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# Global gene expression profiles in skeletal muscle of monozygotic female twins discordant for hormone replacement therapy

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## Summary

**Aging is accompanied by inexorable loss of muscle tissue. One of the underlying causes for this is the massive change in the hormonal milieu of the body. The role of a female sex steroid – estrogen – in these processes is frequently neglected, although the rapid decline in its production coincides with a steep deterioration in muscle performance. We recruited 54- to 62-year-old monozygotic female twin pairs discordant for postmenopausal hormone replacement therapy (HRT,  $n = 11$  pairs; HRT use  $7.3 \pm 3.7$  years) from the Finnish Twin Cohort to investigate the association of long-term, estrogen-based HRT with skeletal muscle transcriptome. Pathway analysis of muscle transcript profiles revealed significant HRT-induced up-regulation of a biological process related to regulation of cell structure and down-regulation of processes concerning, for example, cell–matrix interactions, energy metabolism and utilization of nutrients (false discovery rate  $< 0.15$ ). Lending clinical relevance to the findings, these processes explained a significant fraction of**

**the differences observed in relative proportion of muscle within thigh and in muscle performance ( $R^2 = 0.180–0.257$ ,  $P = 0.001–0.023$ ). Although energy metabolism was affected through down-regulation of the transcripts related to succinate dehydrogenase complex in mitochondria, no differences were observed in mtDNA copy number or oxidative capacity per muscle cross section. In conclusion, long-term use of HRT was associated with subtle, but significant, differences in muscle transcript profiles. The better muscle composition and performance among the HRT users appeared to be orchestrated by improved regulatory actions on cytoskeleton, preservation of muscle quality via regulation of intramuscular extracellular matrix and a switch from glucose-oriented metabolism to utilization of fatty acids.**

**Key words: aging; postmenopause; skeletal muscle; hormone replacement therapy; twin design; transcriptome.**

## Introduction

Natural menopause refers to a state in which normal menstruation spontaneously ceases for 12 consecutive months at 45–55 years of age (McKinlay *et al.*, 1992). The cessation of ovaries to produce estrogens leads to several types of symptoms and consequences, which increase the risk of deterioration of health and decrease quality of life. Interestingly, some reports have provided sound evidence for a link between accelerated decline in muscle strength and the occurrence of menopause (Kallman *et al.*, 1990; Phillips *et al.*, 1993; Samson *et al.*, 2000). Regardless of the cause, low muscle strength is a significant predictor of adverse health events (Rantanen *et al.*, 2003) as well as mobility limitation and disability (Rantanen *et al.*, 1999). Adequate muscle performance is also an important factor responsible for preventing falls and consequent fractures. Moreover, sufficient muscle mass itself is of high importance as skeletal muscle is the primary reservoir of amino acids and also serves as a major site for various metabolic activities contributing to glucose balance and lipid metabolism of the whole body (Nader, 2005; Zurlo *et al.*, 1990).

Hormone replacement therapy (HRT) is a worthwhile option for women to relieve typical menopausal symptoms. A recent meta-analysis of 23 studies documented that estrogen-based HRT exerts beneficial effects on skeletal muscle strength (Greising *et al.*, 2009). In support of these data is the fact that both estrogen receptors (ERs), alpha and beta, are expressed in human skeletal muscle tissue (Lemoine *et al.*, 2003; Wiik *et al.*, 2003). We have shown that short-term, 1-year HRT improves

