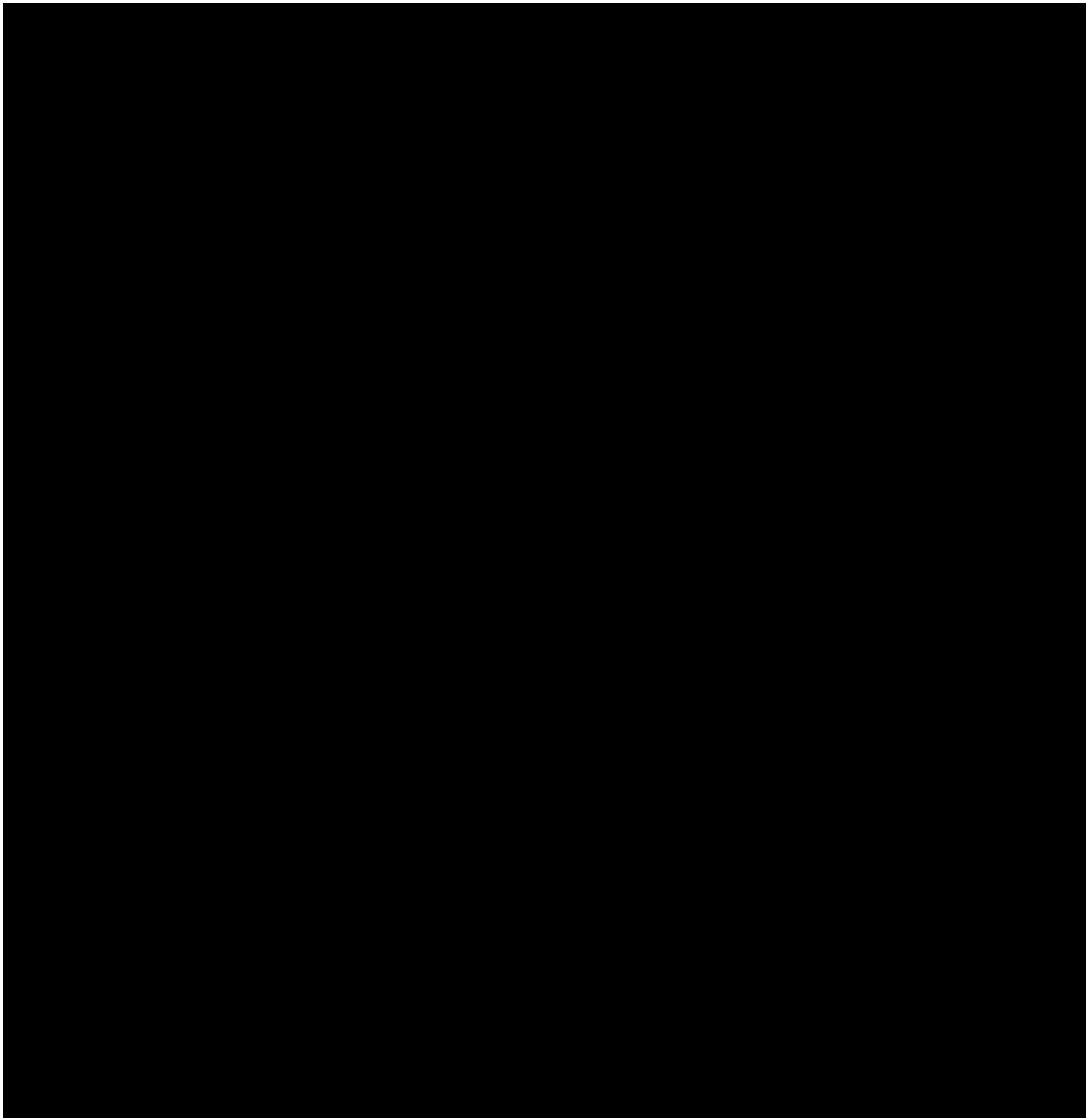
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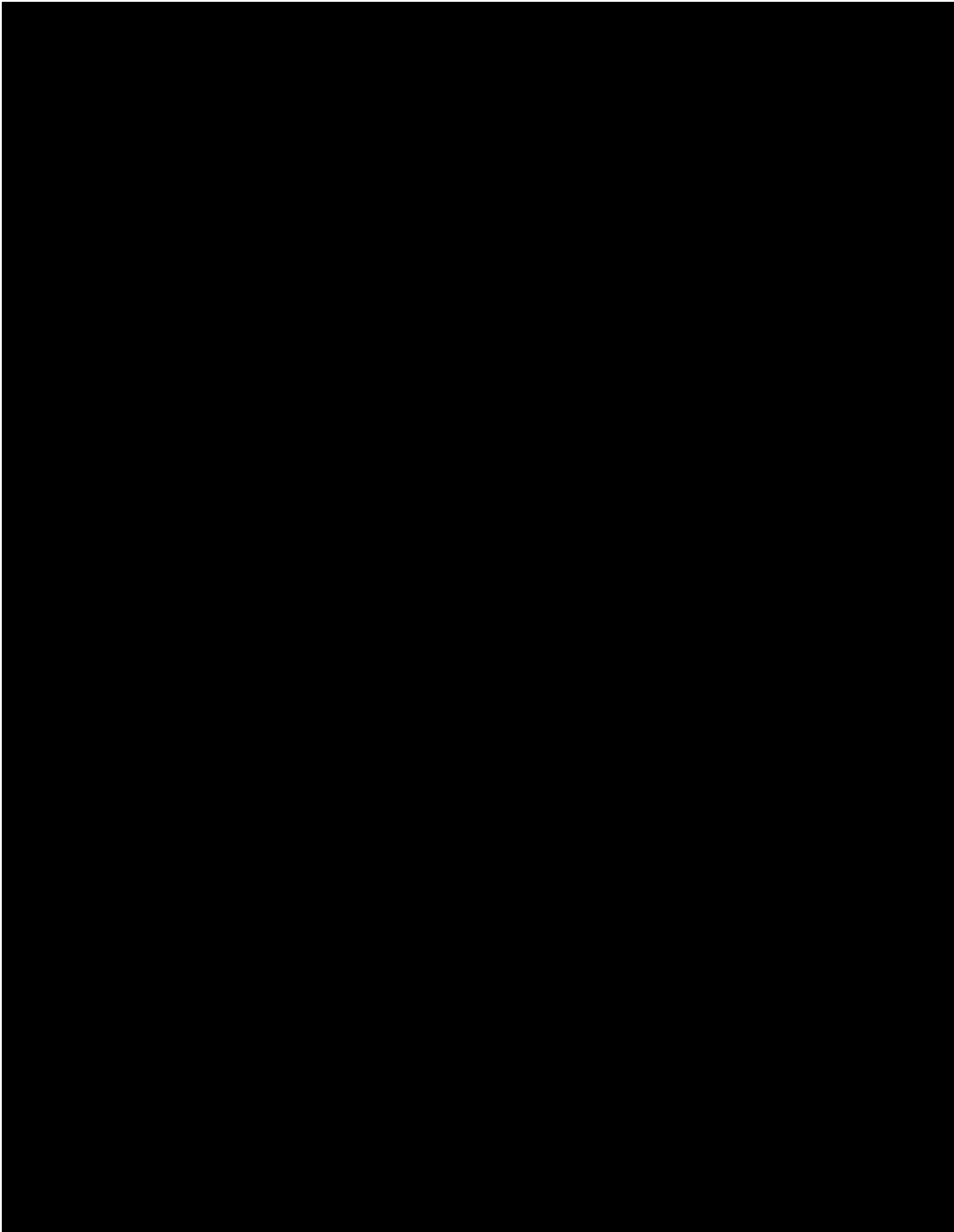
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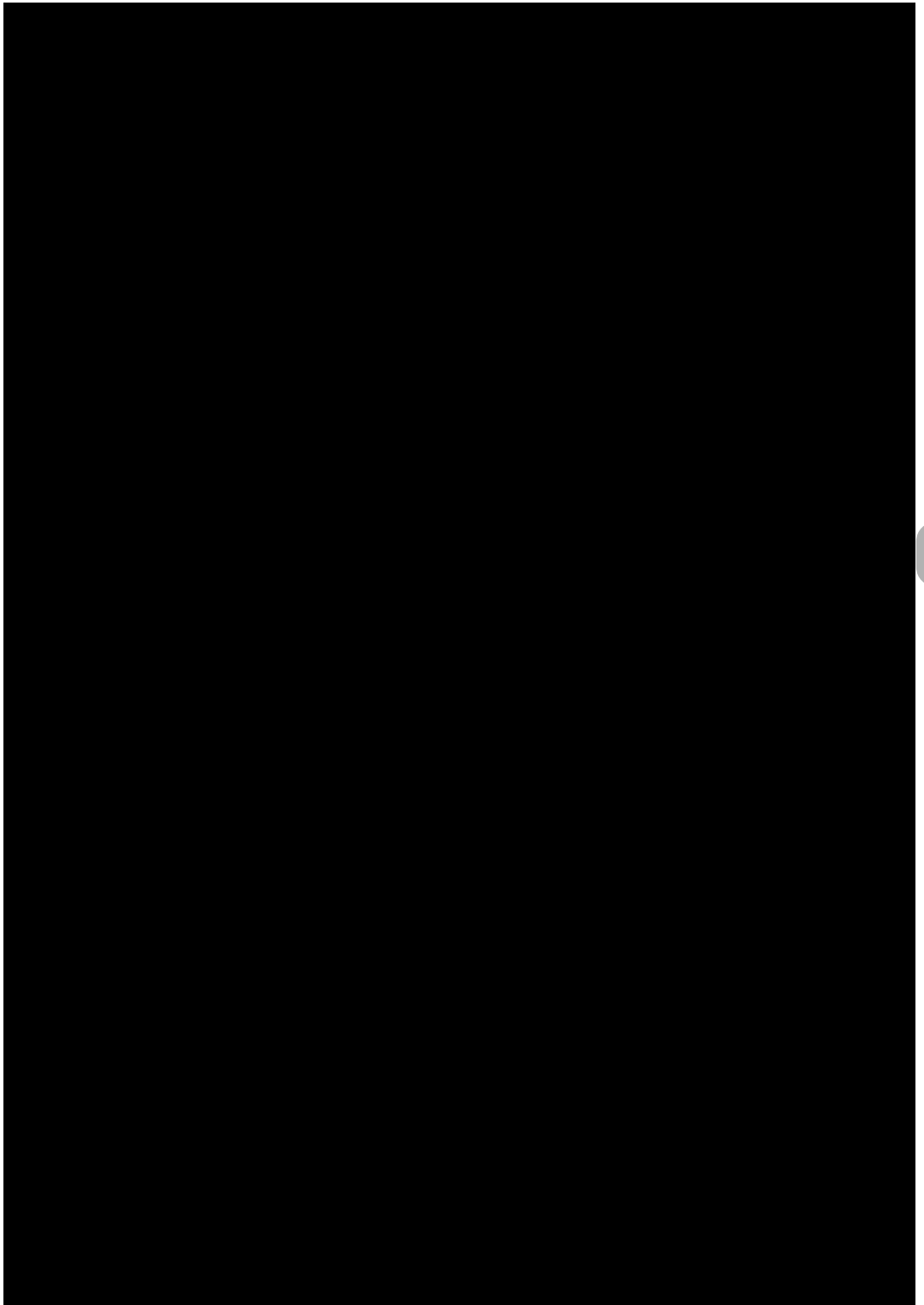
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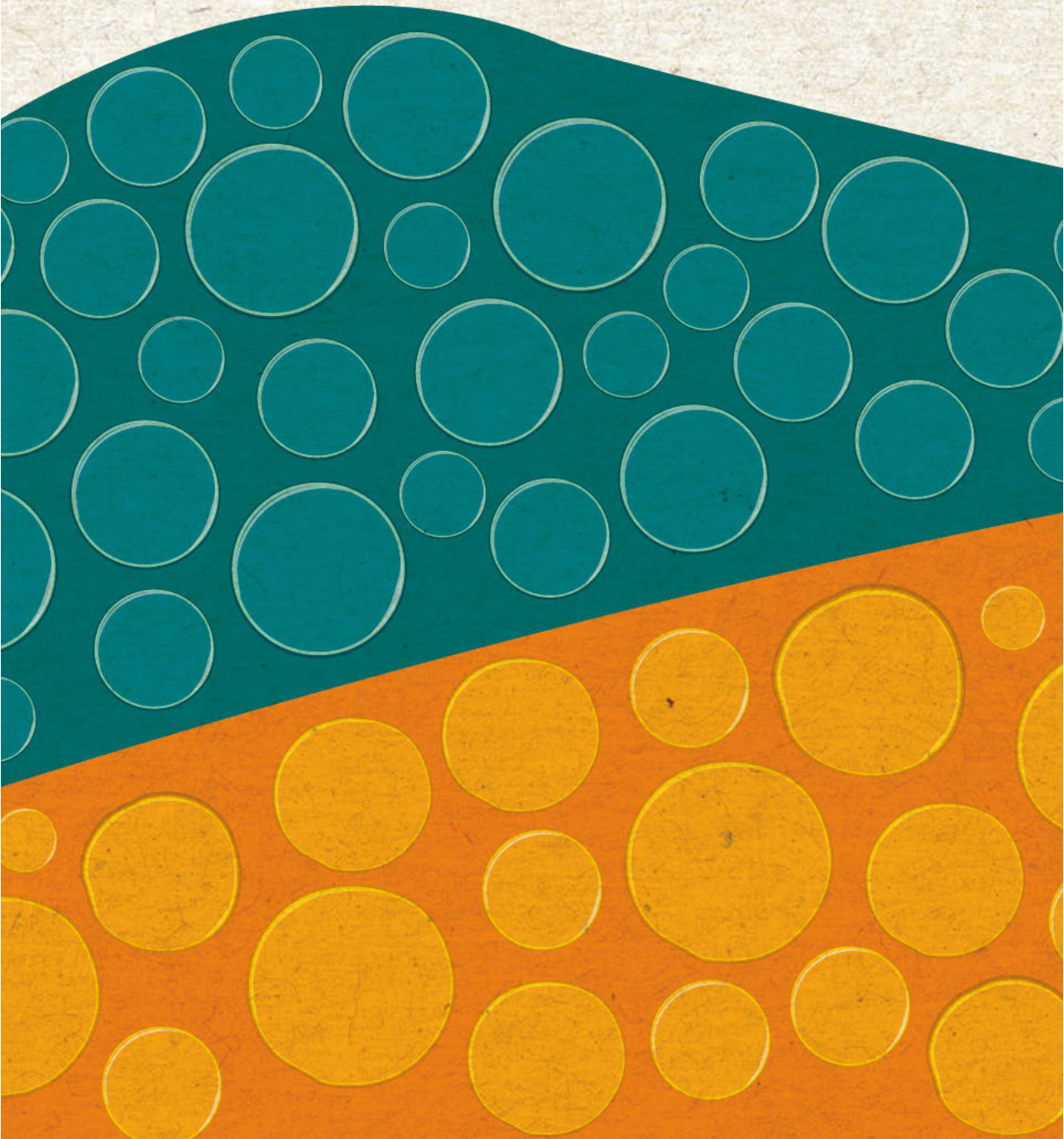
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# CHAPTER 6



