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Circulating miR-21, miR-146a and Fas ligand respond to postmenopausal estrogen-based hormone replacement therapy – a study with monozygotic twin pairs

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Abstract

Biological aging is associated with physiological deteriorations and its' remodeling, which are partly due to changes in the hormonal profile. MicroRNAs are known to post-transcriptionally regulate various cellular processes associated with cell senescence; differentiation, replication and apoptosis. Measured from the serum, microRNAs have the potential to serve as noninvasive markers for diagnostics/prognostics and therapeutic targets.

We analysed the association of estrogen-based hormone replacement therapy (HRT) with selected microRNAs and inflammation markers from the serum, leukocytes and muscle tissue biopsy samples obtained from 54-62 year-old postmenopausal monozygotic twins (n=11 pairs) discordant for the use of HRT. Premenopausal 30-35 year-old women (n=8) were used as young controls. We focused on the hormonal aging and more specifically, on the

interaction between HRT use and the modulation of inflammation associated microRNAs, miR-21 and miR-146a, and classical inflammation markers. Fas-ligand (FasL) was analysed since it functions in both apoptosis and inflammation.

The inflammatory profile is healthier among the premenopausal women compared to the older, postmenopausal twins. The serum miR-21 and miR-146a expression levels and FasL concentrations were lower in the HRT users when compared to their non-using co-twins, demonstrating their responsiveness to HRT. Based on the pairwise FasL analysis, the FasL serum concentration is likely to be genetically controlled. Overall, we suggest that postmenopausal estrogen deficiency sustains the development of “inflamm-aging” in women. Estrogen sensitive, specific circulating microRNAs could be potential, early biomarkers for age-associated physiological deteriorations.

Highlights:

- Unique study design of postmenopausal MZ twins discordant for HRT
- Serum miR-21 and miR-146a expressions are lower in HRT users compared to non-users
- FasL serum concentrations are lower in HRT users and possibly genetically regulated
- Postmenopausal systemic estrogen deficiency partly contributes to the “inflamm-aging”
- Serum miR-21/-146a early indicators of age-associated physiological deteriorations

Keywords: microRNAs, estrogen, hormone replacement therapy, “inflamm-aging”, apoptosis

1 Introduction

Aging is characterized by changes in the hormonal milieu, especially what comes to the sex steroid hormones. Female sex steroids, estrogens, have major importance in reproductive functions, as well as in non-reproductive processes such as maintaining bone mass, participating in brain function and slowing down the processes which

cause vascular damage. The effect of estrogen is delivered by estrogen receptors (ERs), localized in the nucleus or on the cell membranes. ERs are also found in the skeletal muscle cells, making skeletal muscle tissue responsive to estrogens (Lemoine, et al. 2003, Wiik, et al. 2005). There are indications that dramatically decreasing level of circulating 17β -estradiol (E_2) at the time of menopause contributes to age-associated muscle weakness (Phillips, et al. 1993, Sipila, et al. 2001). Our previous studies have shown that the use of estrogen- or estrogen and progesterone -based hormone replacement therapy (HRT) is associated with better muscle quality properties, such as a positive muscle-fat ratio within the muscle compartment, as well as improved muscle performance characteristics like muscle power and mobility (Ronkainen, et al. 2009, Finni, et al. 2011). We also demonstrated in a one-year RCT that HRT counteracts the postmenopause-related transcriptome level changes (Pollanen, et al. 2007) and that several years of HRT causes subtle but relevant changes in the muscle transcriptome in postmenopausal women (Ronkainen, et al. 2010). The most significant changes occurred in the expressions of the genes participating in cell energy metabolism, responses to nutrition, the organization of the cytoskeleton and cell-environment interactions. These findings suggest a positive interplay between long-term HRT and muscle composition and performance. In addition, we determined that after menopause, muscle tissue E_2 does not follow the decline in circulating E_2 concentration, that is, postmenopausal muscle is not locally devoid of E_2 in comparison to premenopausal women. This suggests the distinctive effects of local and systemic estrogen in muscle regulation (Pollanen, et al. 2011).

Several *ex vivo* studies have shown that estrogen deficiency causes spontaneous increase in pro-inflammatory cytokine levels, such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF- α) in circulating monocytes (Pacifci, et al. 1989, Pacifci, et al. 1991), bone marrow macrophages (Jilka, et al. 1992, Bismar, et al. 1995) and osteoblasts (Passeri, et al. 1993). In contrast, *in vivo* studies in tissue samples or circulation have not been able to clearly demonstrate the effects of estrogen deficiency on pro-inflammatory cytokines. However, our previous findings showed that HRT affects the IL-6 and insulin growth factor 1 (IGF-1) pathways suggesting an association between postmenopausal HRT, healthier inflammatory and anabolic condition, together with enhanced muscle quality and performance (Ronkainen, et al. 2009, Pollanen, et al. 2010, Finni, et al. 2011, Ahtiainen, et al. 2012a, Qaisar, et al. 2013). It is commonly known that postmenopausal estrogen deficiency contributes to the aging process and according to our recent findings (Ahtiainen, et al. 2012a, Ahtiainen, et al. 2012b), most likely to the development of age-associated low intensity inflammatory status called "inflamm-aging" (Franceschi, et al. 2000). This chronic

inflammation state is seen as increased levels of pro-inflammatory cytokines in the circulation. Among other classical inflammation parameters, Fas ligand (FasL) is a pro-inflammatory cytokine and its systemic levels have been shown to decrease with age (Kavathia, et al. 2009). It belongs to the TNF family and its main function is the induction of apoptosis in susceptible and Fas receptor expressing cells (Takahashi, et al. 1994). There are two types of FasL: the pro-apoptotic membrane bound form, which is primarily expressed in activated T lymphocytes and immune-privileged organs (Xerri, et al. 1997) –and the soluble one (sFasL), which originates from the membrane-bound FasL by matrix metalloproteinase– mediated cleavage. The physiological role of sFasL is controversial since it has been reported to induce non-apoptotic signals, possibly including NF- κ B-mediated stimulation of cell proliferation, survival or inflammation within an elevated cytokine milieu (Suda, et al. 1997, Mogi, et al. 2001, Serrao, et al. 2001). In addition to FasL, circulating cell free DNA (cfDNA) has quite recently been discovered as a potential marker of inflammation, apoptosis, senescence and malignant conditions (Stroun, et al. 1987, Fatouros, et al. 2006, Jylhava, et al. 2012).