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muscle mass and performance (Sipilä *et al.*, 2001; Taaffe *et al.*, 2005). In addition, one year on HRT during the first years of the postmenopause was associated with smaller amount of changes in muscle transcriptome in comparison with one year on placebo (Pöllänen *et al.*, 2007). Long-term HRT, on the other hand, was associated with better muscle composition and performance. The users also had higher maximal walking speed (MWS) compared to the never-users (Ronkainen *et al.*, 2009). Intriguingly, suggesting a multifaceted role of estradiol in skeletal muscle signaling, studies with murine myoblasts have proposed that a subpopulation of ER $\alpha$  localizes outside the nucleus, more precisely in the mitochondria and the perinuclear compartments (Milanesi *et al.*, 2008), and that – via both ERs – estradiol mediates anti-apoptotic effects through the renowned insulin-like growth factor 1/phosphatidylinositol 3-kinase pathway (Vasconsuelo *et al.*, 2008). Although these documents may indicate that estradiol possibly acts through mitochondria or typical hypertrophic pathways to affect muscle, the molecular level mechanism by which long-term HRT may affect muscle structure and function is poorly understood.

In this study, we utilized a unique collection of genetically identical, HRT-discordant twin pairs to investigate the biological pathways that underlie the effects of long-term HRT on skeletal muscle characteristics. More specifically, the aim was to construct networks of enriched processes in skeletal muscle responding to the long-term use of estrogen-based HRT in comparison with women without any HRT in a genetically controlled setting and dissect whether the differential expression of these transcripts explains the previously reported

(Ronkainen *et al.*, 2009) differences in muscle composition or performance.

## Results

### General characteristics of the participants

Eleven monozygotic (MZ) twin pairs discordant for estrogen-based HRT (one sister is a current user, while her co-twin has never used HRT) aged  $57.6 \pm 1.8$  years (range, 55–62 years) formed the present study sample, a subgroup of our previous publication (Ronkainen *et al.*, 2009). Mean duration of HRT use was  $7.3 \pm 3.7$  years (2–16 years). As expected, the HRT users had higher levels of both total and free 17 $\beta$ -estradiol (E<sub>2</sub>) and total estrone compared to their sisters not on HRT (Table 1). The levels of total and free testosterone (T) or the ratio of total E<sub>2</sub> and T, or free E<sub>2</sub> and T was not statistically significantly different between the users and non-users.

### Transcript profiles reveal significant changes among co-twins using HRT

Pairwise analysis at the level of individual probes revealed that 22 sequences were up-regulated (Table S1) and 33 sequences down-regulated (Table S2) at  $P < 0.001$  in the co-twins using HRT. Also, lists with 30 genes carrying the highest (Table S3) and 30 genes with the lowest fold change (FC) (Table S4) are included. Microarray data mining with enrichment analysis was carried out separately for the up-regulated and for the down-

**Table 1** Physical and biochemical characteristics of the HRT-discordant monozygotic twin pairs

Variable	Non-user (n = 11)	HRT user (n = 11)	Intrapair difference % (95% CI)	P value
<b>Physical characteristics</b>				
Relative muscle area, thigh (%)	49.8 $\pm$ 12.7	54.2 $\pm$ 10.0	11 (3.1–19)	0.013
Relative fat area, thigh (%)	50.2 $\pm$ 12.7	45.8 $\pm$ 10.0	–7.4 (–13 to –1.5)	0.013
Vertical jumping height (cm)	13.0 $\pm$ 4.3	15.1 $\pm$ 4.2	21 (–0.9 to 43)	0.016
Body fat (%)	35.2 $\pm$ 8.9	30.1 $\pm$ 7.1	–11 (–23 to 2.1)	0.026
Maximal walking speed (m s <sup>–1</sup> )	2.0 $\pm$ 0.2	2.1 $\pm$ 0.3	6.3 (–1.4 to 14)	0.074
BMI (kg m <sup>–2</sup> )	28.2 $\pm$ 6.5	25.7 $\pm$ 3.8	–6.7 (–16 to 3.1)	0.09
<b>Biochemical characteristics</b>				
E <sub>1</sub> (pM)	98 $\pm$ 27	900 $\pm$ 1455	760 (–153 to 1673)	0.003
E <sub>2</sub> (pM)	33 $\pm$ 27	173 $\pm$ 203	696 (–147 to 1540)	0.003
Free E <sub>2</sub> (pM)	0.81 $\pm$ 0.58	3.3 $\pm$ 3.3	501 (–33 to 1035)	0.006
SHBG (nM)	43 $\pm$ 15	68 $\pm$ 33	62 (14–109)	0.010
Glucose (mM)	5.2 $\pm$ 0.7	4.9 $\pm$ 0.5	–4.2 (–8.3 to –0.2)	0.041
Testosterone (pM)	639 $\pm$ 269	715 $\pm$ 306	14 (2.1–13)	0.061
Free testosterone (pM)	9.9 $\pm$ 4.7	8.4 $\pm$ 4.7	–12 (–29 to 4.7)	0.075
E <sub>2</sub> /Testosterone	0.06 $\pm$ 0.05	0.27 $\pm$ 0.36	711 (–247 to 1668)	0.087
Free E <sub>2</sub> /Free testosterone	0.10 $\pm$ 0.08	0.54 $\pm$ 0.79	929 (–408 to 2265)	0.104
HbA1c (%)	5.8 $\pm$ 0.3	5.7 $\pm$ 0.3	–1.7 (–4.6 to 1.2)	0.20
Mitochondrial copy number, muscle (%)	377 $\pm$ 107	331 $\pm$ 32	–7.4 (–21 to 5.6)	0.21

