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ARTICLE DRAFT

Hormone therapy associates with better body composition and adipokine/glucose profiles: A study with monozygotic co-twin control design

Running title: HT and improved insulin sensitivity

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Abstract

Objective: To evaluate the possibilities to prevent the metabolic health consequences of postmenopausal hypogonadism with long-term hormone therapy (HT).

Methods: We used a monozygotic co-twin control design including ten twin pairs discordant for HT (aged 56-62 years; duration of HT 2-10 years). Additionally, fourteen premenopausal women with no hormone use were studied (aged 29-35 years) in order to evaluate the differences in metabolic health between the pre- and postmenopausal state. Body composition was determined and waist/hip ratio was used as an estimate for fat distribution. Serum sex steroids, sex hormone-binding globulin, and serum lipid and glucose profiles were analyzed. The serum levels of adiponectin, monocyte chemotactic protein-1 and leptin as well as their local transcript levels in adipose tissue, skeletal muscle and leukocytes were measured.

Results: Long-term HT was associated with healthier amount and distribution of body fat. No difference was seen in serum lipid concentrations between HT users and their identical non-using twin-sisters, but fasting serum glucose and glycated hemoglobin levels were 5 and 3% lower in HT users than non-users. Among the adipokines analyzed, the most notable finding was a 15% lower level of monocyte chemotactic protein-1 in HT users, particularly with respect to its suggested mediator role between obesity and insulin resistance.

Conclusions: Long-term HT is associated with healthier amount and distribution of body fat and better adipocytokine profile with concomitant signs of improved insulin sensitivity.

Key words: menopause, estrogen, adipose tissue, adipokines, glucose metabolism

Introduction

The menopausal transition includes the permanent cessation of the function of the ovaries, leading to a decline in estrogen and progesterone production. Postmenopausal hypogonadism results in changes in body composition and body fat distribution i.e. increased total and central fat (1, 2). Furthermore, the accumulation of abdominal fat is associated with increased insulin resistance (3), predisposing women to increased risk for metabolic syndrome and cardiovascular diseases. Concomitantly with the abrupt decline in estradiol levels, a smaller decline in testosterone levels occurs, leading to a highly increased testosterone-to-estrogen ratio (4). Women with polycystic ovary syndrome represent an example of hormonal pattern of androgen excess, and are related to high prevalence of cardiovascular risk factors and metabolic syndrome (5, 6). Likewise, increasing evidence of harmful health effects of androgen excess on postmenopausal women exists (7-9).

During weight gain, the overload of lipids in adipocytes contributes to the imbalance in the expression of pro- and anti-inflammatory adipokines in adipose tissue (10-12) and further affects the pathogenesis of obesity-related disorders (13). Although adipose tissue is mainly comprised of adipocytes, obesity is also associated with increased accumulation of macrophages into adipose tissue. One of the adipokines responsible for monocyte recruitment into adipose tissue is monocyte chemotactic protein-1 (MCP-1 or CCL2), which attracts monocytes from the circulation into tissues where they transform into macrophages (14). Consequently, the increased infiltration of macrophages into adipose tissue further reinforces the production of inflammatory factors (15). At present, MCP-1 is, in addition to its primary chemoattractant role at sites of inflammation, known to play a critical role in the development of insulin resistance and cardiovascular diseases (16).

A logical approach to the restoration of the hormonal balance at menopause has been estrogen-containing hormone therapy (HT). Besides relieving menopausal symptoms, HT might increase breast cancer risk. Furthermore, debate on the cardiovascular effects is ongoing. Based on several observational studies, HT was thought to be beneficial for cardiovascular system by reducing coronary heart disease events (reviewed in 17). Benefits have been suggested to arise from metabolic changes, e.g. the amount of abdominal fat, dyslipidemia and insulin resistance, (18, 19) and decrease in low-grade inflammatory state (20, 21). Contrary, large experimental trials (WHI study, HERS I and II) (22-24) showed negative cardiovascular effects of HT. Contradiction between these observational and experimental studies can depend on the woman's health status and the type of HT, e.g. timing and duration of the treatment, dosage, type of delivery and hormones involved. The importance of timing, as being the major difference between WHI and observational studies, is currently under consideration in the Kronos Early Estrogen Prevention Study (KEEPS) (17).