MicroRNAs (miRs) are small non-coding RNAs, found in all cell types and body fluids (Lawrie, et al. 2008). They regulate gene expression by binding to mRNA and further repress the translation into proteins (Hamilton and Baulcombe. 1999). MiR-21 is known as an oncomiR due to its overexpression in several human tumours, for instance in breast cancer (Kumar, et al. 2013) and it has also been suggested as a biomarker of “inflamm-aging” (Olivieri, et al. 2012b). According to Olivieri et al. (2012b), plasma miR-21 levels showed age-related differences as well as variations between patients in relation to age-associated conditions, such as cardiovascular disease, and their age-matched controls. Interestingly, miR-21 has been shown to regulate FasL expression (Sayed, et al. 2010). MiR-146a is strongly associated with inflammation and its expression is NF- κ B-dependent and it has been proposed to play an important role in the regulation of innate immune response by regulating the production of cytokines such as IL1-beta (IL-1 β) and TNF- α (Taganov, et al. 2006). MiR-146a targets IL-1 receptor-associated kinase (IRAK-1) and TNF receptor-associated factor-6 (TRAF6), which are both important in signaling associated with immune response (Taganov, et al. 2006). MiR-146a’s increased expression in several cell types during replicative senescence brings out its possible role in the overall aging process (Olivieri, et al. 2012a).

Maintaining the cellular balance under changing metabolic and hormonal states during the aging process involves several pro- and anti-apoptotic factors, as well as other molecular regulators. Postmenopausal women are deficient

for systemic E₂ and, especially, prone for developing muscle weakness and unfavourable body composition. The aim of the present study was to increase understanding about the interactions between the circulating estrogen levels and “inflamm-aging” related factors after menopause. Specifically, the purpose was to investigate the associations between the use of HRT and miR expressions and systemic markers of inflammation. The main studied parameters include serum miR-21, miR-146a and FasL. In addition, the miR and FasL expressions were also measured from skeletal muscle cells and leukocytes.

2 Materials and methods

2.1 Experimental design

This study is part of the 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)” consisting of healthy population including a group of premenopausal women as well as postmenopausal monozygotic (MZ) twin sister pairs with discordance for the use of HRT. A detailed design of the SAWEs- study (Ronkainen, et al. 2009) and the recruitment of the premenopausal women (Pollanen, et al. 2011) have been described previously. Briefly, the study participants were recruited from the Finnish Twin Cohort (n=13888 pairs) (Kaprio and Koskenvuo. 2002). Invitations were sent to all postmenopausal MZ twin sister pairs born from 1943-1952. From the responders, 15 postmenopausal MZ twin sister pairs were willing to participate and were discordant for the use of HRT (mean duration of HRT use 6.9±4.1, range 2–16 years). The premenopausal women were recruited by letter invitation which was sent to two thousand women, randomly selected from the 30-40 years’ age cohort (born in 1967-1977) living in the City of Jyväskylä. 59 women, who had not been treated with hormonal contraceptives or progesterone preparations within the past 5 years, participated in the study. A subgroup of 8 women between ages 30 to 35 was randomly selected for the current study. The exclusion criteria for participation included the following conditions: chronic musculoskeletal disease, type 1 diabetes, type 2 diabetes with medication, diagnosed mental disorder, asthma with oral cortisol treatment, acute cancer, known drug or alcohol abuse/dependence, Crohn's disease, unsuitable hormonal status, acute diseases, haemorrhagic diseases or use of warfarin. The medical conditions, which

were accepted, are mentioned in Table 1 (more detailed version in the supplementary data). Smoking habits (current, former) were evaluated using standard questionnaire and physical activity levels were assessed with the Grimby scale (Grimby. 1986) with slight modifications. The participants were categorized on the basis of their self-reported physical activity into groups labelled; sedentary (no other activities, at the most light walking ≤ 2 times/wk.); moderately active (walking or other light exercise at least 3 times/wk., but no other more intensive activities); and active (moderate or vigorous exercise at least 3 times/wk.). Five of the HRT users were using hormonal replacement preparations containing only E₂ (1-2 mg), six used estrogenic (1-2 mg) + progestogenic compounds and four tibolone (2.5 mg) based treatment. Since the aim of the current study was to investigate the associations of E₂ based HRT, tibolone based HRT users and their co-twins were excluded. Finally 11 pairs participated in this study. Due to the limited amount of tissue samples, the number of observations is less than 11 in some variables, which is mentioned in the tables.

All study subjects participated in the laboratory measurements in 2007 (Ronkainen, et al. 2009, Pollanen, et al. 2011). Body weight and height were measured with standard procedures and the body mass index (BMI) was calculated. Body composition, including lean body mass (LBM) and total body fat percentage, was measured with the multifrequency bioelectrical impedance analyser InBody (720) (

Table 2). Whole blood samples were collected under standard fasting conditions in a supine position to obtain whole blood, serum, plasma and leukocytes. Muscle biopsies were obtained from the *m. vastus lateralis* and the samples for biochemical analysis were snap frozen in liquid nitrogen and stored in -80°C.