# **Estradiol does not affect adipokine expression in human bone marrow adipocytes in vitro**

Marieke Tebbens, Jolanda M.A. Hogervorst, Behrouz Zandieh-Doulabi, Nathalie Bravenboer, Matthias U. Schaefroth, Peter H. Bisschop, Astrid D. Bakker



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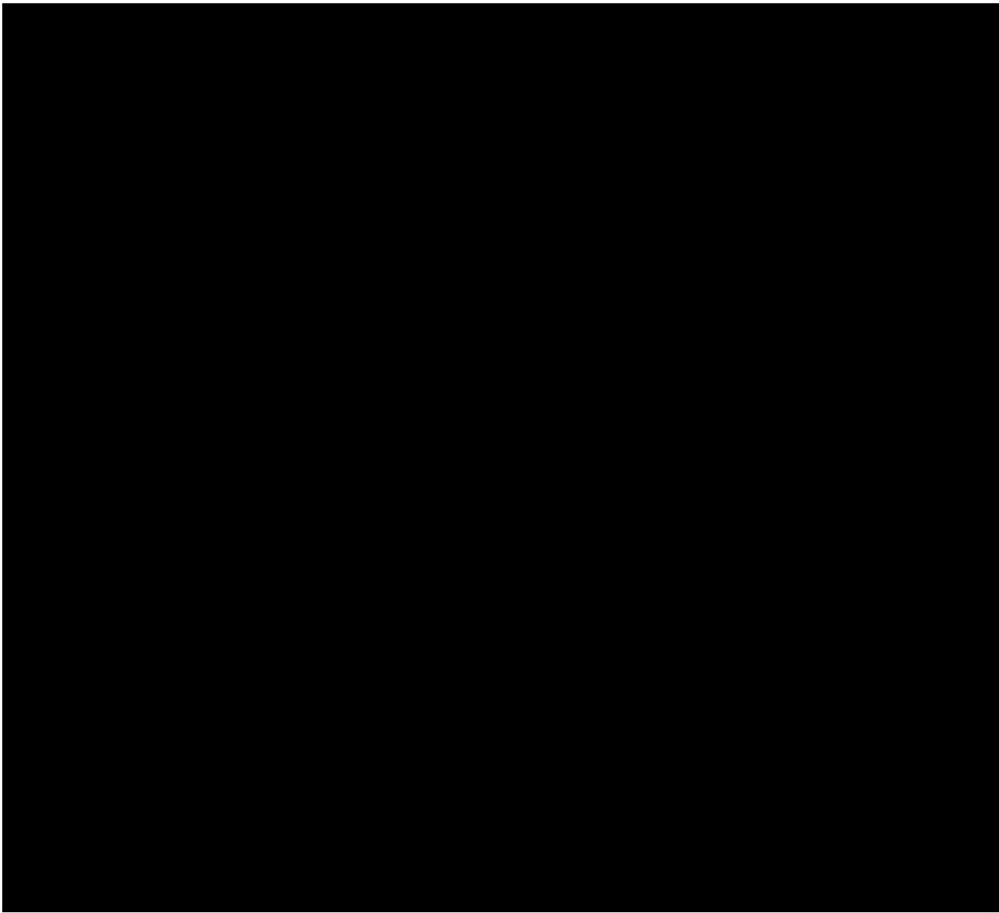
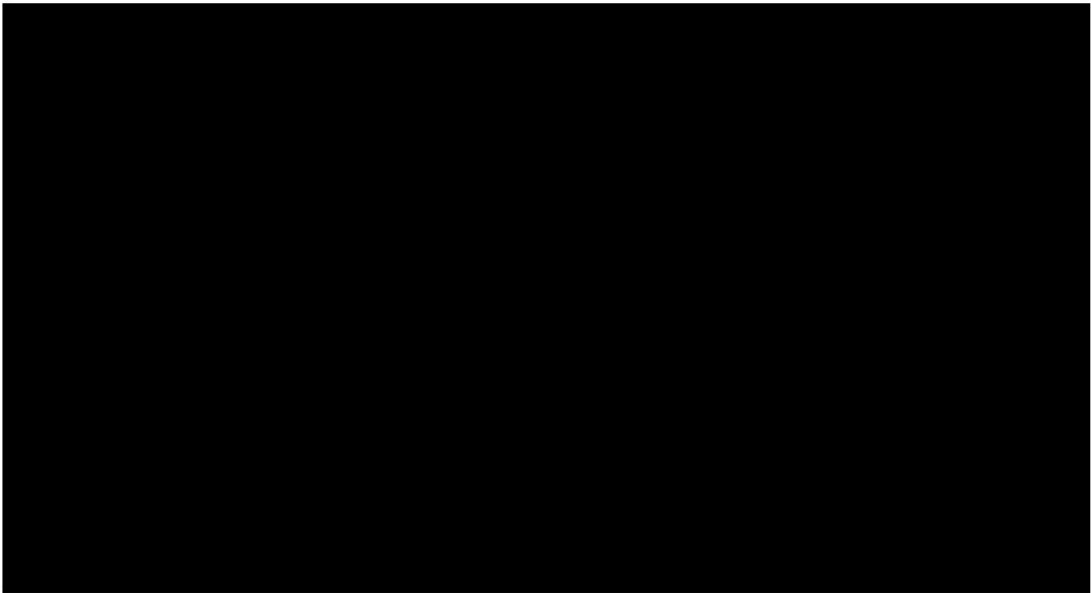
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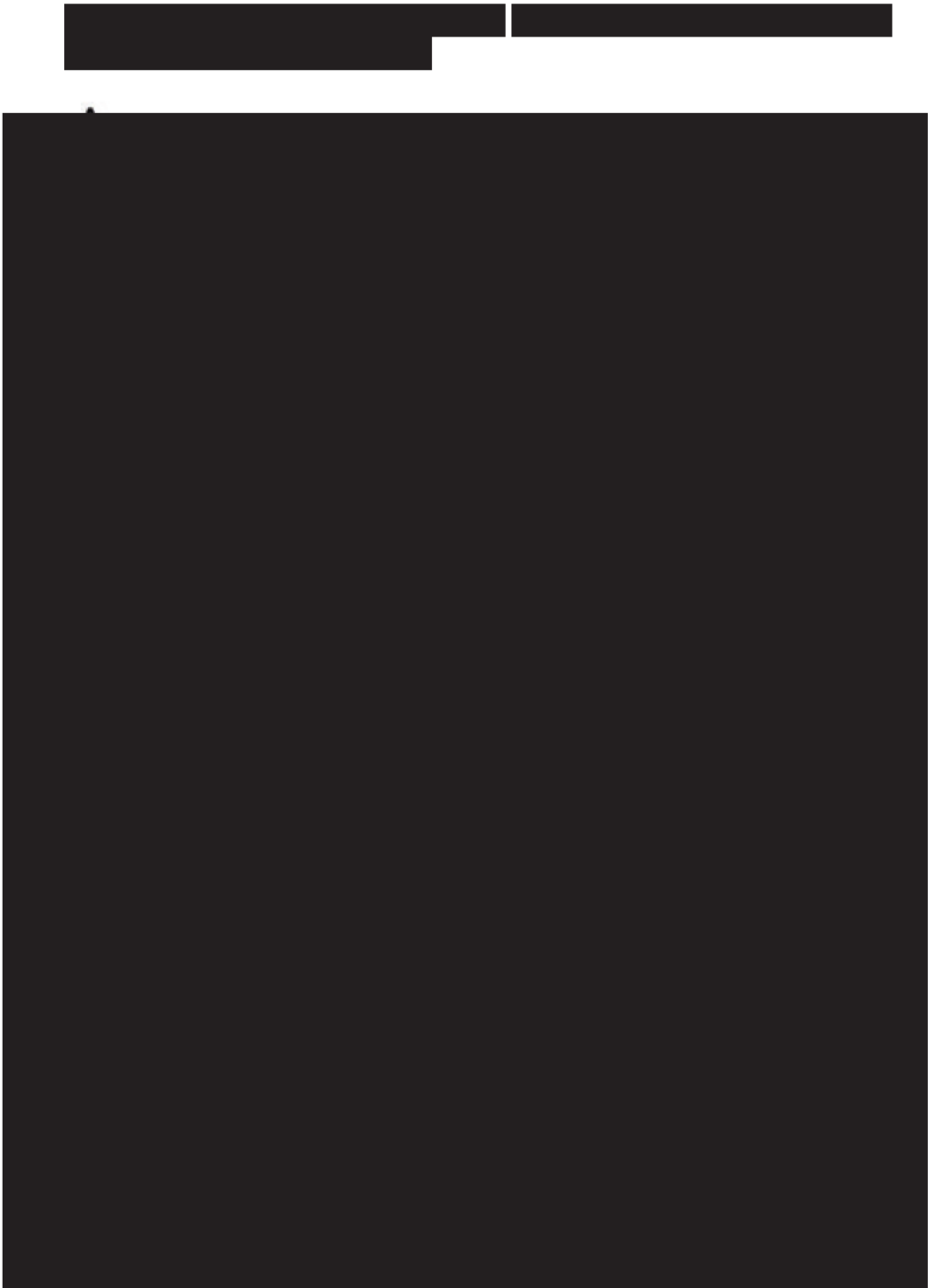
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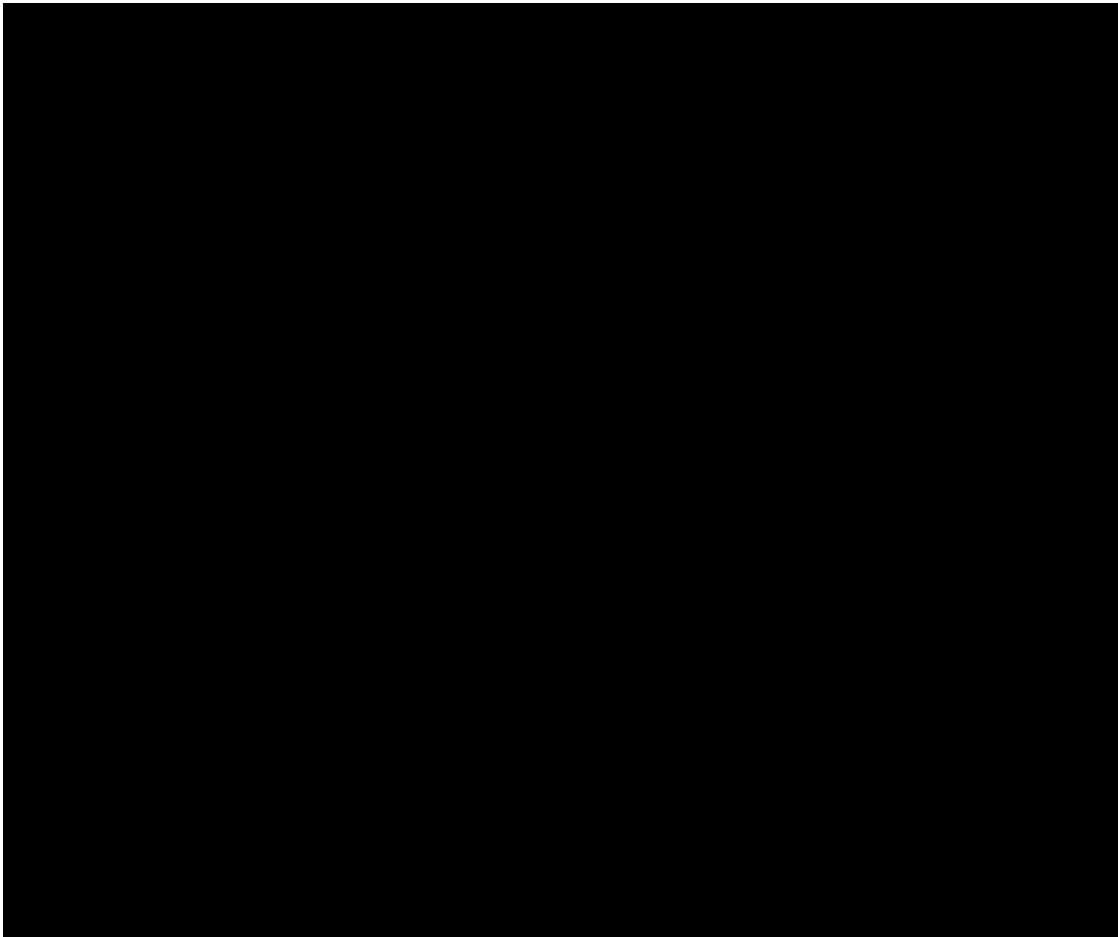