In the present study, we used a monozygotic (MZ) co-twin case-control design, in which co-twins using HT are compared with their genetically identical non-using co-twins. In addition to their genome sequence, MZ co-twins are matched by gender, age, intrauterine environment, and many childhood exposures and experiences. Our aim was to clarify the possible metabolic health consequences of changes in hormonal milieu under the condition of long-term HT use. Body composition, serum sex steroids, sex hormone-binding globulin (SHBG), triglycerides, cholesterols, glucose profile (fasting glucose, glycated hemoglobin (HbA1c) and insulin) and adipokines (adiponectin, MCP-1 and leptin) were analyzed. Also, the local transcript levels of the same adipokines were analyzed in adipose tissue, skeletal muscle and leukocytes. In addition to twin pairs, we recruited a group of premenopausal women not using any hormonal contraceptives to examine the association of metabolic factors with age.

Materials and methods

Study design and participants

This study is a part of a larger research project, "Sarcopenia and Skeletal Muscle Adaptation to Postmenopausal Hypogonadism: Effects of Physical Activity and Hormone Replacement Therapy in Older Women – a Genetic and Molecular Biology Study on Physical Activity and Estrogen-related Pathways" (SAWEs)-study, investigating the molecular events involved in maintaining proper muscle mass and function after menopause (25). The participants for this study were drawn from the Finnish Twin Cohort (n=13 888 pairs) (26). An invitation was sent to all monozygotic female twin pairs born in 1943-1952 (n=537 pairs). Only twin pairs in which one co-twin was a current HT user and the other co-twin was not currently using HT were asked to respond to the invitation. From the total of responders (n=114 pairs), all the twin pairs where one sister had never used HT, while the other sister was a current user, and who reported willingness to participate in the laboratory measurements were contacted (n=21 pairs). Finally, a total of 16 MZ pairs aged 54-62 years discordant for HT use and without any contraindications participated in the study. One twin pair turned out to be dizygotic after genetic marker studies and was excluded from the further analyses. The HT users comprised five women using estradiol-only preparations (1-2 mg), six women on combined treatment including estrogenic (1-2 mg) and progestogenic compounds, and four women using tibolone (2.5 mg). In the present study, our focus was on the estrogen deprivation and replacement (estradiol-only plus combined treatments). Therefore tibolone users were excluded from the study. For one of the HT users, the treatment was clearly not effective, since her circulating estradiol levels did not differ from the levels of the non-users. This pair was also excluded from the present study. Altogether, among 10 pairs, nine women used an oral preparation and one used a transdermal preparation. The mean duration of HT usage was 7.5 ± 3.9 years (range, 2-16 years). For the recruitment of premenopausal women, an invitation was sent to two thousand premenopausal women (representing 39.1 % of the

relevant female base population) randomly selected from the 30- to 40-year-old women (born in 1967-1977) living in the City of Jyväskylä. Finally, a group of 59 premenopausal women aged 30-40 years who met the inclusion criteria (27), and who had not been treated with hormonal contraceptives or progesterone preparations within the past five years, participated in the study. For the present study, a subgroup of 14 women aged 30-35 years was randomly selected from the original sample (PASW Statistical Software, SPSS Inc., IBM, IL, USA). The study follows the guidelines of good clinical and scientific practice as well as Helsinki declaration. Ethics Committee of the Central Finland Health Care District has approved the study on 6.6.2006. An informed consent explaining the possible risks and personal benefits associated with the examinations and a permission to use the data for research purposes and in publications was signed by the participants before the measurements.

Body anthropometry and composition

Body anthropometry was measured between 0700 and 1000 after overnight fasting with the participant wearing only undergarments. Body weight and height was measured and body mass index (BMI) calculated. Percentage body fat was measured by bio-electrical impedance analysis [InBody (720), Biospace Co. Ltd., Seoul, Korea]. Waist circumference was measured midway between the spina iliaca superior and the lower rib margin, and hip circumference at the level of the greater trochanters, both to the nearest centimeter.

Blood and tissue sampling

Muscle (*m. vastus lateralis*) needle biopsies, abdominal subcutaneous adipose tissue biopsies (needle aspiration) and a set of blood samples (whole blood, serum, plasma, leucocytes) were taken under standard fasted conditions in a supine position between 0700 and 0900, as described earlier 25. Adipose and muscle tissue samples together with leukocytes were used for the transcript analyses of adipokines. All samples were stored at –70ºC until analyzed.