All subjects were informed about the possible risks prior to the necessary physical and clinical measurements. Written informed consent, including permission for the use of the gathered data (only for research purposes) was provided by the study subjects. The study protocol was approved by the Ethics Committee of the Central Finland Hospital District (E0606/06) and the study was conducted according to the guidelines of the Declaration of Helsinki.

2.2 RNA extraction and analyses

Total RNA from 100 μ l of serum was isolated after two spins with the Total RNA Purification Kit (Norgen Biotek Corporation, Thorold, ON, Canada) according to the manufacturer's protocol. Synthetic *C.elegans* CmiR-39 was added before RNA extraction into all of the samples for the detection of the RNA recovery. For leukocytes and

muscle, the total RNA was extracted by using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

RNA quality was checked for the microarrays using an Experion electrophoresis station (Bio-Rad Laboratories, Hercules, CA). Transcriptome wide expression analyses were conducted with Sentrix Human-WG6 V3 Expression BeadChip microarrays (Illumina, San Diego, CA) in the Turku Centre for Biotechnology, BTK, University of Turku, as described in more detail by Ronkainen et al. (2010). The Fas and FasL gene transcript expressions were searched from the microarray data for the current study.

2.3 Quantitative PCR of miR-21 and miR-146a

The expression levels of the miR-21 and miR-146a in the serum, leukocytes and muscle biopsy samples were measured by qPCR. The Taqman miR reverse transcription kit and miR assay (Applied Biosystems, Foster City, CA) were used for the qPCR. The total reaction volume for the reverse transcription of the serum RNA samples of the premenopausal women and muscle and leukocyte samples of the MZ twins was 10 μ l, containing 3.35 μ l of sample RNA, 2 μ l of primers, 1.26 μ l of RNase inhibitor (diluted 1:10), 1 μ l of 10x buffer, 1 μ l of 10 mM dNTP's, 0.66 μ l of reverse transcriptase and 0.73 μ l nuclease free H₂O. The incubation was performed at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. The reaction volume for quantitative real-time PCR was 10 μ l, containing 0.5 μ l 20x Taqman MicroRNA Assay (5'-FAM PCR primers and probes), 5 μ l of Taqman® Universal Master Mix 2x (Applied Biosystems) and 1.33 μ l of the reverse transcription product. The reaction protocol was: incubation at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min (Applied Biosystems ABI 7300). For the MZ twin serum samples, RNA reverse transcription was obtained with the TaqMan microRNA RT kit and the reaction volume for the reverse transcription of the serum samples was 5 μ l, which contained 1.67 μ l of sample RNA, 1 μ l of each stem-loop primer, 0.6 μ l of RNA inhibitor (diluted 1:10), 0.5 μ l of 10x buffer, 0.4 μ l of 10 mM dNTP's, 0.3 μ l reverse transcriptase and 0.5 μ l H₂O. The incubation was performed at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. cDNA synthesis was followed by quantitative real-time PCR, while the reaction volume was 5 μ l and contained 0.25 μ l of the 20x Taqman MicroRNA Assay (5'-FAM PCR primers and probes), 2.75 μ l of the TaqMan Fast Universal PCR Master Mix (2x) (Applied Biosystems) and 2.25 μ l of the reverse transcription product. The reaction protocol was as follows: incubation at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min (iCycler, Biorad). The data analysis was performed using the Real Time PCR OpticonMonitor version 2 (MJ Research, Bio-Rad, Hercules, CA).

The Ct settings were automatic and the baseline and threshold were adjusted for Ct determination. Human miR-17 was used as a reference for the normalization of the serum samples and RNU44 for the muscle and leukocyte samples. ΔCt values were calculated as $\Delta Ct = \text{mean } Ct_{\text{miR } X} - \text{mean } Ct_{\text{miR } 17}$ and $\Delta Ct = \text{mean } Ct_{\text{miR } X} - \text{mean } Ct_{\text{RNU44}}$ where X is the studied miR. Each reaction was performed in duplicate and the relative expressions were calculated by using the $2^{-\Delta Ct}$ method.

2.4 Serum cytokine, FasL and hormone measurements

Serum IL-1B, IL-6, IL-10, TNF- α and SHBG concentrations were measured using Immulite®1,000 (DPC, Los Angeles, CA). Circulating human FasL, IL6 receptors IL6R and sgp130 and monocyte chemoattractant protein-1 (MCP-1) concentrations were measured by the Quantikine® ELISA Immunoassay (R&D Systems, Minneapolis, MN, USA). The cfDNA was measured from plasma samples with the Quant-iT™ DNA High-Sensitivity Assay kit and a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA) according to Jylhävä et al (2012). Serum 17 β -estradiol and testosterone levels were measured as described previously by Ankarberg-Lindgren and Norjavaara (2008) and by Turpeinen et al. (2008) respectively.

2.5 Statistical Analyses

All data was tested for normality by using the Shapiro-Wilk significance value. Statistical analyses included either the Independent-Samples T-test or Paired-Samples T-test for the parametric variables or the Mann Whitney U Test or Wilcoxon signed rank test for the non-parametric variables. Intrapair differences (IPD) were calculated as the HRT user value subtracted by the non-user value. The Spearman correlation coefficient was used when measuring associations between variables. The data is shown as the means and standard deviations (SD). Data analyses were carried out by SPSS (IBM SPSS Statistics 20, Chigaco, IL) and matched pair analyses by Stata (version 13.0 StataCorp LP, Texas 77845, USA).