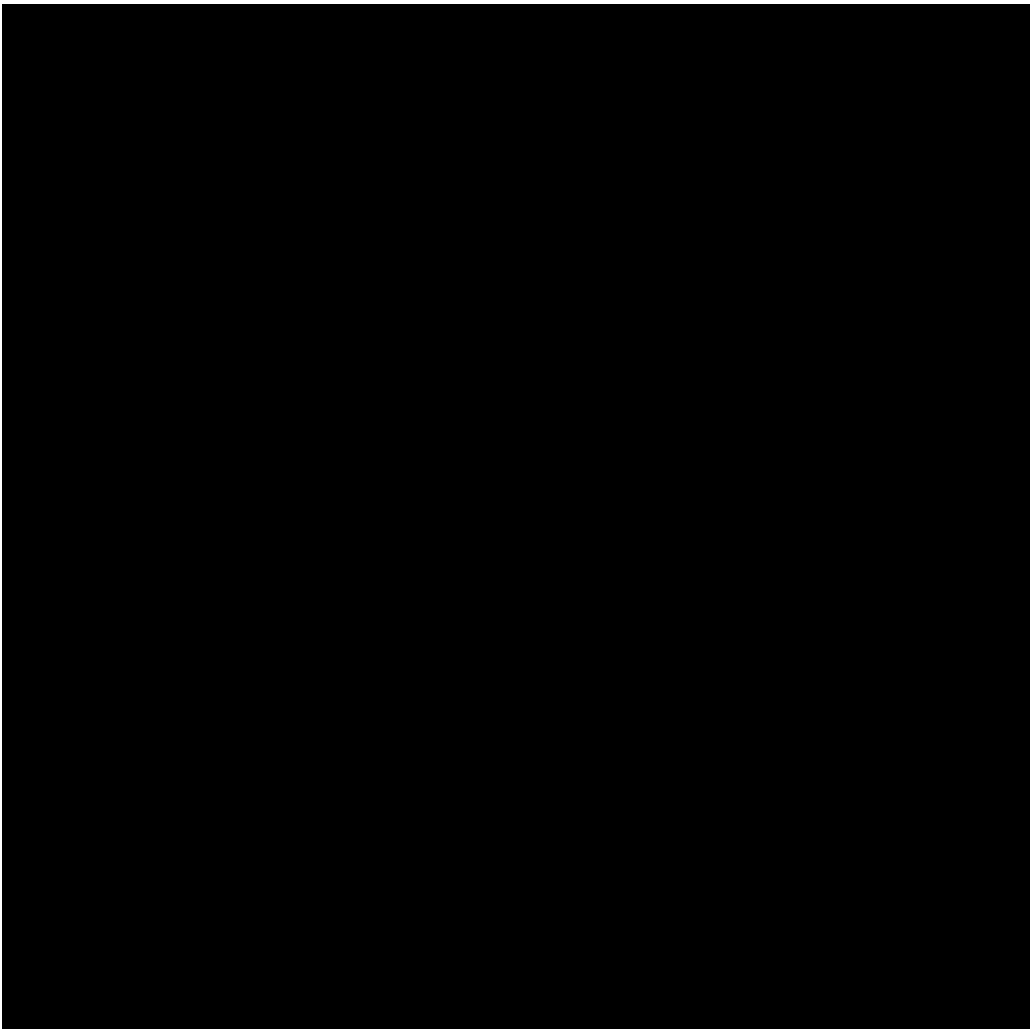




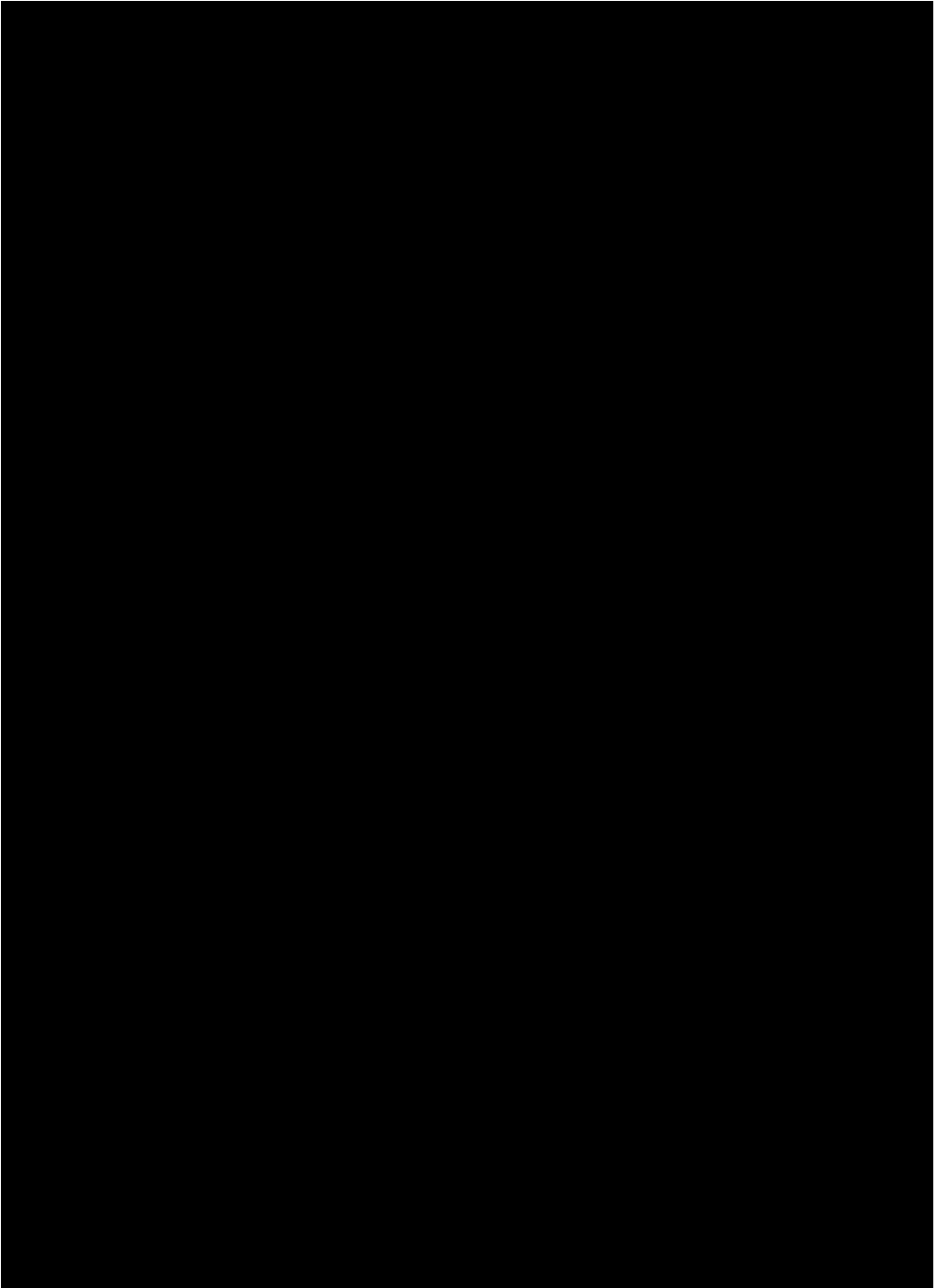


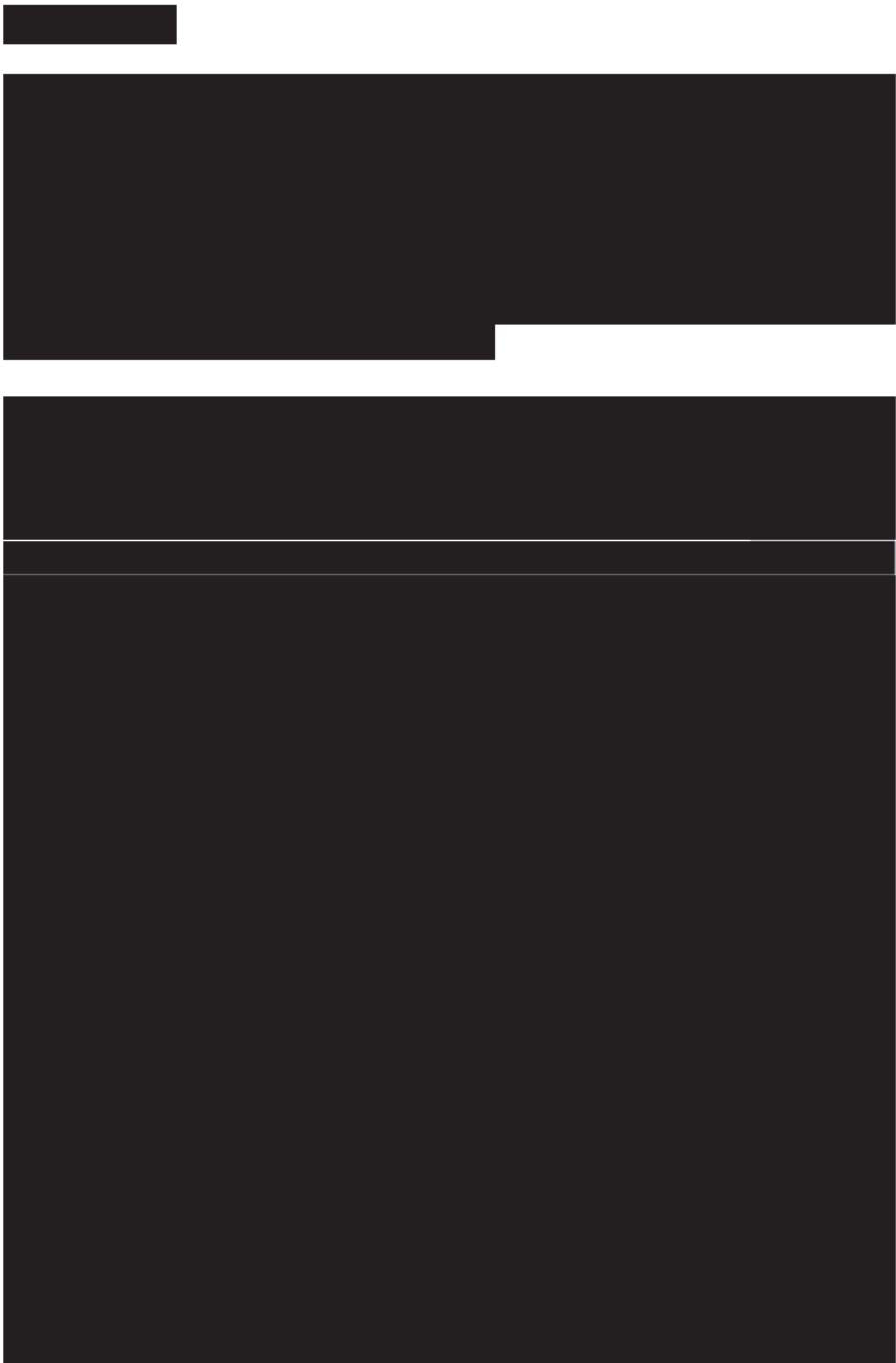


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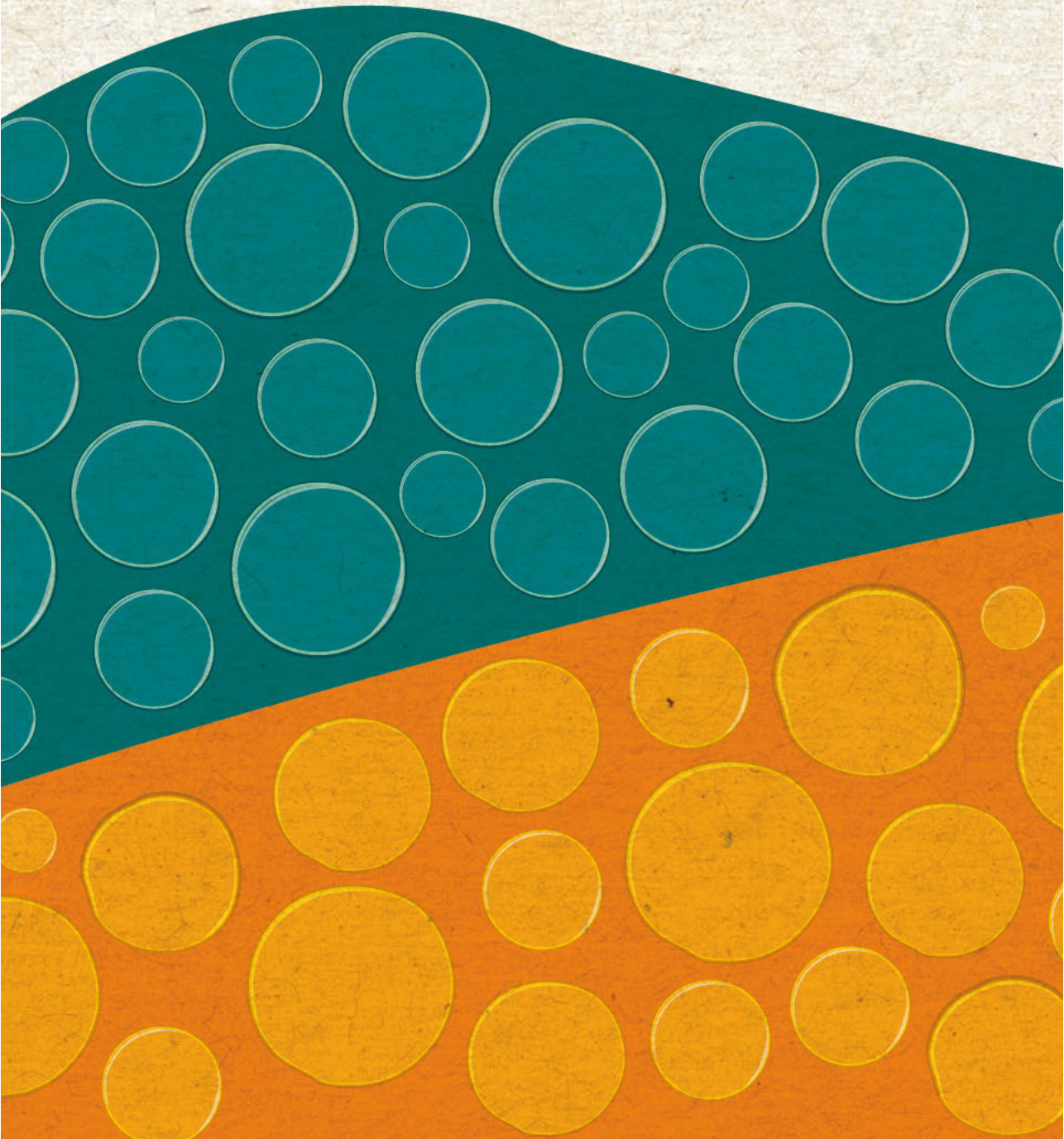
characterization of human bone marrow adipocytes. 2013(1873-2399 (Electronic))