The variables within two sections are arranged according to ascending P value. The data presented are partially reported in a previous publication (Ronkainen *et al.*, 2009).

Data are mean  $\pm$  SD.

BMI, body mass index; SHBG, sex hormone-binding globulin; E<sub>2</sub>, 17 $\beta$ -estradiol; E<sub>1</sub>, estrone; HRT, hormone replacement therapy.

regulated genes each sorted according to *P* value and resulted in identification of one significantly up-regulated biological process and eight down-regulated ones in the co-twins using HRT. The complete list of genes responsible for the enrichment is presented in Table S5. The results are presented from the HRT use point of view, i.e., up-/down-regulated pathways in co-twins using HRT.

### Transcripts of 'regulation of anatomical structure morphogenesis' up-regulated in skeletal muscle of the co-twins using HRT

Gene set enrichment analysis on a preranked gene list with genes carrying positive FC ranked according to *P* value revealed three significantly up-regulated pathways in the co-twins using HRT: 'regulation of anatomical structure morphogenesis' with nine genes responsible for the enrichment [false discovery rate (FDR) *q* value = 0.061], 'regulation of cell shape' (FDR *q* value = 0.015) and 'regulation of cell morphogenesis' (FDR *q* value = 0.018), the latter two with six identical genes with core enrichment. Because the latter two were included in the first one, only 'regulation of anatomical structure morphogenesis' is reported in Table 2 and discussed further. One of the genes in the top-ranked gene subset encodes a protein entitled roundabout, axon guidance receptor, homolog 1 (*ROBO1*), a member of the neural cell adhesion molecule (NCAM) subfamily, characterized as a single-pass transmembrane receptor and regarded as a guide for neuronal migration (Kidd *et al.*, 1998). Another gene responsible for the enrichment encoded microtubule-associated protein tau (*MAPT*), a cytosolic phosphoprotein, which functions in stimulating and stabilizing the assembly of microtubules from tubulin. Also, two genes encoding CDC42 effector proteins (Rho GTPase binding) 1 and 4, which mediate the organization of actin cytoskeleton, were found in this up-regulated pathway.

Correlation analysis corrected for clustered sampling and including all the participants revealed that the mean expression of this single up-regulated pathway explained 19% of the varia-

tion observed in the relative proportion of muscle within thigh (*P* = 0.001, Fig. 1A).