3 Results

3.1 Phenotype characteristics

The participants' age, anthropometry, body composition and the systemic steroid hormone levels with expected differences between the premenopausal women, non-user twins and HRT users are presented in the Table 2. There

were no differences in the BMI or LBM between any of the studied groups. However, the body fat percentage was greater among the non-users compared to the HRT users. The mean age of the premenopausal women was 32.0 (± 1.6) years and MZ twins 57.2 (± 1.8) years.

Table 3 shows the concentrations of the classical inflammation markers and how they differ between the different groups. All of the studied inflammation markers were lower in the premenopausal women compared to the both postmenopausal groups. However, statistical significance was identified only for s-TNF- α ($p < 0.001$ in non-user comparison, $p = 0.023$ in HRT user comparison) and s-MCP-1 ($p = 0.047$ in non-user comparison) while s-cfDNA was in the borderline of significance ($p = 0.054$ in non-user comparison).

3.2 Aging and HRT modulation of miR-21, miR-146a and FasL

Table 4 shows the serum miR-21, miR-146a levels and FasL concentrations and their differences between the premenopausal women and the non-user and HRT user twin sisters and muscle and leukocyte miR-21 and miR-146a levels and their differences between the non-user and HRT user twin sisters. Serum miR-21 levels are lower in the premenopausal women compared to non-users ($p = 0.001$) and HRT users ($p = 0.001$). Serum miR-146a levels were higher in premenopausal women than in HRT users ($p < 0.05$). FasL is significantly higher in the premenopausal women compared to the HRT users ($p < 0.033$). The serum miR-21 and miR-146a levels were significantly lower in the HRT using twins, than in their non-using co-twins ($p = 0.018$ and $p = 0.039$ respectively). The muscle miR-146a was lower in the HRT users compared to the non-users ($p = 0.012$). Instead, no differences in the leukocyte miR-21 and miR-146a values were detected between the HRT users and non-users (Table 4). The miR-21 transcript levels were highest in the muscle and those of the miR-146a in the leukocytes. FasL transcript levels were higher in the leukocytes than in the muscle and the serum FasL concentration was significantly lower in the HRT users than non-users ($p = 0.021$) (Table 4).

3.3 Intrapair correlations of systemic miR-21, miR-146a and FasL

Figure 1 shows the intrapair correlations of the serum miR-21, miR-146a transcript levels and serum FasL concentration between the HRT using and non-using co-twins. A significant intrapair correlation was detected in the miR-21 transcripts, but not in miR-146a transcripts. The serum FasL intrapair correlation was very strong ($r = 0.838$,

p=0.001) and the detected intrapair correlations were independent from the time of HRT usage (in years) (Figure 1) or the age of the participants (data not shown). Individual intrapair differences and correlations of the other measured parameters used in this study are presented in the supplementary data.

3.4 Associations of serum miR-21 and miR-146a transcripts and FasL concentration with classical inflammation markers among the MZ twins

Table 5 presents the correlations of the systemic miR-21, miR-146a transcript levels and FasL concentrations with the measured inflammation parameters and with each other. The miR-21 had a strong negative association with the MCP-1 when all of the twins were included in the analysis, miR-21 correlated positively with the miR-146a in non-users ($r=0.755$; $p=0.007$) but not among the HRT users, while no correlation between the miR-21 and FasL was detected. The miR-146a correlated negatively with the cfDNA in the HRT users ($r= -0.709$; $p=0.015$) but not among the non-users. The mir-146a had a strong negative association with the MCP-1 among non-users ($r= -0.709$; $p=0.015$), but not in the HRT users and the miR-146a did not correlate with sFasL. In addition, the serum miR-146a intrapair difference correlated positively with the leukocyte miR-146a intrapair difference ($r=0.733$; $p=0.025$) (data not shown). A positive correlation of the serum FasL and TNF-alpha concentration was detected in the HRT users ($r=0.815$; $p= 0.002$) but a negative correlation was found in the non-users ($r=-0.773$; $p=0.005$). The FasL also correlated negatively with serum IL6 among non-users ($r= -0.665$; $p=0.026$), while the FasL and sgbp130 had a positive association among HRT users ($r=0.709$; $p=0.022$).

Table 1: Medical history and lifestyle characteristics of the premenopausal women as well as the postmenopausal HRT using twins and their non-using co-twins.

Variable	Premenopausal women (n)	Non-users (n)	HRT users (n)
Hysterectomy and ovariectomy	-	1	4
Hysterectomy	-	1	0
Medication for hypertension	-	4	5
Medication for hypercholesterolemia	-	3	3
History of basal cell carcinoma/melanoma	-	1	1
Smoking			
Never	1	8	6
Former	4	1	1
Current	2	2	4
Physical activity			
Sedentary		0	0
Moderately active	7	2	5
Active	1	9	6

Table 2: Body anthropometry and hormone status of the premenopausal women and the non-user and HRT user twins.