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# CHAPTER 7



# **Gender-Affirming Hormone Treatment Induces Facial Feminization in Transwomen and Masculinization in Transmen: Quantification by 3D Scanning and Patient-Reported Outcome Measures**

Marieke Tebbens, Nienke M. Nota, Niels P. T. J. Liberton, Brigitte A. Meijer, Baudewijntje P. C. Kreukels, Tim Forouzanfar, Rudolf M. Verdaasdonk, Martin den Heijer

Published in the *Journal of Sexual Medicine*, 2019



## ABSTRACT

**Introduction:** Hormone treatment induces feminization of the body in transwomen and masculinization in transmen. However, the effect of hormone treatment on facial characteristics is still unknown.

**Aim:** We aimed to study whether hormone treatment induces facial feminization and masculinization and how this potential change affects satisfaction and self-esteem.

**Methods:** In this single-center cohort study, we included 27 transwomen and 15 transmen who received standardized hormone treatment in the Center of Expertise on Gender Dysphoria, VU University Medical Center Amsterdam. Facial 3-dimensional images were obtained at baseline and at 3 and 12 months. At each image, 22 facial landmarks were placed. Furthermore, the FACE-Q Satisfaction with Facial Appearance Overall and the Rosenberg self-esteem scale were obtained at the same measurement points.

**Main Outcome Measures:** The main outcome measures included the relative local shift of skin in millimeters in the 22 landmarks in the transverse (x-axis), coronal (y-axis), and sagittal (z-axis) anatomic axes, the color maps, and the outcomes of the questionnaires.

**Results:** After 12 months, cheek tissue in transwomen increased, with 0.50 mm (95% CI 0.04 – 0.96) in the x-axis and 1.08 mm (95% CI 0.31 – 1.85) in the z-axis. Tissue in the jaws decreased with 0.60 mm (95% CI 1.28 – 0.08) in the x-axis and 0.18 mm (95% CI 0.03 – 0.33) in the y-axis. Cheek tissue in transmen decreased with 0.45 mm (95% CI 1.00 – 0.11) in the x-axis and 0.84 mm (95% CI 1.92 – 0.25) in the z-axis. These changes already started after 3 months. An increase in satisfaction with the facial appearance was found in both transwomen and transmen. There were no changes in reported self-esteem.

**Clinical implication:** These results could lead to more realistic expectations of facial changes. Furthermore, our results suggest that the face continues to change for at least a year, which could suggest that performing facial feminization surgery after 1 year of hormone treatment might be too early.

**Strength & Limitations:** This study is the first that provides insight into the facial changes in transgender individuals receiving hormone treatment, and it introduces

an objective method to examine (small) facial changes. Our study is limited by the poor reliability of the landmarks, the difficulty of facial fixation, and the lack of gender-specific questions in the questionnaires.

**Conclusions:** Hormone treatment in transwomen induces an increase in cheek tissue and a decrease in jaw tissue. In transmen a tendency of decrease in cheek tissue and an increase in jaw tissue was found. These changes are in the direction of the desired gender.

## INTRODUCTION

Gender affirming hormone treatment induces feminization and masculinization of the body through various mechanisms. For example, change in body fat distribution leads to a more gynoid body shape in transwomen (male sex assigned at birth, female gender identity) and a more android body shape in transmen (female sex assigned at birth, male gender identity) <sup>1,2</sup>. In addition, hormone treatment stimulates breast growth in transwomen <sup>3</sup>, deepening of the voice in transmen <sup>4</sup>, and a change in muscle mass in both transwomen and transmen <sup>5</sup>. Another important aspect of gender appearance is the face. Transgender individuals often report to experience feminization or masculinization of the face. Ainsworth et al. <sup>6</sup> have examined the influence of facial feminization surgery on the quality of life in transgender individuals. They found a significant improvement of quality of life after surgery, emphasizing the importance of a face matching the experienced gender.

Sex differences in the face have been long known and studied <sup>7,8</sup>. One of the first quantitative methods used to study facial differences was anthropometry, consisting of the measurement of facial distances and angles <sup>9</sup>. Another quantitative method is cephalometry, which uses imaging techniques to measure facial distances and angles <sup>10</sup>. More recently, 3-dimensional (3D) imaging techniques have been used to study facial dimorphism, offering the possibility to study soft tissue as well as bone structures <sup>11-13</sup>. Skeletal structure plays an important role in facial differences between women and men. Skeletal differences underlie the larger and wider face, frontal bossing, wider zygomatic arches, more prominent nasal bones and larger mandibles in men <sup>14-16</sup>. However, differences in soft tissue also contribute to sexual differences. Women have, among other things, more prominent and rounder cheeks, less prominent jaw angles and a greater upper vermillion height in comparison to size of the mouth <sup>17-20</sup>.

This study aims to investigate the effect of gender affirming hormone treatment on facial features in transwomen and transmen, using 3D imaging. Satisfaction with facial features and self-esteem will be measured as well. We hypothesize that hormone treatment will induce changes in facial soft tissue. We expect an increase in tissue in the cheek area and a decrease in tissue around the jaw in transwomen and a decrease in tissue in the cheek area and an increase tissue around the jaw in transmen. We hypothesize that these changes will begin in the first months of treatment, simultaneously with the changes in fat distribution in the body <sup>5</sup>.

## MATERIALS AND METHODS

### Subjects and study design

We performed a prospective single center cohort pilot study in the Amsterdam UMC, Vrije Universiteit. Between September 2015 and January 2016, all adult transgender individuals who were planned to start gender affirming hormone treatment in our center, were invited to participate in the study. All subjects were diagnosed with gender dysphoria according to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders 21. The Medical Ethics Testing Committee of the Amsterdam UMC reviewed our research protocol and approved it. All participants signed informed consent and could withdraw their participation at any time during the study. We excluded subjects with current or previous use of gender-affirming hormones and gonadotropin-releasing hormone analogues, who did not speak Dutch, who did not follow the standard treatment regimen, and who had a history of epileptic seizures (to prevent possible seizures due to flashes of the 3D scanner). Finally, 27 transwomen and 15 transmen were included and scanned at baseline. After 3 months, 4 transwomen and 1 transman were lost to follow-up, and, after 12 months, another transwoman and 5 transmen were lost to follow-up. 3 transwomen were excluded because they underwent facial feminization surgery or had fillers placed, and 1 because she did not start the hormones at baseline due to the wish of semen preservation. This resulted in the analysis of 19 transwomen and 14 transmen after 3 months and 15 transwomen and 9 transmen after 12 months. The number of participants invited to participate and the acceptance rate are not available.

### Treatment protocol

Transwomen were treated with the anti-androgen cyproterone acetate (CPA) 25-50mg daily combined with transdermal estradiol patches (EP) 50-100mcg per 24 hours or oral estradiol tablets (ET) 2-4mg daily. Transmen were treated with either transdermal testosterone gel (TG) 50mg daily, intramuscular short-acting testosterone esters (TE) 250mg once every 2-3 weeks, or intramuscular long-acting testosterone undecanoate (TU) 1000mg once every 10-12 weeks.

### Landmarks

22 soft tissue facial landmarks (Table 1) were defined, according to Ferrario *et al.*<sup>22</sup>, to quantify the facial deviation. Most changes are to be expected in soft tissue. Therefore we defined the cheek and gonion landmarks as landmarks of interest.

**Table 1.** Definition and abbreviation of the used soft tissue landmarks

| Landmarks                    | Definition                                                                                                        |
|------------------------------|-------------------------------------------------------------------------------------------------------------------|
| <i>Midsagittal landmarks</i> |                                                                                                                   |
| Trichion (tr)                | The most cranial midpoint on the forehead                                                                         |
| Nasion (n)                   | The most posterior midpoint of the nasofrontal suture                                                             |
| Pronasale (prn)              | The post posterior midpoint of the nose                                                                           |
| Subnasale (sn)               | The midpoint of the angle between the columella nasi and the upper lip                                            |
| Labiale superius (ls)        | The midpoint of the upper vermillion                                                                              |
| Labiale inferius (li)        | The midpoint of the lower vermillion                                                                              |
| sublabiale (sl)              | The most posterior midpoint on the labiomental fold                                                               |
| Pogonion (pg)                | The most anterior midpoint of the chin                                                                            |
| <i>Bilateral landmarks</i>   |                                                                                                                   |
| Frontotemporale (ft)         | The point just above the eyebrows in the vertical line of the exocanthion                                         |
| Exocanthion (ex)             | The point at the outer commissure of the eye fissure                                                              |
| Cheek (chk)                  | The point where the line between the exocanthion and cheilion and the line between the tragus and alar crest meet |
| Tragus (t)                   | The most lateral point on the tragus                                                                              |
| Alar crest (ac)              | The most lateral point in the alar curve                                                                          |
| Cheilion (ch)                | The point at the labial commissure                                                                                |
| Gonion (go)                  | The most lateral point on the mandibular angle                                                                    |

## Imaging and processing

Facial 3D images were obtained at baseline, at 3 months and 12 months. The images were captured using a surface scanner, Artec Spider (Artec Group, Luxembourg City, Luxembourg). The Spider has a resolution up to 0.1mm, an accuracy up to 0.05mm and a maximum capture rate of 8 frames/sec. The scans were made with and processed in Artec Studio software (Artec Group). The same settings were maintained throughout all the scans; the features we tracked were geometry and texture, the scanning speed was set at 8 frames/sec, and the depth of field was between 170 – 300mm. Subjects were asked to sit in a chair with their head in a neutral position and keep an expression as neutral as possible. Tanning goggles were used to protect the eye from the bright blue light flashes emitted by the scanner. The scanning time was typically 10-60 seconds/scan. Multiple scans (3 – 10) were made until the entire face was scanned. After scanning the subjects, processing was performed to convert the multiple scans to one mesh. A mesh is a polygonal model, composed of various-sized triangles, easy to process and store. To process the scans into meshes, we used the standard scan acquisition and workflow process outlined in the manufacturer-provided user guide (Artec Group, 2013). A fine registration was run on all scans individually, after which noise

and background (including clothes, hair and neck) were manually removed. Global registration was performed to attain a maximum registration error of 0.2mm. The scans were aligned with rigid alignment, using 3 shared points. Global registration was then performed on all combined scans, again to attain a maximum registration error of 0.2mm. Fast fusion was executed to combine the scans to generate a mesh, which was saved as a STereoLithography file. Artec software was also used to generate average meshes of the meshes of all transwomen and all transmen at 1 time point. The meshes were aligned with "best match" and fast fusion was executed, averaging all the meshes into 1 average mesh.

The STereoLithography files were uploaded and analyzed with GOM inspect V8 software (GOM, Braunschweig, Germany). The origin of the coordinate system was set on the mid-point between both endocanthion points, the point at which the inner ends of the upper and lower eyelid meet. The transverse, coronal and sagittal anatomical axes were defined as the x-axis, y-axis and z-axis, respectively. The section of the x-axis was defined as the line between the endocanthia. The section of the y-axis was defined to be parallel to the line between the glabella and pogonion. For each subject, the mesh at baseline was compared with the mesh at 3 months and 12 months (if available). The meshes at baseline were marked as nominal elements and the meshes at 3 months or 12 months as actual elements. The meshes were aligned with the option "local best-fit", selecting the entire mesh. The landmarks were placed on the nominal element (baseline mesh) with "surface points" and projected on the actual element (3 months or 12 months mesh). The displacement in mm in the x, y and z-axis was calculated by the software. Thereafter, deviations between the meshes were visualized in color on the actual element (color maps).

### **Repeatability and reliability**

The repeatability of the scans was tested by determining the intraday variability. This variability was tested by scanning 5 transwomen and 3 transmen twice on the same day. The reliability of the landmarks was determined by the intraobserver variability, which was measured by placing the landmarks twice on the same mesh in 4 transwomen and 2 transmen by the same observer. Intraclass correlation coefficients (ICC), two-way mixed effects, were calculated per landmark. Interobserver variability was not tested, since only 1 observer was involved. This observer was not blinded to the measurement points, since the second or third mesh needed to be compared to the first mesh.