Lipid and glucose measurements

Serum total cholesterol, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C) and triglycerides were measured by enzymatic colorimetric assays (KoneLab^{TM}) by KoneLab20 Clinical Chemistry Analyzer (Thermo Scientific, Vantaa, Finland). Serum glucose was determined by the hexokinase method (Thermo Scientific) by a KoneLab20 Clinical Chemistry Analyzer and serum HbA1c by a fully automated ion-exchange HPLC assay (Bio-Rad Variant II, Bio-Rad Laboratories, UK).

Hormone and cytokine measurements

Serum hormone measurements were done as described previously (25). In brief, serum estrone (E_1) was determined as a dansyl derivative by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with an API 4000 mass spectrometer, as described previously (28). Serum 17β-estradiol (E_2) levels were assessed in duplicate by extraction RIA qualified for low serum steroid levels, as described previously (29). The levels of serum testosterone (T) were measured as described previously (30). SHBG concentrations were measured with solid-phase, chemiluminescent immunometric assays (Immulite 1000, Diagnostic Products, Los Angeles, CA). E_2 and T levels together with SHBG levels were used in calculating the respective free hormone levels as described previously (31, 32). Blood concentrations of adiponectin, MCP-1 and leptin were measured by Quantikine® immunoassays (R&D Systems, Minneapolis, USA). Serum insulin was measured with Immulite 1000 Insulin reagent and an Immulite® 1000 Analyzer (DPC, Los Angeles, CA).

RNA analyses

Total RNA from muscle and leukocytes was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and from fat tissue using an RNAqueous Micro-kit (Applied Biosystems). For the microarray, RNA quality was determined using an Experion electrophoresis station (Bio-Rad Laboratories, Hercules, CA), and Sentrix Human-WG6 V3 Expression BeadChip microarrays (Illumina, San Diego, CA) were used for the transcriptome-wide expression analysis (Turku Center for Biotechnology, BTK, University of Turku). Local transcript levels for *adiponectin*, *MCP-1* and *leptin* (NM_004797.2,

NM_002982.3 and NM_000230.1, respectively) were picked out from microarray data of adipose tissue, muscle and leukocytes utilizing R software (http://www.R-project.org) together with BioConductor development software (http://www.bioconductor.org).

Statistical analyzes

Data are reported as means ± SD and *P* values ≤0.05 were considered significant. The inter-group comparisons were performed between the premenopausal women and postmenopausal non-users, while paired comparisons were conducted between the postmenopausal MZ twin sisters, i.e. the HT users and non-users. The statistical analyses for the group comparisons included either parametric independent samples t-test and paired-sample t-test, or non-parametric Mann-Whitney and Wilcoxon Signed Ranks tests, depending on the normal distribution of the means tested by the Shapiro-Wilk-test. The current study and the variables measured were based on clear hypothesis driven and theoretically reasoned study questions, which defined the above-mentioned statistical analyzes. The adjustment for body composition was done for serum lipid, glucose and insulin values by dividing with the percentage fat values, after which the differences between the groups and twin pairs were analyzed. The strength of the associations between the variables was assessed by Pearson's correlation coefficient (*r*). The data analyses were carried out with the PASW Statistic Software (SPSS Inc., IBM, IL, USA).

Results

Participant characteristics

The physical characteristics and body composition of the study participants are presented in Table 1. Among postmenopausal twin sisters discordant to HT, the percentage of fat was significantly lower in the HT users (p=0.02) and their waist and hip circumference were smaller, but not statistically significant (p=0.06 for both), when compared to non-users. The observed trend for smaller BMI in HT users than non-users (25.3 ±3.9 vs. 28.1 ±6.8; p=0.09) was not seen in 1990 (23.0 ±1.7 vs.

23.7±3.1; p=0.393), when the mean age of participants was 40 years. When the postmenopausal women were compared with the premenopausal women, the postmenopausal women had a higher waist/hip ratio (p= 0.04) and tended to have higher percentage body fat and waist circumference (p=0.09 and 0.08, respectively) than the premenopausal women.

Systemic status of sex steroids and SHBG

In the HT-using women, the serum levels of E_1, E_2, T and SHBG were, as expected, significantly higher (p<0.01, <0.01, 0.04 and <0.01, respectively) than in their identical twin sisters not using HT (Table 2). Consequently, the T/E_2 ratio in the HT users was only one-fourth of that in the non-users (p<0.01). In postmenopausal women, the serum E_1 and E_2 levels were 68 and 87 % lower and testosterone levels 45% lower compared to the premenopausal women (*p*<0.01). In addition, the testosterone/estradiol ratio (T/E_2) was 4-fold higher in the postmenopausal women than premenopausal women (p< 0.01). The serum SHBG levels were lower in the postmenopausal than in premenopausal women, but the difference did not reach statistical significance (p= 0.06).