Variable	Premenopausal women (n=8)	Non-users (n=11)	HRT users (n=11)	P-value pre-non user	P-value pre-HRT user	P-value HRT user – non-user	Intrapair Difference (95% CI) (HRT user - non-user)
Age	32.0±1.6	57.2±1.8	57.2±1.8	0.000	0.000		
BMI (kg/m ²)	25.9±4.5	28.2±6.5	25.7±3.8	0.396	0.935	0.091	-6.7 (-16.4 to 3.1)
LBM (kg)	45.5±3.7	46.3±4.6	46.2±3.4	0.337	0.230	0.37	0.1 (-5.0 to 5.3)
Body fat %	29.8±7.0	35.2±8.9	30.1±7.1	0.169	0.774	0.026	-10.6(-23.4 to 2.1)
FSH (IU/l)	6.2±2.86	93.2±16.7	62.8 ±23.6	0.000	0.000	0.006	-31.8 (-47.7 to -15.9)
E2 (pmol/l)	496.5±311.9	33.3±27.4	172.9±203.2	0.004	0.014	0.003	696.4 (-146.7 to 1539.5)
Free E2	11.5±6.6	0.81±0.58	3.31±3.27	0.002	0.002	0.006	500.7 (-33.3 to 1034.6)
E1 (pmol/l)	369.4±195.3	97.7±26.8	899.6±1454.7	0.006	0.324	0.003	759.9 (153.0 to 1672.8)
T (pmol/l)	1016.3±303.9	639±269	715±306	0.011	0.048	0.061	13.6 (-10.9 to 40.9)
Free T	14.7±5.3	9.9±4.7	8.4±4.7	0.053	0.014	0.075	-12.0 (28.7 to 4.7)
SHBG (nmol/l)	49.3±17.6	42.6±14.6	68.2±33.1	0.638	0.070	0.010	61.7 (14.2 to 109.3)

BMI: body mass index, LBM: lean body mass
 Values are the mean ± standard deviation. The P values were obtained by the independent (premenopausal vs. non-user and premenopausal vs. HRT user) or paired samples T-test (co-twin analyses). Intrapair differences were calculated as the HRT user value subtracted by the non-user value.

Table 3: The measured inflammation parameters of the premenopausal women and MZ twins discordant for long-term hormone replacement therapy.

Variable	Premenopausal women (n=7)	Non-users (n=10-11)	HRT users (n=10-11)	P-value pre-non user	P-value pre-HRT user	P-value HRT user- non-user	N (twin pair)	Intrapair Difference (95% CI) HRT user - non-user
s-CRP (mg/l)	0.88±1.1	1.42±0.97	1.14±0.92	0.270	0.576	0.480	11	-0.28 (-2.00 to 2.20)
s-TNF-α (pg/ml)	6.10±1.56	10.56±2.18	10.42±4.65	0.000	0.023	0.941	11	-0.15 (-7.3 to 14.40)
s-IL-1B (pg/ml)	0.15±0.19	0.28±0.61	0.37±0.56	0.577	0.309	0.717	11	0.09 (-1.27 to 1.79)
s-MCP-1 (pg/ml)	267.65±51.69 (n=4)	406.97±136.39	361.51±116.24	0.047	0.110	0.080	11	-45.46 (-150.54 to 141.90)
s-IL-10 (pg/ml)	0.89±1.66	2.48±3.51	2.38±3.01	0.251	0.223	0.604	11	-0.10 (-1.50 to 0.62)
s-IL-6 (pg/ml)	1.05±0.70	2.22±1.48	2.00±1.20	0.108	0.195	0.676	11	-0.22 (-2.94 to 3.55)
s-IL-6R (ng/ml)	32.26±8.64	44.11±8.84	39.55±8.72	0.018	0.095	0.000	10	-4.56 (-7.9 to -1.50)
s-sgp130 (ng/ml)	278.58±36.90	345.61±29.23	312.01±47.23	0.001	0.139	0.027	10	-33.59 (-89.59 to 58.44)
s-cfDNA (µg/ml)	0.61±0.07	0.69±0.09	0.65±0.07	0.054	0.211	0.135	11	-0.04 (-0.19 to 0.08)

Values are the mean ± standard deviation. The P values were obtained by the independent samples T test in comparisons between premenopausal women and non-users and premenopausal women and HRT users, and the paired samples T-test in comparisons between the co-twins. Intrapair differences (IPD) were calculated as the HRT user value subtracted by the non-user value.

Table 4: Serum (s-) and tissue miR relative expressions in arbitrary units (AU) and serum.

Variable	Premenopausal women (n=7)	Non-users (n=8-11)	HRT users (n=8-11)	P-value pre-non-user	P-value pre-HRT user	P-value HRT user – non-user	N (twin pair)	Intrapair Difference (95% CI) HRT user – non-user
s-miR-21	0.72±0.22	2.21±0.96	1.60±0.67	0.001^M	0.001^M	0.018^W	11	-0.61 (-1.59 to 0.44)
s-miR-146a	0.78±0.44	0.49±0.26	0.31±0.11	0.089	0.030	0.039	11	-0.17 (-0.60 to 0.13)
muscle miR-21	-	8.21±5.10	4.21±4.03	-	-	0.169	8	-4.00 (-13.15 to 8.65)
muscle miR-146a	-	0.15±0.06	0.07±0.03	-	-	0.012^W	8	-0.08 (-0.19 to -0.04)
leukocyte miR-21	-	1.77±2.54	1.05±0.63	-	-	0.374	9	-0.72 (-6.5 to 1.0)
leukocyte miR-146a	-	1.17±0.68	1.81±1.48	-	-	0.263	9	0.64 (-1.58 to 3.18)
s-FasL (pg/ml)	107.09±12.76	85.61±33.72	71.68±32.43	0.160 ^M	0.033^M	0.021^W	11	-13.92 (-46.84 to 11.32)
muscle FasL mRNA	-	80.96±8.67	81.65±5.38	-	-	0.784	11	0.69 (-10.06 to 16.97)
leukocyte FasL mRNA	-	184.03±51.69	205.64±48.03	-	-	0.260 ^W	9	21.61 (-37.10 to 150.72)

Values are the mean ± standard deviation. The P values were obtained by the independent sample T test or Mann Whitney U test in comparisons between premenopausal women and non-users and premenopausal women and HRT users, and the paired samples T-test or Wilcoxon Signed Rank test in comparisons between co-twins. Non-parametric Mann Whitney U and Wilcoxon Signed Rank tests were used only for non-normally distributed variables and their use is indicated by M or W, respectively. Intrapair differences (IPD) were calculated as the HRT user value subtracted by the non-user value.