## Questionnaires

All participants were asked to complete 2 questionnaires during their visits. The FACE-Q Satisfaction with Facial Appearance Overall <sup>23</sup>, adapted for a Dutch sample, was used to measure changes in the satisfaction with facial appearance. The FACE-Q is a validated tool, developed to measure the satisfaction and quality of life in patients who undergo aesthetic facial procedures and contains >40 scales <sup>24,25</sup>. The FACE-Q Satisfaction with Facial Appearance Overall, contains 10 items about the satisfaction of different aspects of the face. The total score has a range between 10 – 40 and can be converted to a 0 – 100 scale with a conversion table. A higher score is associated with more satisfaction of the facial appearance overall. The Rosenberg self-esteem scale <sup>26</sup>, adapted for a Dutch sample, was used to measure changes in self-esteem. The scale consists of 10 items and gives insight into the different aspects of self-esteem of the participants. The scale generates a score ranging from 0 – 30; a higher score is associated with a higher self-esteem.

## Statistical analysis

The statistical analyses were performed in STATA (Stata Statistical Software: Release 14; StataCorp LP, College Station, TX, USA). Histograms, q-q-plots and the Shapiro-Wilk test were used to assess whether the data was normally distributed. Means with 95% CI were used to describe continuous, normally distributed data. Medians with interquartile ranges (IQR) were used to describe continuous, non-normally distributed data. The deviation in each landmark between baseline and 3 months and baseline and 12 months was described as mean with 95% CI. The deviation in the landmarks of interest were analyzed with a paired two-sample t-test. Because of the exploratory nature of the analyses, no correction was made for the multiple analyses. The correlation between change in weight, age, and the deviation in the landmarks was analyzed with linear regression. Mean absolute deviation was used to determine the intraday variation. The ICC was used to assess the intraobserver variation. The paired two-sample t-test was used to analyze the change in outcome of the FACE-Q questionnaire and the Rosenberg self-esteem questionnaire. Linear regression was performed to analyze the relation between the deviations and change in outcome in the questionnaires. A *P* value < .05 was considered significant in all tests.

## RESULTS

### Deviations

The baseline characteristics of the participants are shown in Table 2. The deviations in transwomen and transmen between baseline and 3 months and baseline and 12 months in all the landmarks are presented in Supplemental Tables 1 and 2. The deviations in the regions of interest are presented in Table 3. In transwomen, after 3 months, the cheek landmarks showed a lateral deviation in the x-axis, and an anterior deviation in the z-axis, which increased after 12 months. The gonion landmarks (jaw region) deviated medial in the x-axis and caudal in the y-axis after 3 months, and this was maintained after 12 months. In transmen, after 3 months, the combined cheek landmarks showed no deviation in the x-axis; however, after 12 months a medial deviation was found. In the z-axis a small posterior deviation was found after 3 months, which increased after 12 months. The combined gonion landmarks showed a tendency to shift medial in the x-axis after 3 months; however, after 12 months, this effect was not found.

As described above, average meshes were made of all transwomen combined and all transmen combined at each time point. Color maps were made to display the difference between the average meshes in colors (Figure 1). The color map of the comparison between the average mesh of transwomen at baseline and the average mesh at 12 months is presented in Figure 1A (frontal view) and Figure 1B (sagittal view). The areas around the cheeks are colored yellow, orange and red, indicating a positive deviation between 0.60 – 2.00mm. The area around the gonion landmark appears light and dark blue, indicating a negative deviation of 0.40 – 2.00mm. The upper lip and nose show a light blue color, indicating a negative deviation between 0.40 – 0.60mm. The color map of transmen is shown in Figure 1C (frontal view) and Figure 1D (sagittal view). The areas around the cheeks are colored light and medium blue, indicating a negative deviation between 0.60 – 1.60mm. The areas around the gonion appear green, indicating no deviation. The areas around the mouth and nose display mostly yellow, which represents a positive deviation between 0.60 – 1.00mm.

**Table 2.** Baseline characteristics. Data are represented as n (%), unless otherwise specified. Medication is medication prescribed at baseline.

| Characteristics                                    | Transwomen (n = 19) | Transmen (n = 14)  |
|----------------------------------------------------|---------------------|--------------------|
| Age (years) at baseline, median [IQR]              | 28 [22 – 32]        | 22 [21 – 25]       |
| Current smoker                                     | 5 (26.3)            | 3 (21.4)           |
| BMI (kg/m <sup>2</sup> ) at baseline, median [IQR] | 23.8 [21.2 – 28.2]  | 25.9 [22.0 – 31.7] |
| <i>Medication</i>                                  |                     |                    |
| Cyproterone acetate                                | 19 (100)            | -                  |
| Estradiol tablets                                  | 10 (52.6)           | -                  |
| Estradiol patches                                  | 9 (47.4)            | -                  |
| Testosterone gel                                   | -                   | 8 (57.1)           |
| Testosterone esters                                | -                   | 5 (35.7)           |
| Testosterone undecanoate                           | -                   | 1 (7.1)            |

Abbreviations: IQR = interquartile range, BMI = body mass index

## Questionnaires

The outcomes of the questionnaires are presented in Table 4. Not all participants filled out the questionnaires; hence, 14 transwomen and 9 transmen were included in the analysis of the questionnaires. In transwomen the FACE-Q scores increased from 42.9 [IQR 34.1 – 51.6] at baseline to 48.5 [IQR 40.1 – 56.8] after 3 months and 54.4 [IQR 40.1 – 56.8] after 12 months. The Rosenberg self-esteem (RSE) scores remained the same throughout the study. In transmen, the FACE-Q scores increased from 52.4 [IQR 44.2 – 60.6] at start to 59.9 [IQR 53.2 – 66.5] after 3 months and 59.9 [IQR 54.1 – 65.6] after 12 months. The RSE scores remained constant throughout the study.

Simple linear regression was carried out to investigate the relationship between the deviation in landmarks of interest and change in outcome of the questionnaires. No relationships were found between the deviation in landmarks and either the FACE-Q or RSE scores in both transwomen and transmen.

**Table 3.** Deviations in landmarks of interest in transwomen and transmen. Data are presented as mean [95% confidence intervals].

|                   | x-axis                |                       | y-axis               |                      | z-axis               |                      |
|-------------------|-----------------------|-----------------------|----------------------|----------------------|----------------------|----------------------|
|                   | 3 months              | 12 months             | 3 months             | 12 months            | 3 months             | 12 months            |
| <i>Transwomen</i> |                       |                       |                      |                      |                      |                      |
| Cheeks combined   | 0.19 [0.03 – 0.34]    | 0.50 [0.04 – 0.96]    | 0.02 [-0.02 – 0.06]  | 0.12 [-0.09 – 0.33]  | 0.31 [0.05 – 0.57]   | 1.08 [0.31 – 1.85]   |
| Gonion combined   | -0.76 [-1.25 – -0.27] | -0.60 [-1.28 – -0.33] | 0.20 [0.10 – 0.30]   | 0.18 [-0.03 – 0.40]  | -0.00 [-0.05 – 0.05] | -0.04 [-0.17 – 0.09] |
| <i>Transmen</i>   |                       |                       |                      |                      |                      |                      |
| Cheeks combined   | -0.09 [-0.23 – 0.04]  | -0.45 [-1.00 – 0.11]  | -0.05 [-0.11 – 0.02] | -0.09 [-0.32 – 0.14] | -0.17 [-0.42 – 0.08] | -0.84 [-1.9 – 0.25]  |
| Gonion combined   | 0.28 [-0.44 – 0.99]   | -0.05 [-1.65 – 1.55]  | -0.11 [-0.40 – 0.17] | -0.07 [-0.63 – 0.48] | 0.04 [0.00 – 0.07]   | 0.04 [-0.12 – 0.21]  |

Positive values indicate a deviation away from the origin and negative numbers indicate a deviation towards the origin.

**Table 4.** Results FACE-Q and RSE in transwomen (n=14) and transmen (n=7). Data are presented as mean [95% confidence intervals].

| Questionnaire     | Baseline           | 3 months            | 12 months           |
|-------------------|--------------------|---------------------|---------------------|
| <i>Transwomen</i> |                    |                     |                     |
| FACE-Q            | 42.9 [34.1 – 51.6] | 48.5 [40.1 – 56.8]  | 54.4 [40.1 – 56.8]* |
| RSE               | 16.6 [13.4 – 19.7] | 17.6 [13.5 – 21.7]  | 17.1 [13.6 – 20.7]  |
| <i>Transmen</i>   |                    |                     |                     |
| FACE-Q            | 52.4 [44.2 – 60.6] | 59.9 [53.2 – 66.5]* | 59.9 [54.1 – 65.6]* |
| RSE               | 18.6 [13.5 – 23.7] | 19.7 [15.4 – 24.0]  | 20.1 [15.3 – 25.0]  |

\*  $p$ -value < 0.05, measured with a two-sample paired t-test. A higher score is associated with a higher satisfaction or self-esteem.

### Effect of age, changes in weight and type of medication

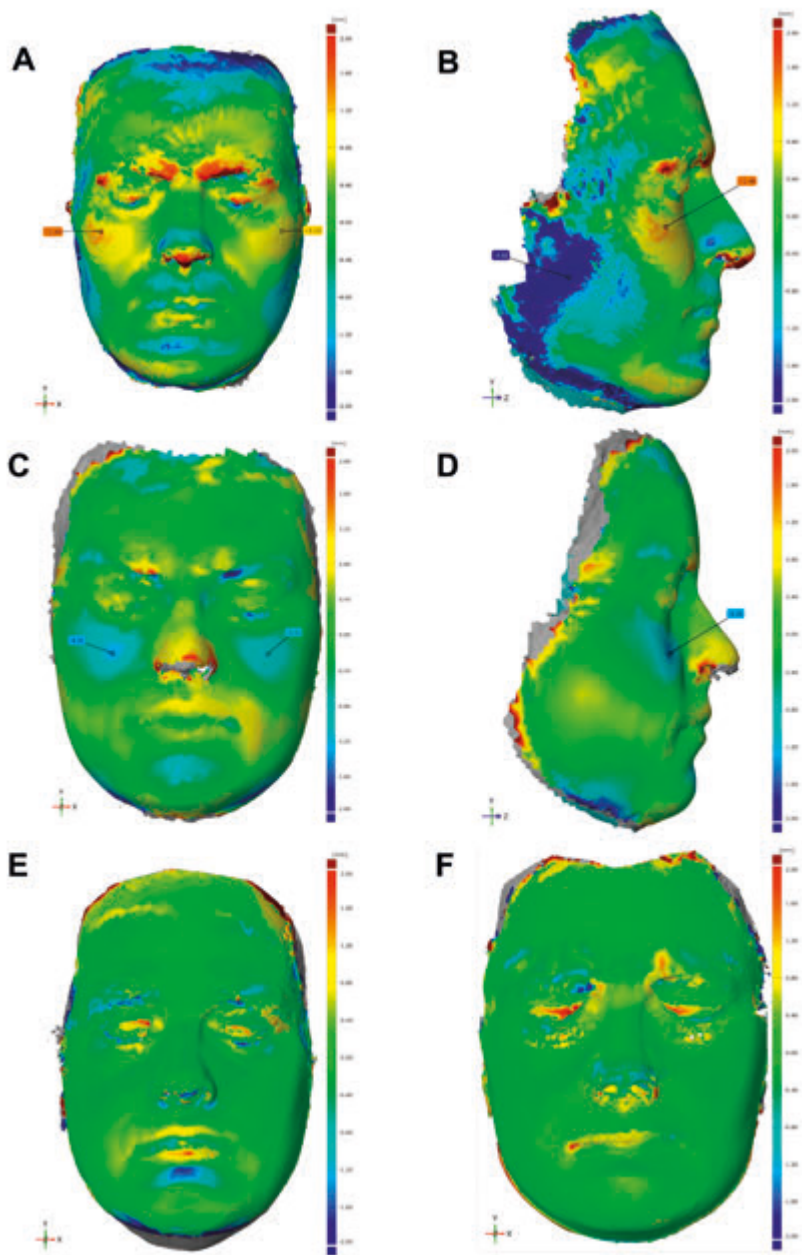
To examine the effect of age, changes in weight and type of medication on the deviation in landmarks, linear regression was performed on the landmarks of interest. In our population, no linear relationship was found between the landmarks of interest and age. A positive linear relationship was found between changes in weight after 12 months and a deviation away from the origin in the gonion landmark in the x- and y-axis in transmen and the z-axis in transwomen. Stratification was performed for difference in weight after 12 months of  $\leq 0$  (no weight gain) and  $> 0$  (weight gain). Transmen showed a deviation in the x-axis of 1.25mm [95%CI 0.26 – 2.23] in the weight gain group and -0.67mm [95%CI -3.69 – 0.36] in the no weight gain group, in the y-axis the deviation was 0.47mm [95%CI -0.17 – 1.11] in the weight gain group and -0.51mm [95%CI -0.95 – -0.07] in the no weight gain group. Transwomen with weight gain deviated 0.09mm [-0.03 – 0.21] in the y-axis and the group without weight gain -0.17mm [95%CI -0.36 – 0.02].