Systemic and local levels of adiponectin, MCP-1 and leptin

In the HT-using women, serum MCP-1 levels were 15% lower than in their non-using identical co-twins (p<0.01, Table 3). Also, serum leptin levels were lower in the HT users compared to non-users, but the difference did not reach statistical significance (p=0.08). The differences in MCP-1 and leptin levels were abolished after adjustment for percentage body fat. Instead, the adjustment for percentage fat increased the difference in serum adiponectin levels between the HT users and non-users (p=0.08). The expression of *adiponectin* gene in the adipose tissue of the HT users tended to be higher than the corresponding expression in their identical co-twins (p= 0.07). In leukocytes, *MCP-1* was expressed less in the postmenopausal HT users than in their non-using co-twins (*p*=0.05). No difference was seen in serum adiponectin and leptin levels between the postmenopausal and premenopausal women. Serum MCP-1 showed higher, but not significantly different (p= 0.08) values in the postmenopausal than premenopausal women.

Lipid and glucose profiles

No concentration differences were observed in any of the measured serum lipids between the postmenopausal twin-women with and without HT (Table 4). Instead, serum glucose levels were 5% lower and HbA1c levels 3% lower in HT-women than in their identical co-twins without HT ($p= 0.04$ and 0.05, respectively). There were no significant differences in insulin levels between the HT users and non-users. In the postmenopausal women, serum total cholesterol levels were 14% higher and LDL-C levels 20% higher than in the premenopausal women ($p= 0.03$ for both). After adjustment for percentage body fat, these differences were no longer significant. Serum glucose, HbA1c and insulin, i.e. indicators of glucose profile, were higher in the postmenopausal women than premenopausal women ($p<0.01$, <0.01 and 0.05, respectively). As found with the lipid values, the differences were no longer significant after adjustment for percentage body fat.

Discussion

In the present monozygotic co-twin control study we showed that postmenopausal long-term estrogen-based therapy associated with healthier adipokine and glucose profiles. Our results showed that while the premenopausal sex steroid balance was maintained by HT, the users had a healthier amount and distribution of body fat, significantly lower systemic levels of MCP-1, fasting glucose and HbA1c in comparison with their non-using identical sisters.

Our results support the well-known phenomena of increased body and abdominal adipose tissue, increased insulin resistance and harmful changes in lipid concentrations in women in the post- compared to premenopausal era (33, 34). Consequently, the adjustment for percentage body fat abolished the differences between the pre- and postmenopausal women. Furthermore, our study shows that long-term HT users have better body composition and smaller waist circumference,

suggesting less visceral fat than their non-using co-twins. The similar BMI between these co-twins at the age of 40 is in line with a very rare discordance in BMI observed altogether in MZ twins (35, 36). Therefore, we can assume that our observations arise from the HT-discordance between co-twins.

Estrogen is known to promote the accumulation of subcutaneous fat (37) instead of visceral fat (38), and the postmenopausal loss of estrogen has been associated with increased intra-abdominal fat (39). During the past few years, increasing attention has been paid to the role of postmenopausal androgen increase in the hormonal milieu of women and to the changes in body fat distribution (7). The SWAN (The Study of Women´s Health Across the Nation) investigators have shown that the progressive androgenicity (i.e. increased levels of bioavailable testosterone and/or decreased levels of SHBG) during postmenopausal years is a strong predictor of visceral fat accumulation and may increase the risk for metabolic syndrome and cardiovascular diseases (8, 9, 40). Serum SHBG level, which determines the bioavailability of sex hormones, decreases after menopause (41, 42). In line with that our HT users had higher serum levels of SHBG than their non-using co-twins, and the T/E_2 ratio was only one fourth of that in non-users, mirroring the premenopausal ratio. Despite of the limitations of our study with respect to disentangling the discrete input of sex steroids, we suggest that the HT-maintained balance in steroid levels has a notable role in promoting a healthier amount and distribution of body fat (see Table 1).