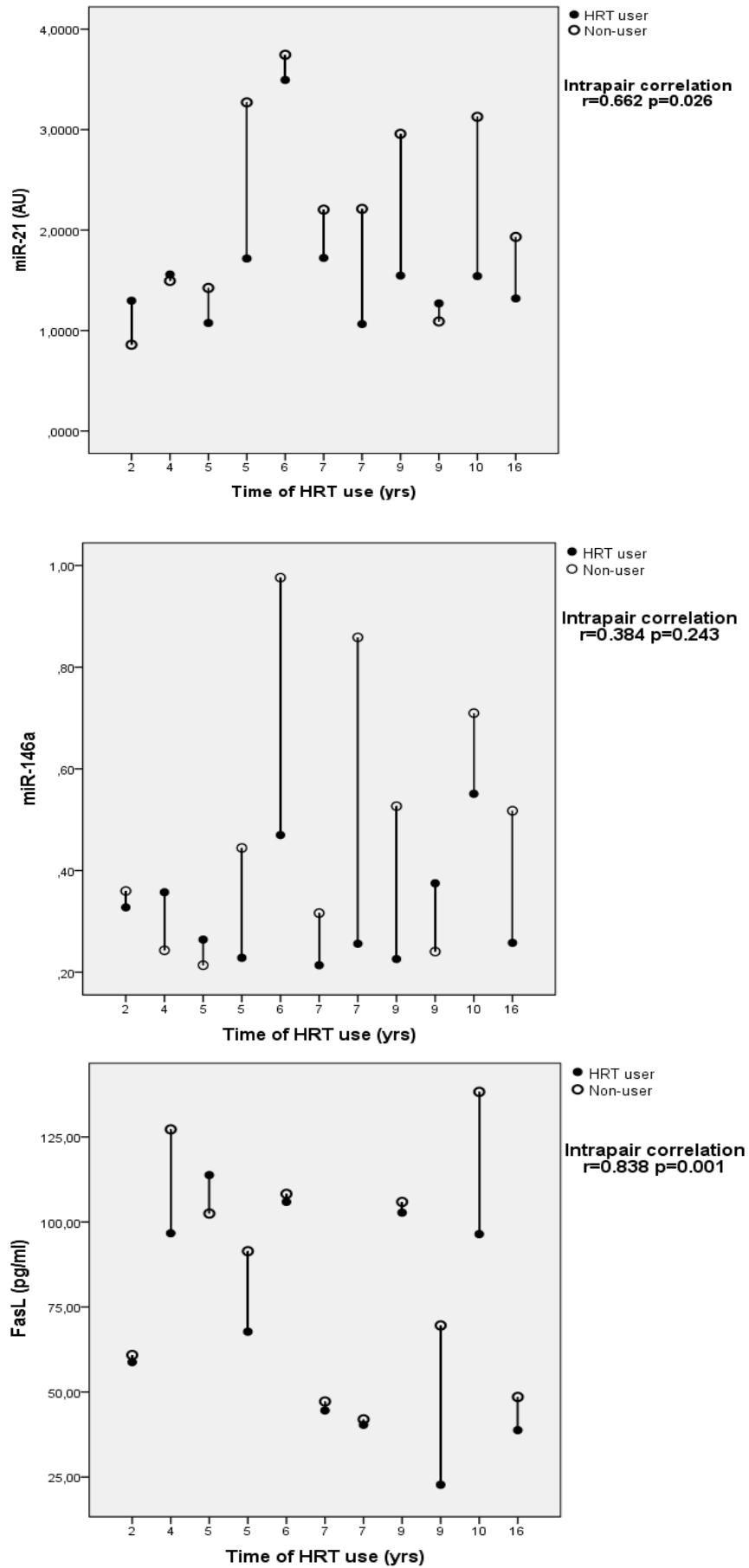


Figure 1: Pairwise values in relation to the time of HRT use in years, and overall intrapair correlations of circulating miR-21, miR-146a transcripts and FasL serum concentrations

Table 5: Spearman correlations of circulating miR-21 and miR-146a transcript levels and FasL concentrations with measured inflammation parameters. #n=10 pairs.