Stratification was performed to investigate the effect of different types of medication prescribed at baseline. Most participants stayed on the same medication throughout the year; only 1 transmen changed from TG to TU after 3 months and another transmen changed from TE to TU after 9 months. In transmen,

a difference between TG and TE users was seen in the cheek landmarks. TG users showed a deviation in the cheek landmarks of -0.99mm [95%CI -1.63 to -0.34] in the x-axis and -1.88mm [95%CI -3.10 to -0.66] in the z-axis. TE users showed a deviation of -0.01 [95%CI -0.58 – 0.56] in the x-axis and 0.015 [95%CI -1.18 – 1.21] in the z-axis. Only 1 participant received TU, therefore TU is not included in this analysis. An unpaired t-test was performed on the differences between TG and TE users and the difference in the x-axis was 0.98mm, with a two-sided  $\alpha$  of 0.06 and in the z-axis 1.90mm with a two-sided  $\alpha$  of 0.06. In transwomen, a difference between CPA in combination with ET and CPA in combination with EP was seen in the cheek landmarks. EP users deviated 0.68mm [95%CI 0.17 – 1.19] in the x-axis and 1.34 mm [95%CI 0.44 – 2.24] in the z-axis. ET users deviated 0.10mm [95%CI -0.40 – 0.60] in the x-axis and 0.45mm [95%CI -0.41 – 1.31] in the z-axis. These differences were not statistically significant.

### **Repeatability and reliability**

The intraday variability, measuring the repeatability, is presented in color maps in Figure 1E for transwomen and Figure 1F for transmen. In both transwomen and transmen, the color maps are mostly green, indicating no deviation. The majority of deviations is seen in the eye and mouth region, indicating both increase and decrease in tissue. Mean absolute deviations were calculated for each pair of scans with a median of 0.22 mm [IQR 0.16 – 0.27]. The ICCs of each landmark, indicating the reliability, are presented in supplemental Table 3. The median of the ICCs was 0.80 [IQR 0.34 – 0.95].



**Figure 1. Color maps of the differences between average meshes.** Differences in mm, displayed in colors, projected on the latest mesh. Red indicates a negative deviation and blue a positive deviation, as displayed in the legend. The labels indicate the greatest deviation in that area. **A**, Frontal color map transwomen baseline – 12 months. **B**, Sagittal color map transwomen baseline – 12 months. **C**, Frontal color map transmen baseline – 12 months. **D**, Sagittal color map transwomen baseline – 12 months. **E**, Intra-day differences in transwomen. **F**, Intra-day differences in transmen.

## DISCUSSION

### Main findings

In this study, we examined the effect of gender-affirming hormone treatment on facial features. We demonstrated that, already after 3 months, the face starts to change under the effect of sex steroids in transwomen, as well as transmen. In trans- women, the cheeks become more prominent, and the jaw region becomes less prominent. In transmen, opposite effects were found, with the cheeks tending to be less prominent and the jaw region more prominent. These findings are in line with our hypothesis and fat redistribution effects <sup>1,5</sup>. The other 20 landmarks showed no deviations after 12 months, except for the trichion landmark. This landmark deviated anterior in transmen, however no deviation was seen in the landmark after 3 months and also the color map showed no sign deviation.

Even though the other 19 landmarks showed no deviations after 12 months, small deviations were seen after 3 months in some landmarks. After 3 months, in transwomen, some axis of the tragus, alar crest, cheilion, and pronasal landmarks deviated, and, in transmen, some axis of the exocanthion, alar crest, and pogonion landmarks deviated. However, after 12 months all of these deviations disappeared. This could imply that these deviations are due to movement of the face rather than actual facial changes. Ozsoy et al. <sup>27</sup> assessed the effect of a sitting, standing, or supine body position on the same soft tissue landmarks with the same scanner and found significant deviations between different body positions, indicating the sensitivity of the scanner for small movements of the face. Moreover, this study found the bilateral landmarks to be less accurate than the midsagittal landmarks, which enhances our findings. Furthermore, Sforza et al. <sup>28</sup> studied facial movements during different facial expressions, using 3D imaging. The movement of closing the eyes caused deviations of landmarks around the mouth of 2-4mm and smiling led to a deviation of 2-4mm in landmarks around the eyes. This indicates again how subtle movements can cause deviations in landmarks.

### Repeatability and reliability

To test the repeatability of the scans, we assessed the intraday variability. In both transwomen and transman, intraday color maps were mostly green colored, indicating no deviations. Most deviations in the color maps were seen in the mouth and eye regions, the areas with the most deviation during facial expressions <sup>28</sup>. This supports the hypothesis that the deviations found after 3 months are caused by movement of the face. Mean absolute deviations were calculated for each pair of scans with an median of 0.22 mm (IQR 0.16 – 0.27). Ozsoy et al. <sup>29</sup> studied

the symmetry of the face and excluded scans with a mean absolute deviation > 0.30mm. This indicates a good repeatability of our scans.

The reliability of the landmarks was tested by calculating the intraclass correlation coefficients (ICC per landmark are presented in supplemental Table 3). The median of the ICCs of each landmark was 0.80 (IQR 0.34 – 0.95), which indicates a good reliability<sup>30</sup>. The landmarks with a poor reliability (ICC < 0.5) in  $\geq 2$  axes were the labius superior and inferior, the right exocanthion, both cheeks, both cheilions, and both gonion points. The landmarks with excellent reliability (>0.9) in  $\geq 2$  axes were the nasion, pronasal, sublabial, both frontotemporal points, exocanthion left, tragus right, and both alar crest points. Unfortunately, this indicates a poor reliability of the landmarks of interest and questions the absolute deviations. However, the landmarks deviated in the same direction at 3 and 12 months, suggesting an actual change. Furthermore the color maps present the same deviations in the same direction, supporting the results of the landmarks.

### **Differences among medication types**

In our study, we found a larger decrease of cheek tissue in TG users in comparison to TE users and more increase in cheek tissue in EP users in comparison with ET users. These results do not seem to be in line with previous research. Klaver et al.<sup>2</sup> found that body fat in transmen decreased most in TE users, followed by TU users, and then TG users. In transwomen body fat increased most in ET users and did not increase in EP users. This difference could be due to differences in hormone levels instead of medication type. This is difficult to study, because hormone levels change during hormone treatment, especially in TE and TU users, but also in TG and estrogen users. Another explanation could be the difference in function and phenotype of sub- cutaneous fat depots in the body and the face<sup>31</sup>. Due to the small numbers in each group, this subgroup analysis and the subgroup analyses on age and weight were not powered to detect significant differences.

### **Questionnaires**

In transwomen the FACE-Q scores increased 13.1 points, and, in transmen, the score increased with 8 points. The clinical impact of this increase remains difficult to define. The FACE-Q questionnaire is developed to evaluate outcome of facial aesthetic surgery and scores for a general population are not available. Previous studies find an increase in FACE-Q scores from 41.2 to 72.9 points after a combination of fillers, onabotulinum toxin A and bimatoprost<sup>32</sup>, from 48.4 to 72.9 points after orthognathic surgery<sup>33</sup>, from 47.2 to 73.7 points after rhinoplasty<sup>34</sup>. The scores at baseline are comparable with our findings before hormone

treatment. The increase in FACE-Q scores after 1 year of hormone treatment appears to be approximately half of the increase after cosmetic interventions. The individual amount of deviation in the landmarks of interest did not correlate with the change in FACE-Q score. Only in transwomen, less decrease in jaw tissue was correlated with a higher FACE-Q score, which is the opposite of what we expected.

The scores of the Rosenberg self-esteem questionnaire remained the same for transwomen and transmen after 3 and 12 months. We anticipated that self-esteem would increase with a higher satisfaction; however this was not the case. These results are not in line with previous research; Gorin-Lazard et al. found a significantly higher self-esteem in transgender persons treated with 12 months of hormones than without this treatment <sup>35</sup>. That study included 67 transgender persons of whom 73% received hormone treatment. Furthermore, they used a different outcome measure, the Social Self-Esteem Inventory. Further research is necessary to determine whether hormone treatment increases self-esteem.

### **Strengths and limitations**

The strength of this study is the introduction of a method for the objectification of facial changes due to gender-affirming hormone treatment. To our knowledge, this is the first study to investigate these effects. The number of landmarks assessed enables a precise analysis of all aspects of the face. Additionally, the color maps provide a visualization of the changes overall. The method is a promising tool for further research. Our study has several limitations. First, as described above, the landmarks of interest have a poor reliability; this method should be improved. Second, this method is able to detect very small displacements of the face, detecting the smallest movements, including facial expressions. Fixation of the face, including the facial expression, is fairly impossible. Despite our efforts, movement of the skin was found in areas with more muscles, located mostly around the mouth and eyes. Third, we did not compare the results of the participants with a control group. Finally, the FACE-Q questionnaire was designed to measure the satisfaction of patients after aesthetic procedures, but not after facial changes induced by hormone treatment. The participants of this study perceive an imbalance between their actual face and the face of their experienced gender, but they might be satisfied with the symmetry or freshness of their face. A questionnaire that contains questions about the experienced or perceived femininity or masculinity of the face could provide more insight into the satisfaction with the face.

### **Clinical implications**

The results of this study could lead to more realistic expectations of facial changes in transgender persons. Furthermore, we found deviations in the landmarks of interest between the 3 months and 12 months mesh, suggesting that the face continues to change for at least a year and possibly longer. This could implicate that performing facial feminization surgery after 1 year of hormone treatment might be too early.

### **Future research**

Future research is needed on long-term effects, to examine when the facial changes will stabilize and to indicate where in the process facial feminization surgery should be performed, if desired. Moreover, facial proportions do not only differ between the sexes, but also between ethnic and racial groups <sup>36</sup>; future research should examine this. Furthermore, the relation between androgen and estrogen levels and facial changes should be studied. Finally, 3D scanning can only provide information about the surface of the face; other imaging techniques should be used to determine changes in specific tissues.

### **Conclusion**

This study indicates that, in both transwomen and transmen, the face changes under the influence of gender-affirming hormone treatment. These facial changes are into the direction of the desired sex characteristics, inducing facial feminization in transwomen and facial masculinization in transmen. 3D facial scanning techniques are promising and could, with some adjustments, detect small facial changes. Furthermore, gender-affirming hormone treatment increases the satisfaction with facial appearance in both transwomen and transmen. Further research is needed to provide more insight into long-term effects, changes in specific tissues, and the association with self-esteem.

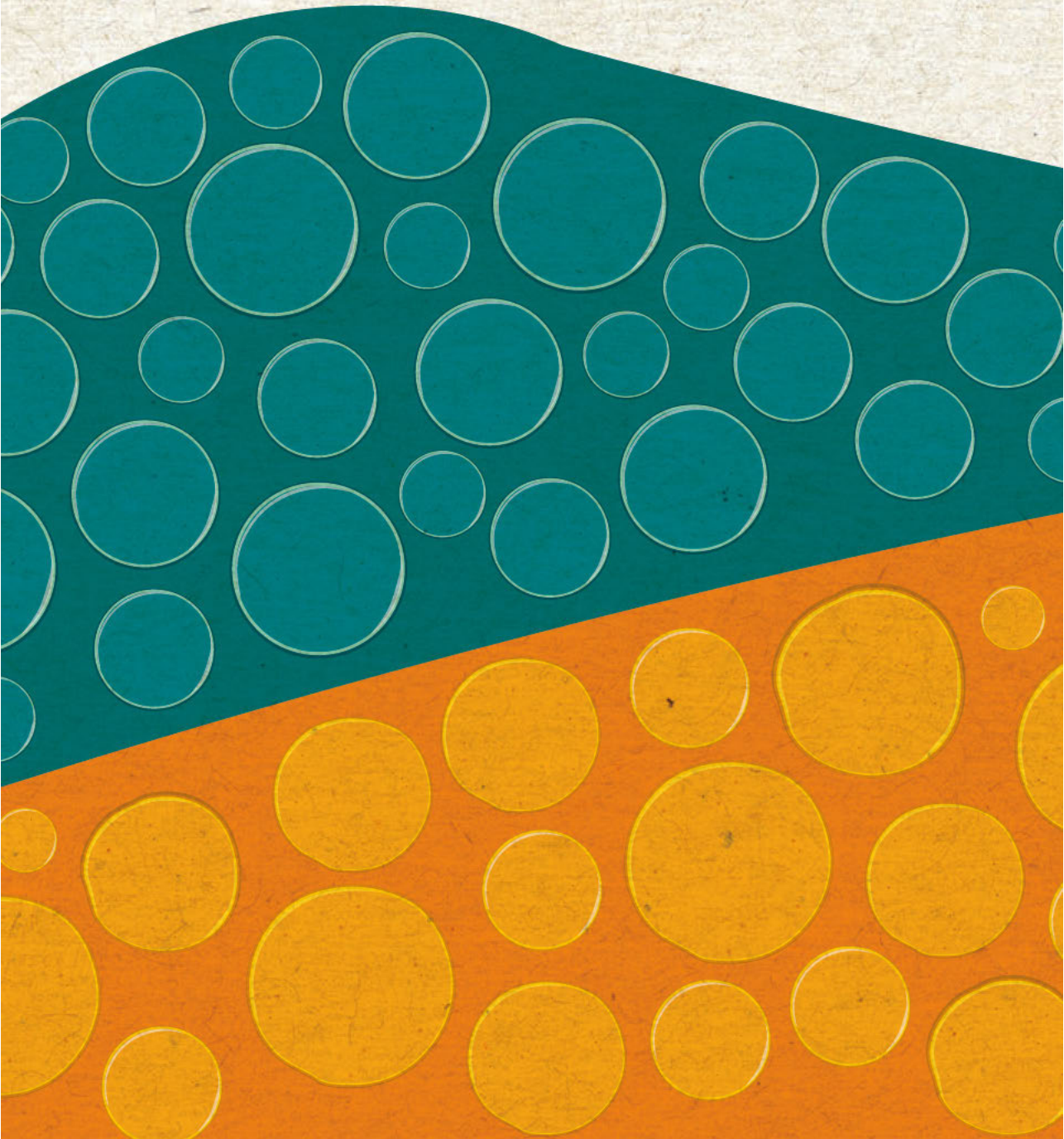
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Hormone treatment induces facial feminization and masculinization in trans persons

# CHAPTER 8



## Summary and general discussion



## SUMMARY OF MAIN FINDINGS

In **chapter 2** we showed that serum estrone concentrations are not associated with change in body fat and breast development in trans women receiving either oral or transdermal estradiol. Moreover, no difference in clinical outcomes was observed between oral and transdermal administration of estradiol, despite the fact that oral estradiol was clearly associated with much higher estrone levels compared with transdermal estradiol. In **chapter 3** we demonstrated that in trans women, suppressing testosterone and increasing estradiol increases subcutaneous fat and decreases the VAT/SAT ratio and liver fat. In trans men, the increase in testosterone concentrations in combination with lower estradiol concentrations results in an increase in visceral fat, VAT/SAT ratio and liver fat. In **chapter 4** we demonstrated that DXA estimations of visceral fat volume are strongly correlated with MRI quantified visceral fat, both cross-sectional and longitudinal, in both trans women and trans men. However, longitudinally, we did find a systematic bias, indicating that DXA-estimated visceral fat underestimates the actual change in visceral fat, as determined by the validated technique of MRI. In **chapter 5**, we observed a rapid increase in bone marrow fat fraction after testosterone and estradiol suppression in trans women. Furthermore, the bone marrow fat fraction decreased just as rapidly after start of estradiol treatment and remained around baseline values throughout the study. In trans men, on the other hand, we observed a rapid increase in bone marrow fat fraction after testosterone treatment and suppression of estradiol by triptorelin and anastrozole. In trans men without anastrozole, the bone marrow fat fraction increased only after 12 weeks and decreased to baseline after 52 weeks. In **chapter 6** we demonstrated that estradiol does not affect number of adipocytes, area of lipid droplets or adipokine expression in cell culture. Finally, in **chapter 7** we observed that gender affirming hormone treatment induces facial feminization in trans women and facial masculinization in trans men. These changes were mostly seen in the cheeks, the area that consists mostly of adipose tissue.