There is increasing evidence to show that visceral adiposity is causally linked to chronic low-grade inflammation, hence contributing to obesity-linked metabolic dysfunction (43, 44). Adiponectin is an anti-inflammatory factor, which has been suggested to protect from obesity-linked metabolic disorders (45-47). It is the most abundant adipokine in circulation, and, contrary to others, the serum levels are decreased in obesity (48). Adiponectin is thought to mediate the effects on insulin

sensitivity through interactions with adiponectin receptors in the skeletal muscle and liver (43). By reducing the secretion of several cytokines from adipocytes, adiponectin has been shown to prevent the induction of insulin resistance in human skeletal muscle cells (49). Subcutaneous adipose tissue has also been shown to express adipokines associated with dysregulated glucose metabolism (50, 51). Studies on the effects of HT on plasma adiponectin levels show either no change in systemic adiponectin levels in HT users (52, 53) or decreased levels compared to non-users (54). In the present study, we wanted to see whether differences exist in systemic and/or local mRNA levels of certain adipokines known to be related to metabolic dysfunction. We found that both the local transcript levels in subcutaneous adipose tissue and serum adiponectin levels tended to be higher in HT users than non-users. Also, the adiponectin levels correlated negatively with the waist/hip ratio in all groups (data not shown).

Increasing evidence has been reported on the importance of the local action of adiponectin in adipose tissue. *In vitro* studies on human adipocytes have shown that adiponectin acts as an autocrine regulator by reducing the release of several adipose cell secretory factors, e.g. IL-6, IL-8 and MCP-1 (49). This corresponds well with the clearly lower serum levels of MCP-1 in the HT users compared to non-users in the present study, as well as the previously reported lower levels of IL-6 in the same participants (55). The differences in serum MCP-1 and leptin levels between the HT users and non-users were abolished after adjustment for percentage body fat, suggesting that the observed beneficial differences in these adipokines in HT users are likely due to their having a smaller relative amount of fat compared to non-users. In contrast, the difference in adiponectin levels between non-users and HT users became clearer after adjustment for percentage body fat, suggesting the existence of other factors regulating the production and/or secretion of adiponectin. A plausible candidate is the lower androgenicity in HT users, as testosterone treatment was shown to have an inhibitory effect on adiponectin secretion in hypogonadal men (56-58). Also, acute

administration of insulin has been shown to decrease circulating adiponectin concentrations (59, 60), although the chronic effects are unknown. Regardless of the regulator(s), it is tempting to suggest that postmenopausal years with long-term HT use result in higher levels of adiponectin than years without, and could be a sign of beneficial health effect.

MCP-1 is the most prominent secretory factor in inducing insulin resistance. In fact, it is able to reduce insulin signaling and insulin-stimulated glucose uptake at physiological concentrations in contrast to the other adipokines showing such effects only at supraphysiological concentrations (61). It was suggested by Sell et al. (2006), that even moderate changes in BMI could cause an increase in MCP-1 levels high enough to promote the development of insulin resistance. If so, then a rather small decrease in BMI can be assumed to decrease MCP-1 levels enough to restrain insulin resistance. Regarding the possible role of MCP-1 as a link between obesity and insulin resistance, our observation of lower MCP-1 serum levels together with slightly lower glucose and HbA1c levels in long-term HT users may be important. The existing data on the effects of HT on insulin resistance is mixed, but in aggregate HT is thought to be beneficial for glucose metabolism (18, 62). This is reasonable, as several studies have linked estrogen and estrogen receptors to the maintenance of metabolic control (63).

Conclusion

In sum, our results suggest that long-term HT is associated with healthier amount and distribution of body fat. Interestingly, while no difference was found in serum lipid concentrations between the groups, fasting serum glucose and HbA1c levels were lower among HT users than non-users. In the adipokines, the most important finding was a 15% lower level of monocyte chemotactic protein-1 in HT users than non-users, with respect to its mediator role between obesity and insulin resistance.

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Table legends

Table 1. *Body composition of premenopausal women and postmenopausal MZ twin pairs discordant for long-term HT.*

Table 2. *Serum sex steroid and SHBG levels of premenopausal women and postmenopausal MZ twin pairs discordant for long-term HT.*

Table 3. *Serum adipocytokine levels and local transcript levels of corresponding genes in premenopausal women and postmenopausal MZ twin pairs discordant for long-term HT.*

Table 4. *Serum lipid and glucose profiles of premenopausal women and postmenopausal MZ twin pairs discordant for long-term HT.*