	Non-user		HRT user		ALL	
	r	p	r	p	r	p
miR-21 correlations						
CRP (mg/l)	0.252	0.455	0	1	0.282	0.246
TNF- α (pg/ml)	-0.164	0.631	0.264	0.432	0.089	0.690
IL-1B (pg/ml)	-0.463	0.151	-0.028	0.935	-0.015	0.946
MCP-1 (pg/ml)	-0.945	0.000	-0.591	0.056	-0.665	0.001
IL-10 (pg/ml)	-0.333	0.318	-0.278	0.408	-0.321	0.254
IL-6 (pg/ml)	-0.583	0.060	-0.173	0.612	-0.281	0.131
IL-6 receptor (ng/ml)	-0.559	0.093	-0.018	0.960	-0.155 [#]	0.519
sgbp130 (ng/ml)	0.393	0.257	0.055	0.881	0.295 [#]	0.243
cfDNA (μ g/ml)	-0.082	0.811	0.155	0.650	0.005	0.983
miR-146a	0.755	0.007	-0.082	0.811	0.518	0.007
FasL (pg/ml)	0.318	0.340	0.318	0.340	0.324	0.126
miR-146a correlations						
	r	p	r	p	r	p
CRP (mg/l)	0.384	0.243	0.338	0.309	0.354	0.168
TNF- α (pg/ml)	-0.245	0.467	0.050	0.884	-0.030	0.907
IL-1B (pg/ml)	0.032	0.927	-0.088	0.796	-0.040	0.851
MCP-1 (pg/ml)	-0.709	0.015	-0.091	0.790	-0.337	0.099
IL-10 (pg/ml)	-0.355	0.284	0.232	0.492	-0.061	0.807
IL-6 (pg/ml)	-0.260	0.441	0	1	-0.041	0.857
IL-6 receptor (ng/ml)	-0.219	0.544	0.564	0.090	0.280 [#]	0.109
sgbp130 (ng/ml)	0.287	0.422	-0.018	0.960	0.154 [#]	0.496
cfDNA (μ g/ml)	-0.018	0.958	-0.709	0.015	-0.133	0.452
FasL (pg/ml)	0.073	0.832	0.155	0.650	0.154	0.464
FasL correlations						
	r	p	r	p	r	p
CRP (mg/l)	-0.137	0.687	-0.247	0.465	-0.157	0.358
TNF- α (pg/ml)	-0.773	0.005	0.815	0.002	0.181	0.201
IL-1B (pg/ml)	-0.600	0.051	0.451	0.164	-0.142	0.439
MCP-1 (pg/ml)	-0.236	0.484	-0.273	0.417	-0.197	0.375
IL-10 (pg/ml)	0.178	0.601	0.205	0.545	0.191	0.499
IL-6 (pg/ml)	-0.665	0.026	-0.145	0.670	-0.320	0.090
IL-6 receptor (ng/ml)	0.049	0.894	-0.091	0.803	0.033 [#]	0.907
sgbp130 (ng/ml)	0.098	0.789	0.709	0.022	0.525[#]	0.040
cfDNA (μ g/ml)	-0.009	0.979	-0.064	0.853	0.033	0.894

4 Discussion

The purpose of this study was to investigate if estrogen or estrogen and progesterone containing postmenopausal HRT interplays with miR regulation, specifically with miR-21 and miR-146a. In addition, we measured several inflammation and apoptotic markers, including FasL, from the circulation, in order to understand whether the postmenopausal lack of systemic E₂ or, contrarily, HRT, contributes to the age-associated pro-inflammatory condition, named “inflamm-aging.” The current study showed that serum miR-21 and miR-146a levels, as well as FasL concentrations, were significantly lower in HRT users than in their non-using co-twin sisters, indicating their responsiveness to HRT. The results also suggest that the FasL serum concentration may be genetically regulated, although environmental influences cannot be totally ruled out.

Women in this study were relatively young compared to the classical aging research settings where the study participants would be at a more mature age. However, in the current study, we wanted to see if potential early indicative markers of “inflamm-aging” could be detected from the circulation of postmenopausal MZ twins in relation to systemic estrogen deficiency. Premenopausal women were used as young control group to demonstrate how the aging itself affects the hormonal and inflammatory profiles. As it is known, skeletal muscles of postmenopausal women are prone to physiological malfunction predisposing them to sarcopenia. Our previous findings support the idea that a decrease in systemic E₂ contributes to developing age-associated conditions, seen as weaker skeletal muscle quality properties. In the current MZ twin study, we showed that serum miR-21, miR-146a and FasL are modulated by HRT. As previously demonstrated, FasL is a target of miR-21 (Sayed, et al. 2010). The connection between estrogen, miR-21 and FasL has previously been shown in bone in which, with the suppression of mir-21 expression, estrogen induces FasL production followed by osteoclastic apoptosis (Sugatani and Hruska. 2013). This connection made us to hypothesize that when miR-21 expression is low with higher systemic estrogen (premenopausal women and postmenopausal HRT users), FasL concentration is high and vice versa. However, this hypothesis holds true only in premenopausal women and in postmenopausal non-users. The association between these parameters with estrogen status in the serum was more complicated, since both parameters were low in the HRT users. The association between FasL and miR-146a can also be recognized since the FasL receptor, Fas, has been shown to be a target of miR-146a in bone marrow-derived mesenchymal stem cells (Suzuki, et al. 2010). A connection between miR-21 and miR-146a and female sex steroids can be emphasized, as it has been demonstrated

that both of these miRs are highly present in the plasma of breast cancer patients (Kumar, et al. 2013). In addition, a similar pattern was recently shown by Liu et al. (2014) in cervical cancer derived exosome miRs. In the current study the serum miR-21 levels were more similar between the premenopausal women and HRT users than non-users supporting a counteracting role of HRT towards the development of “inflamm-aging” of the HRT. In addition, our results on the FasL are in accordance with the previous findings where FasL concentration has been shown to decrease with age (Kavathia, et al. 2009). However, no effect of HRT is detected for FasL or miR-146a if the value of young controls is regarded as a reference. However, complex age-related trajectories are expected for systemic inflammatory markers according to the “health status”, and the reference value for younger population could be significantly different from those of the elderly subjects.

MiRs, especially inside exosomes, are quite stable in the circulation, and have been suggested to play an important role in intercellular communication (Kosaka, et al. 2010). Some studies have also shown that macrophages actively intake exosomes containing miR molecules, which could be another way of delivering the regulatory message of the miRs from one cell to another (Lasser, et al. 2011). According to Vinciguerra et al. (2009) the up-regulation of miR-21 by excessive amounts of circulating fatty acids results in the down-regulation of phosphatase and tensin homolog (PTEN) expression in hepatocytes leading to further liver diseases. These results interestingly suggest miR-21’s potential role in the development of metabolic diseases. Within the studied MZ twins, both the body- and muscle compositions have been shown to be healthier among the HRT users (Ronkainen, et al. 2009). When comparing the expression levels of miR-21 in the different tissues used in the current study, the greatest potential reservoir of miR-21 seems to be skeletal muscle (Table 4). For miR-146a, the highest expression level was in the leukocytes. A positive correlation between the serum miR-146a and leukocyte miR-146a intrapair differences (data not shown) could indicate that at least part of the circulating miR-146 originates from the leukocytes. This seems reasonable since miR-146a has been strongly linked to inflammation. Table 5 shows that negative correlations among miR-21 or miR-146a and various inflammation markers are not as strong among HRT users as they are among non-users. This data is relatively difficult to interpret, due to the limited sample size; nevertheless, it clearly shows that HRT alters the associations between these inflammation associated molecules in the serum.