## Discussion

In this thesis I assessed the effect of sex steroids on body fat distribution in transgender persons. In trans women, suppressing testosterone and increasing estradiol increases subcutaneous fat, both abdominal and facial; and decreases the VAT/SAT ratio, liver fat content and amount of bone marrow fat. In trans men, increasing testosterone and decreasing estradiol concentrations increases the amount of visceral fat, the VAT/SAT ratio, liver fat content and the amount of bone marrow fat, and has no effect on subcutaneous fat. Table 1 summarizes the current

knowledge (including results from this thesis) on changes in different fat depots during several states of change in hormone concentrations. This shows that our results are mostly in line with previous studies. These studies combined suggest that visceral, liver and bone marrow fat respond similarly to changes in hormone concentrations. Furthermore, these results suggest that estradiol is the major sex steroid regulating body fat distribution in both women and men. An increase in estradiol is associated with a decrease in visceral, liver and bone marrow fat and a decrease in estradiol is associated with an increase in visceral, liver and bone marrow fat. Our only finding that is not in line with previous studies, is that we did not observe a decrease in visceral fat in trans women in chapter 4. This might be explained by the actual change in serum estradiol concentrations in trans women. In chapter 4, we hypothesized that visceral fat might have a threshold to respond to estradiol concentrations. In a small post analysis we observed that in trans women with a mean estradiol concentration above 300 pmol/L (n=3), visceral fat volume decreased significantly after one year, while in trans women with a mean estradiol concentration below 300 pmol/L (n=5), visceral fat volume increased significantly. However, these groups are too small to draw any conclusions. The hypothesis that visceral fat has a threshold for estradiol would be in line with the mechanism in bone mineral density, where Wiepjes et al <sup>1</sup> suggested a threshold of 200 pmol/L estradiol.

**Table 1.** Changes in sex steroid concentrations and fat tissues in several states of change in hormone concentrations.

|                     | Trans women        | Trans men       | Postmenopausal women | Postmenopausal women + HRT | Hypogonadal men   | Aromatase deficient men |
|---------------------|--------------------|-----------------|----------------------|----------------------------|-------------------|-------------------------|
| Estradiol           | ↑ <sup>30</sup>    | ↓ <sup>30</sup> | ↓ ↓ <sup>31,32</sup> | ↑ <sup>33</sup>            | ↓                 | ↓ ↓                     |
| Testosterone        | ↓ <sup>30</sup>    | ↑ <sup>30</sup> | = <sup>31,32</sup>   | = <sup>34</sup>            | ↓                 | =                       |
| Fat tissue          |                    |                 |                      |                            |                   |                         |
| <i>Subcutaneous</i> | ↑ <sup>30,35</sup> | = <sup>30</sup> | ↑ <sup>2</sup>       | ↓ <sup>2</sup>             | ↑ <sup>3,36</sup> | ↑ <sup>3</sup>          |
| <i>Visceral</i>     | = <sup>16,30</sup> | ↑ <sup>30</sup> | ↑ <sup>2</sup>       | ↓ <sup>33,37</sup>         | ↑ <sup>3,36</sup> | ↑ <sup>3</sup>          |
| <i>Liver</i>        | ↓ <sup>30</sup>    | ↑ <sup>30</sup> | ↑ <sup>38,39</sup>   | ↓ <sup>38,39</sup>         | ↑ <sup>40</sup>   | ↑ <sup>41-44</sup>      |
| <i>Bone marrow</i>  | ↓ <sup>45</sup>    | ↑ <sup>45</sup> | ↑ <sup>46</sup>      | ↓ <sup>47</sup>            | ↑ <sup>48</sup>   | ?                       |

Abbreviations: HRT: hormone replacement therapy. ↑: increase in this variable, ↓ decrease in this variable, = no change in this variable. References are shown between brackets.

Table 1 further shows that subcutaneous fat responds differently to changes in estradiol in trans gender persons compared to postmenopausal women (with or without hormone replacement therapy (HRT)), hypogonadal men and aromatase

deficient men. In these last groups, an increase in estradiol is associated with a decrease in subcutaneous fat <sup>2</sup> and a decrease in estradiol with an increase in subcutaneous fat <sup>2,3</sup>. However, the increase in subcutaneous fat in trans women is in line with the increase in subcutaneous fat in girls during puberty, when they are first exposed to high concentrations of estradiol <sup>4</sup>. This could indicate that the increase in subcutaneous fat in trans women is temporary and a one-time phenomenon, however, no long term studies on subcutaneous fat in trans women have been performed until now. The stable subcutaneous fat volume in trans men could be explained by gradual increase of abdominal subcutaneous fat with aging in both women and men <sup>5</sup>. Unfortunately, we included no control groups in our study in chapter 4 and can therefore not distinguish between the effects of sex steroids and time.

Obesity and fat distribution are influenced by multiple variables such as socioeconomic status, education and ethnicity, which makes it difficult to determine whether the difference in fat distribution between women and men is due to differences in hormones or genetics. However, the findings in this thesis in combination with the changes in different fat depots during several states of change in hormone concentrations, indicate that estradiol has a major role in the regulation of body fat distribution in women and men. In **chapter 6** we observed that estradiol has no direct effect on adipocyte number, lipid droplet area and adipokine expression in bone marrow adipocytes. A possible explanation is that estradiol exerts its effect on adipose tissue earlier in the differentiation of adipocytes <sup>6,7</sup>. Another explanation is that the effect of estradiol on mature adipocytes is mediated through another cell type. Finally, the effect of dramatic changes in estradiol levels on adipose tissue such as occur after the menopause in women, may in fact be mediated through shifts in hormone levels other than estradiol. For example, the rapid increase in marrow adipose tissue during menopause may be caused by a rise in follicle stimulating hormone (FSH). FSH induces accelerated formation of lipid droplets in 3T3-L1 preadipocytes *in vitro*, and accelerated lipid droplet formation in primary human abdominal adipocytes <sup>8</sup>. As far as I know, the effect of changes in FSH on body fat distribution during hormone treatment has not been studied in transgender persons.

Multiple other possible mechanisms by which estradiol influences the body fat distribution have been proposed. Estradiol suppresses appetite and increases energy expenditure by enhancing anorectic signals such as leptin, cholecystokinin and apolipoprotein A-IV and by decreasing orexigenic signals such as ghrelin and melanin-concentration hormone (reviewed in <sup>9</sup>). This is supported by

the observations in animals and humans that energy intake changes with the menstrual cycle and that this is the lowest when estrogen concentrations peak (reviewed in <sup>10</sup>). Further, a decrease in estradiol is associated with a decrease in energy expenditure and fat oxidation <sup>11</sup>.

### Clinical implications

In this thesis I have examined the effects of estradiol and testosterone on body fat distribution in transgender persons, receiving hormone treatment. Firstly, this thesis shows that testosterone treatment and thereby lowering estradiol concentrations in transmen is associated with an increase in visceral fat, liver fat content and bone marrow fat. As mentioned before, in cis men, visceral and liver fat are associated with insulin resistance and metabolic and cardiovascular risks. However, previous studies in trans men did not find a clear increased risk of cardiovascular disease <sup>12,13</sup>. It should be stated that these studies had a mean follow-up of 8.1 and 3.6 years per person, respectively, and most participants were young, while the risk of cardiovascular disease increases with age <sup>14</sup>. And even though a previous study in trans men did observe an unfavorable change in lipid profile <sup>15</sup>, a recent study by Klaver et al., observed that these unfavorable changes in lipid profile and decreased insulin resistance, were not related to the amount of increase in visceral fat <sup>16</sup>. However, these studies are all short-term and therefore the cardiometabolic risks of increased visceral fat in trans men remain largely unknown. Therefore it is important for transgender persons and clinicians to be aware of this increase in visceral fat and clinicians should incorporate lifestyle advice and body weight management in their treatment of transgender persons. In **chapter 4**, we measured liver fat content. At baseline, already two trans men met the criterion for liver steatosis (liver fat content  $\geq 5\%$ ) and two trans men increased in liver fat content during the study and met the liver steatosis criterion after 3 or 12 months. This means the prevalence of liver steatosis in our cohort of trans men was 22%. In the Netherlands, the prevalence of liver steatosis is 20% <sup>17</sup>. However, in this large Dutch cohort, persons with suspected liver steatosis had a median age of 47 years, a median BMI of 30.8 kg/m<sup>2</sup> and liver steatosis was estimated by using the Fatty Liver Index (FLI), with an accuracy of 0.84 <sup>18</sup>. In our cohort the median age in trans men was 22 years old and the median BMI was 25.4 kg/m<sup>2</sup>. The four trans men who had a liver fat content  $\geq 5\%$  were aged between 19 and 25 years and had a BMI between 27.5 and 36.4 kg/m<sup>2</sup>. Besides the cardiometabolic risks, the main risk of liver steatosis is progression into fibrosis, cirrhosis or hepatocellular carcinoma <sup>19</sup>. There are currently no global consensus guidelines to screen for liver fibrosis in patients considered at high risk <sup>20</sup>. This is due to uncertainties about diagnostic tests, treatment options and lack of evidence

of the long-term benefits and cost-effectiveness <sup>20</sup>. Furthermore, our short-term study is the first to assess liver fat content in trans gender persons. It is unknown whether this increase in liver fat content will continue, stabilize or even decrease again. Therefore, screening for liver steatosis has currently no place in monitoring hormone treatment in trans men.

Previous studies have shown that visceral fat and bone marrow fat are positively associated and are both negatively associated with bone mineral density (BMD) <sup>21,22</sup>. Furthermore, liver fat is also negatively associated with BMD <sup>23</sup>. Luckily, Wiepjes et al, studied trans men who used hormone treatment for 10 years and found no change in BMD <sup>1</sup>. Therefore, with the knowledge of this moment, the increase in bone marrow fat does not seem to have any major clinical implications for trans men. Finally, this thesis is the first to show that gender affirming hormone treatment induces facial feminization in trans women and facial masculinization in trans men. These results contribute to more accurate expectations by transgender individuals and can be implemented in information provided by clinicians. Furthermore, our study suggest that these facial changes continue after one year of hormone treatment, which has implications for the best timing for facial feminization surgery.

## **Future research**

In this thesis, I focused on the short-term (weeks) effects of sex steroids on body fat distribution in transgender persons. It remains unknown whether the changes in adipose tissue will stabilize or not. Most health risks associated with adipose tissue, e.g. cardiovascular disease and osteoporosis, are most prevalent later in life. Since the transgender persons participating in studies in this thesis were mostly in their twenties, it is important to perform long-term follow-up studies to assess the risk of major health issues later in life. Further, future research should focus the relation between health risks such as increased visceral fat, bone marrow fat and liver fat, and the actual complications, in trans gender persons. For now, it remains unknown if an increase in these risk factors yields the same risks in cis persons and transgender persons.

As mentioned above, another topic of interest for future research could be the effect of FSH on body fat distribution. A previous *in vitro* study, demonstrated that FSH induces a more rapid formation of lipid droplets in preadipocytes, and upregulates genes involved in synthesis of fatty acids and triglycerides in these cells <sup>8</sup>. Recently, a clinical study by Veldhuis-Vlug et al., found that in postmenopausal women, higher FSH concentrations are associated with more

bone marrow fat and lower total body fat <sup>24</sup>. This indicates that FSH is another important sex steroid in the regulation of marrow adipocytes. The effect of FSH has also been studied in other fat depots, Liu et al., found that FSH increased adipocyte size in animal and human adipose tissue and more in visceral fat than in subcutaneous fat <sup>8</sup>. Future studies in transgender persons should assess whether FSH plays a role in body fat distribution and *in vitro* studies should elucidate the effect of FSH on adipokine expression.