### Pathways related to interactions between cells and their environment down-regulated in the co-twins using HRT

A total of ten gene sets were significantly down-regulated in the co-twins using HRT (Table 2). The most significantly down-regulated biological process was 'cell matrix adhesion' (FDR *q* value = 0.053) with 20 genes included in the top-ranked gene subset. Also, nine other down-regulated gene sets were found, of which 'maintenance of localization' and 'maintenance of protein localization', as well as 'cell matrix adhesion' and 'cell substrate adhesion' were identical. The latter ones of both pairs were included in the first gene sets shown in Table 2. 'Cell matrix adhesion' included, for example, a gene encoding sarcoglycan, epsilon (*SGCE*), a membrane-associated glycoprotein, expressed in a variety of tissues and representing an important component mediating membrane–matrix interactions also in skeletal muscle (Ettinger *et al.*, 1997), and a gene encoding fibulin 5 (*FBLN5*), essential in elastogenesis (Nakamura *et al.*, 2002; Yanagisawa *et al.*, 2002). Other important players in the adhesion of cells to its environment included genes encoding proteins such as RAS p21 protein activator 1 (*RASA1*), collagen type XVII alpha 1 (*COL17A1*), lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE1*), integrin beta 1 binding protein 1 (*ITGB1BP1*) and actinin alpha 1 (*ACTN1*).

Correlation analysis revealed a significant negative correlation in that 20% of the variation observed in vertical jumping height was explained by the mean expression of 'cell matrix adhesion' (*P* = 0.008, Fig. 2A).

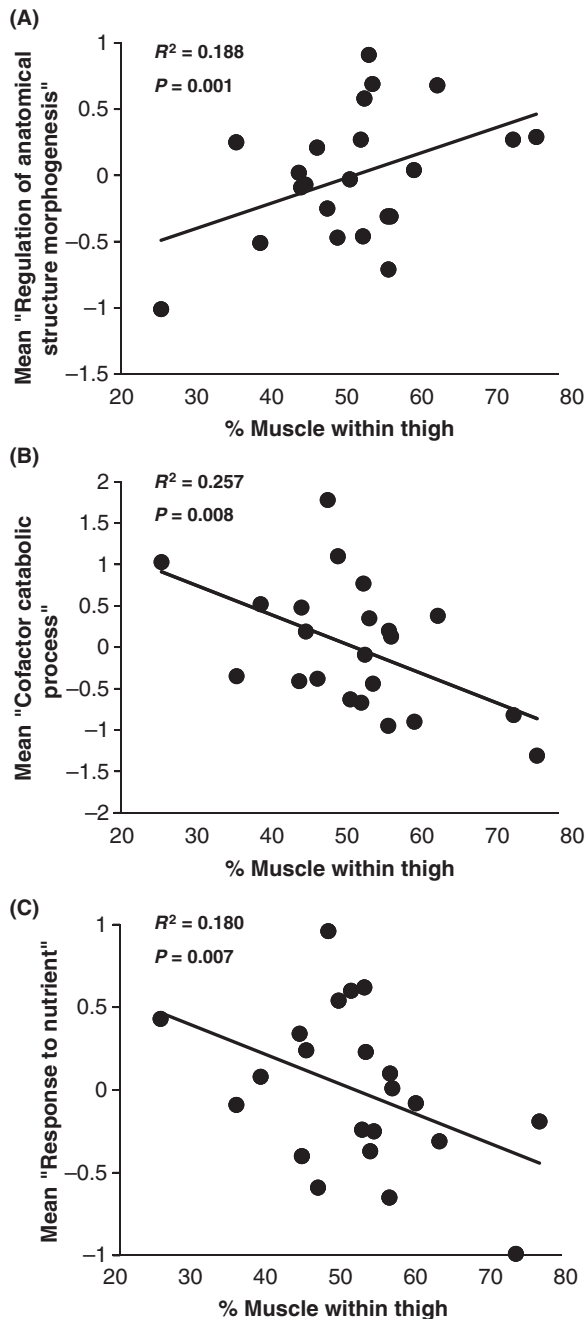
### Mitochondria-related genes down-regulated in the co-twins using HRT

'Cofactor catabolic process' with, for example, genes encoding four subunits – A, B, C and D – of the succinate dehydrogenase