MCP-1, also known as CCL2, is one of the main chemokines and it is produced in various cell types, however, mainly in monocytes and macrophages (Yoshimura, et al. 1989a, Yoshimura, et al. 1989b). MCP-1 secretion is activated by

signals and molecules associated with oxidative stress, cell growth or inflammation (such as pro-inflammatory cytokines), and its function is to recruit and direct monocytes and macrophages. We have shown previously that the systemic MCP-1 concentration is 15% lower in HRT users than in non-users (Ahtiainen, et al. 2012b); additionally, MCP-1 has also been suggested to play a role as a link between obesity and insulin resistance, which has been presented in our previous study (Ahtiainen, et al. 2012b). We have shown that serum levels of IL-6 receptors, including the membrane-bound receptor IL-6R and soluble receptor sgp130, were lower in HRT users than the non-user co-twins, suggesting the E₂ responsiveness in IL-6 signalling (Ahtiainen, et al. 2012a). These previous findings, and the current findings on TNF- α and MCP-1 concentration differences between premenopausal women and MZ twins, together with the current serum miR-21, miR-146a and FasL results between HRT users and non-users, suggest the onset of “inflamm-aging” at the time of menopause while slightly better inflammatory status according to some, but not all of the inflammation markers was observed among the HRT users when compared to the non-users. However, correlations among the mentioned inflammatory parameters and serum miRs, are not so straightforward, and most likely, at least one additional regulatory layer especially among HRT users is involved. In the current study, a negative correlation between systemic MCP-1 and miR-21 was quite strong, especially among non-users: the MCP-1 concentration explained 78.2% of the miR-21 level in the serum. Among HRT users, the percentage was only 23.2. A miR-146a and MCP-1 correlation had a similar trend but it was not as strong as with the miR-21 and MCP-1. According to Li et al. (2013), by targeting IRAK1 and TRAF-6 transcripts, miR-146a suppresses the induction of pro-inflammatory cytokines such as IL-1 β , IL-6, TNF α and MCP-1 in mycobacteria infected macrophages. Our finding, the lower the serum miR-146a level the higher the MCP-1 concentration in non-users suggests that E₂ deficiency increases the negative correlation between these two molecules providing further support for the role of HRT in the inflammatory response.

The inflammatory status can also be monitored by the level of serum cfDNAs which has been linked to systemic inflammation in aged people due to its positive association with a commonly used inflammation marker, the high sensitivity C-reactive protein (hsCRP), and negative correlation with high density lipoprotein (HDL) (Jylhava, et al. 2012). In the current study, cfDNA was lower in the premenopausal women compared to the non-users, however, it did not quite reach the borderline of significance (p=0.054). The serum cfDNA did not differ significantly between the HRT using and non-using co-twins, while the cfDNA levels of the HRT using twin sisters correlated negatively with

their serum miR-146a levels. This suggests a link between miR-146a regulation and cfDNA release, and is in agreement with the conclusion that HRT can partially counteract the developing age-related systemic inflammation.

Our current genetically controlled study arrangement enabled us to investigate the differences between HRT users and non-users individually, without sequence-level genetic variability. Also, the environmental factors affecting MZ twins' development remain similar through intrauterine time and childhood. The heritability of structural traits, such as muscle composition, is generally relatively high. When taking these mentioned aspects into account, the power of this matched pair design– study was greater when compared to the traditional case-control study designs. Despite the differences in HRT use within pairs, the MZ twins were the most similar for FasL ($r=0.84$), with smaller but still substantial similarity for miR-21 ($r=0.66$) and miR-146a ($r=0.38$). This is likely due to genetic influences, but shared environmental influences during adulthood cannot be excluded given the data on the MZ pairs alone. The group of studied premenopausal women was relatively small which has to be recognized when interpreting the results especially for variables with high genetic regulation. However, our findings support the phenomenon of “inflamm-aging” and its partial suppression by postmenopausal HRT.

In conclusion, systemic miR -21 and -146a and FasL are responsive to HRT. The expression levels of both of these circulating miRs and the FasL concentrations are lower in HRT users compared to non-users. These miRs have major potential to act as new more sensitive biomarkers of HRT's effects. Since the postmenopausal women in the current study were relatively healthy and quite young (mean age 57.2 ± 1.8 years), the aging-related increments in the levels of the traditional inflammation markers, a condition known as “inflamm-aging”, were not fully detectable although a trend for worse inflammation status in postmenopausal women compared to premenopausal control group (mean age 32.0 ± 1.6 years) was identified. However, the modulation of circulating inflamma-miRs, which we were able to observe, might precede the detectable development of “inflamm-aging” and they could be early biomarkers at the breaking up point between chronological and biological age. In fact, no differences were observed in the amounts of traditional inflammation markers between the HRT users and non-users, however, significant differences in specific serum miR transcript levels, associated with inflammation, suggest that miRs could be used as early indicators of developing age-associated conditions, such as “inflamm-aging”. Nevertheless, a deeper understanding of the interplay between the miRs, inflammation markers and estrogenic regulation is needed to solve the complex regulatory system. Serum miRs provide easy access, and

novel and potential information about the regulatory changes occurring in different tissues. Their use in diagnostics and drug targeting in the near future is promising.

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