Finally, estradiol seems to be protective against an increase fat depots associated with increased health risks such as visceral fat, liver fat content and bone marrow fat. However, treatment with estradiol is associated with increased risks of venous thrombosis, breast cancer, albeit small increases <sup>25</sup>. Therefore, future research should focus on compounds that have an agonistic effect on estrogen receptors in adipose tissue, but antagonistic effects on other tissues, such as breast tissue. Selective estrogen receptor modulators (SERMs) are a family of compounds that are capable of exerting estrogenic agonist or antagonist effects different tissues <sup>26</sup>. Raloxifene for instance, is a SERM that acts as an estrogenic antagonist in breast tissue, but acts as an estrogenic agonist on other tissues such as bone, where it prevents bone loss by negatively modulating osteoclasts, and positively affecting osteoblasts <sup>27</sup>. An animal study showed that Raloxifene significantly decreased body weight, visceral fat and adipocyte size <sup>28</sup>. Furthermore, a randomized controlled trial in postmenopausal women demonstrated that Raloxifene shifted fat distribution from android to gynoid and reduced visceral adiposity <sup>29</sup>. Future research should further unravel the precise mechanism of action of these SERMs and assess potentially adverse effects.

## Conclusions

In conclusion, this thesis has shown that in trans women, suppressing testosterone and increasing estradiol results in an increase in subcutaneous fat, both abdominal and facial; and a decrease in VAT/SAT ratio, liver fat and bone marrow fat. In trans men, an increase in testosterone concentrations in combination with a decrease in estradiol concentrations results in an increase in visceral fat, VAT/SAT ratio, liver fat and bone marrow fat, and no significant changes in subcutaneous fat. These results suggest that estradiol is the major sex steroid regulating body fat distribution in women and men. However, estradiol does not seem to directly affect mature adipocytes with respect to adipocyte number, lipid droplet area or adipokine expression. The effect of testosterone on body fat distribution acts mainly through the aromatization of testosterone into estradiol.

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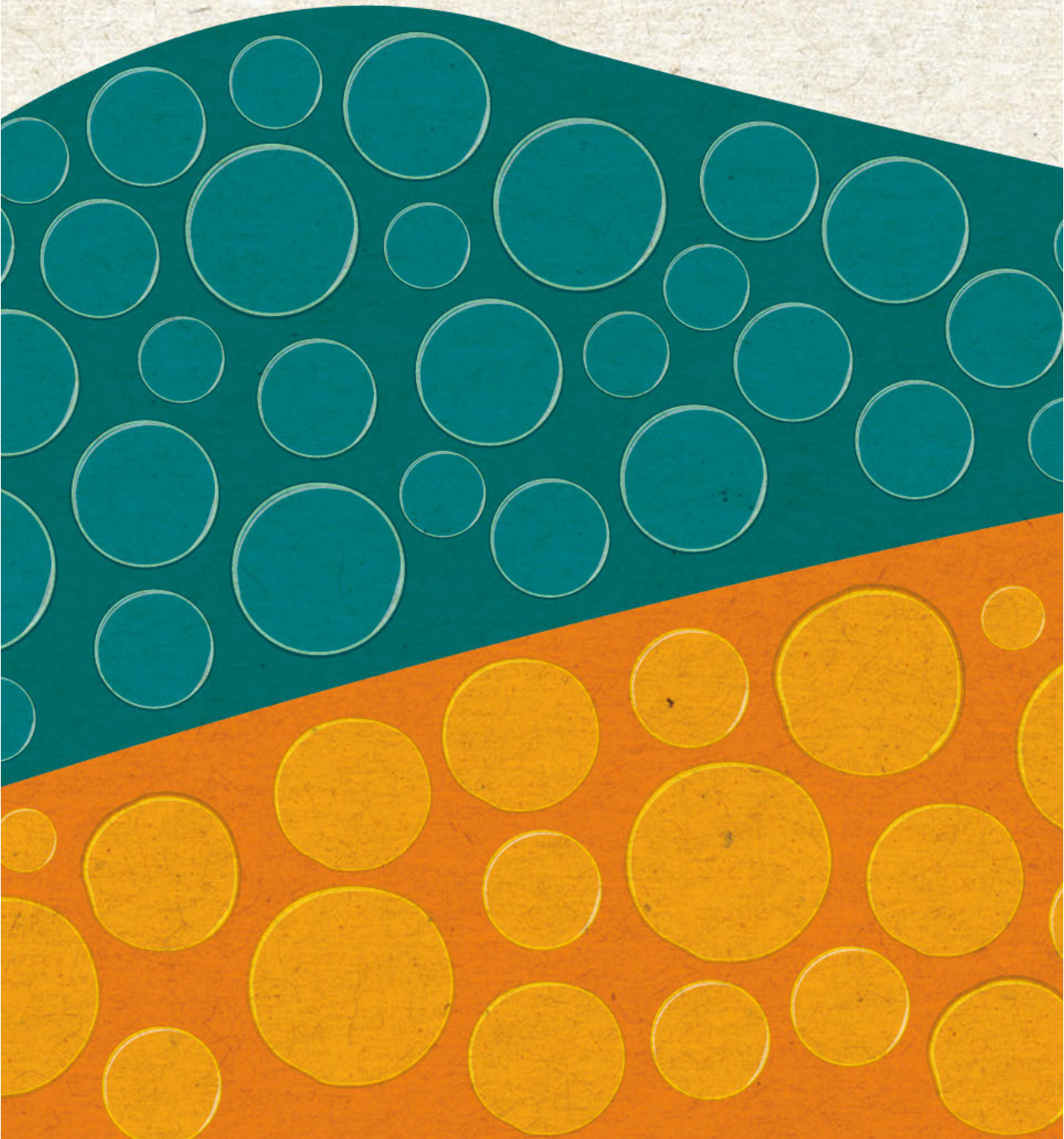
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# ADDENDUM





## SUMMARY

### Introduction

It has been well established that the distribution of regional body fat is associated with major health problems. Visceral adipose tissue and liver fat content are associated with cardiovascular disease and bone marrow adipose tissue is associated with osteoporosis. The regulation of body fat distribution is multifactorial and a major group of regulators are sex steroids. Sex steroids or sex hormones are a group of steroid hormones that include estrogens, androgens and progestogens. In this thesis we focus on estrogens and androgens, more specifically estradiol and testosterone. The ratio between these hormone concentrations differs for women and men. Women typically have a high serum concentration of estrogens that fluctuates with the menstrual cycle and a low serum concentration of androgens. In contrast, men have a stable low serum concentration of estrogens and a high serum concentration of androgens. In transgender persons receiving gender affirming hormone treatment, the ratio of these sex steroids changes during their transition. Transgender persons are people where the sex assigned at birth does not match the experienced gender. In this thesis we use the terms trans women, males assigned at birth, identify as female; and trans men, females assigned at birth, identify as male. Gender affirming hormone treatment has a large impact on adipose tissue distribution. The overall objective of this thesis is to investigate the effects of sex steroids on body fat distribution in transgender persons. This provides us with the opportunity to not only separate the effects of genetics and sex steroids on body fat distribution, but also the effects of estradiol and testosterone. In this thesis, we first examine which estrogens exert effect on body fat distribution in **chapter 2**. We then assess the effects of sex steroids on liver fat content and visceral fat in **chapter 3** and compare two methods to estimate visceral fat in **chapter 4**. In **chapter 5** we assess the effect of sex steroids on bone marrow fat *in vivo* and in **chapter 6** we assess the effects of estradiol on bone marrow adipocytes *in vitro*. Finally, in **chapter 7**, we study the effect of sex steroids on soft tissue in the face.

## Main findings

In **chapter 2** we showed that estrone concentrations are not associated with change in body fat or breast development or in trans women receiving either oral or transdermal estradiol. Moreover, no difference in clinical outcomes was observed between oral and transdermal administration of estradiol, despite the fact that oral estradiol was clearly associated with much higher estrone levels compared with transdermal estradiol. This suggests that only estradiol and not estrone exerts effect on body fat distribution. In **chapter 3** we demonstrated that in trans women, suppressing testosterone and increasing estradiol results in an increase in subcutaneous fat and a decrease in VAT/SAT ratio and liver fat. In trans men, we showed that the increase in testosterone concentrations in combination with lower estradiol concentrations results in an increase in visceral fat, VAT/SAT ratio and liver fat. In **chapter 4** we demonstrated that DXA is a valid method to estimate visceral fat in trans women and trans men at baseline and after 12 months of hormone treatment. However, the change in visceral fat after 12 months of hormone treatment is underestimated by DXA, compared to MRI. In **chapter 5**, we demonstrated a rapid increase in bone marrow fat fraction after testosterone and estradiol suppression in trans women, followed by a rapid decrease after start of estradiol treatment. In trans men, on the other hand, we observed a rapid increase in bone marrow fat fraction after testosterone treatment and suppression of estradiol by triptorelin and anastrozole. In trans men without anastrozole, the bone marrow fat fraction increased only after 12 weeks and decreased to baseline after 52 weeks. Together these results suggest that estradiol and not testosterone is the major sex steroid regulating the bone marrow fat fraction in both women and men. In **chapter 6** we demonstrated that this effect of estradiol on bone marrow fat is not caused by a change in number of adipocytes, lipid droplet area or adipokine expression. The effect of estradiol on bone marrow fat might be exerted earlier in the differentiation of adipocytes or via other cell types. Finally, in **chapter 7** we observed that gender affirming hormone treatment induces an increase in cheek tissue in trans women and a decrease in cheek tissue in trans men. The cheeks consist mostly of subcutaneous adipose tissue and therefore we can conclude that sex steroids exert the same effect on subcutaneous tissue in the abdomen and the face.

## LIST OF PUBLICATIONS

**Tebbens M**, Heijboer AC, T'Sjoen G, Bisschop PH, den Heijer M. *The Role of Estrone in Feminizing Hormone Treatment*, The Journal of Clinical Endocrinology & Metabolism, Volume 107, Issue 2, February 2022, Pages e458–e466, doi.org/10.1210/clinem/dgab741

**Tebbens M**, Nota NM, Liberton NPTJ, Meijer BA, Kreukels BPC Forouzanfar T, Verdaasdonk RM, den Heijer M. *Gender-Affirming Hormone Treatment Induces Facial Feminization in Transwomen and Masculinization in Transmen: Quantification by 3D Scanning and Patient-Reported Outcome Measures*. The Journal of Sexual Medicine 2019;16(5):746-754. doi:10.1016/j.jsxm.2019.02.011

## CURRICULUM VITAE

Marieke Tebbens was born on the 4th of February 1991 in Apeldoorn, The Netherlands. She graduated from the Stedelijk Gymnasium Apeldoorn in 2008 with the profile Nature and Health. In 2008 she moved to Groningen to start her medical school and experience the joys of student life. During her bachelor she joined IFMSA (the International Federation of Medical Student Associations) in the workgroup SCOPH (Standing Committee on Public Health). As president of this workgroup, she was involved in organizing medical movie nights, recruiting new blood donors and organizing an international IFMSA meeting. Further, she went on a clinical internship, hosted by IFMSA, to Marilia, Brazil. In 2011 she decided to study philosophy, a field which had her interest for a long time. In 2012 she finished her bachelor 'Philosophy of a specific discipline' with a bachelor thesis on 'The experience of a wanted act'. In 2012 she started with her master in medicine, first in the UMCG (University Medical Center Groningen) and later in the MST (Medical Spectrum Twente) in Enschede. For her social medicine internship, she went to Mangochi, Malawi, where she conducted interviews and educated school children about malaria and HIV. In 2015 she moved to Amsterdam, to perform research in transgender medicine for her master thesis titled 'Facial feminization and masculinization: The effects of cross-sex hormone treatment on facial features in individuals with gender dysphoria'. After she graduated her masters in medicine in 2016, she worked as a medical doctor on the internal ward. In 2017 she was drawn back to research and started a PhD program in the VU medical center, supervised by Martin den Heijer, Peter Bisschop and Astrid Bakker. During her PhD program, Marieke worked as a medical doctor in the Center of Expertise on Gender Dysphoria and was a member of the PhD students committee. Currently, Marieke lives in Amsterdam and works as a medical doctor.

## PORTFOLIO OF EDUCATION

| Date       | Activity                                                | EC    |
|------------|---------------------------------------------------------|-------|
| 11-11-2021 | Oral presentation NVCB                                  | 1,00  |
| 06-11-2020 | Oral presentation WPATH                                 | 2,00  |
| 28-10-2020 | V20: principes van epidemiologische data-analyse        | 3,00  |
| 23-04-2019 | The art of scientific writing                           | 2,00  |
| 09-04-2019 | Writing a Scientific Article                            | 3,00  |
| 12-03-2019 | Training lab skills (AMC)                               | 0,50  |
| 19-12-2018 | MRI veiligheidstraining (AMC)                           | 0,50  |
| 22-11-2018 | AMS - Research Integrity                                | 2,00  |
| 05-11-2018 | Oral presentation WPATH (Argentina)                     | 2,00  |
| 18-09-2018 | Epidemiologisch onderzoek: opzet en interpretatie (V10) | 4,00  |
| 08-09-2018 | ECTS - PhD training course                              | 2,00  |
| 05-04-2018 | Oral presentation PhD retreat                           | 1,00  |
| 21-03-2018 | Training lab skills (ACTA)                              | 0,50  |
| 16-02-2018 | Annual meeting MOVE, visitor                            | 0,50  |
| 26-01-2018 | Oral presentation DEM                                   | 2,00  |
| 19-05-2017 | GNK - BROK                                              | 2,00  |
| 01-04-2017 | AMS - Received supervision by (daily) supervisor        | 2,86  |
| Total      |                                                         | 30,86 |

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