**Table 2** Up- and down-regulated pathways in the muscle of co-twins using HRT (FDR *q* value ≤ 0.15)

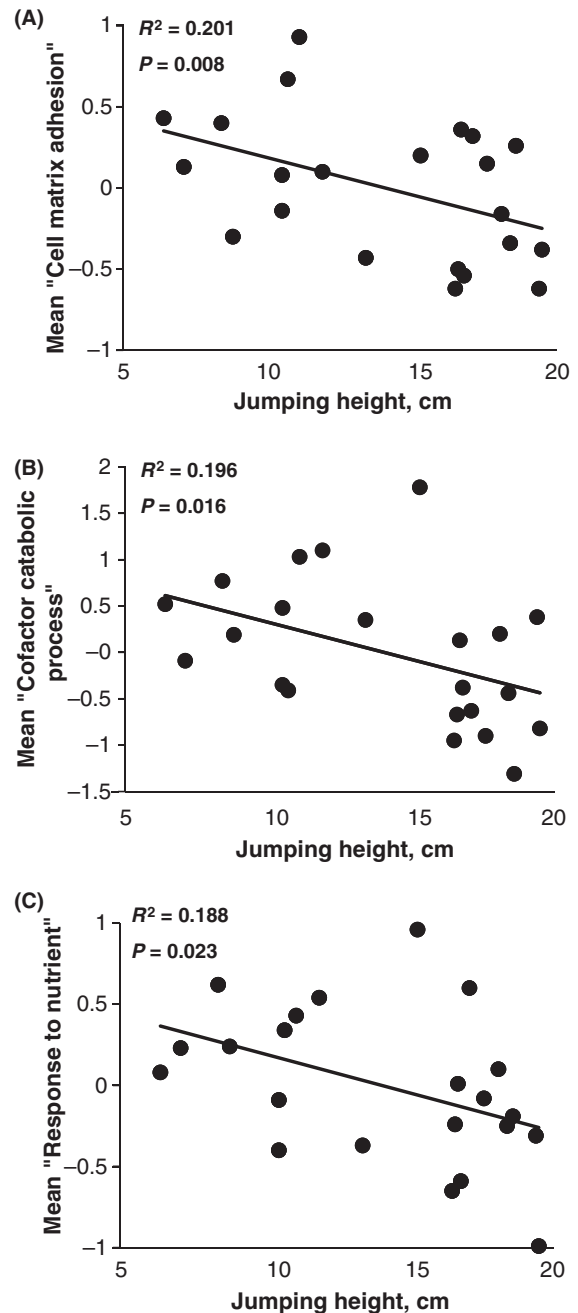
Gene set name	Genes with core enrichment/ genes in the list/ genes in the set	ES	NES	Nominal <i>P</i> value	FDR <i>q</i> value
Up-regulated gene sets in co-twins using HRT					
Regulation of anatomical structure morphogenesis	9/15/26	0.660	1.890	0.0004	0.061
Down-regulated gene sets in co-twins using HRT					
Cell matrix adhesion	20/25/38	0.611	1.911	< 0.0001	0.053
Cofactor catabolic process	6/9/10	0.705	1.798	0.002	0.091
G1 phase	6/8/15	0.721	1.778	0.002	0.101
Maintenance of localization	9/15/22	0.615	1.765	0.001	0.103
Regulation of homeostatic process	8/10/14	0.684	1.800	0.002	0.107
Respiratory gaseous exchange	6/9/15	0.707	1.809	0.003	0.119
Vitamin metabolic process	4/9/17	0.680	1.734	0.004	0.132
Response to nutrient	9/12/17	0.624	1.716	0.004	0.146

ES, enrichment score; NES, normalized ES; FDR, false discovery rate; HRT, hormone replacement therapy.



**Fig. 1** The relationships between relative proportion of muscle within thigh and normalized mean expression values of enriched biological processes titled 'regulation of anatomical structure morphogenesis' (A), 'cofactor catabolic process' (B) and 'response to nutrient' (C).

(SDH) complex was down-regulated in the co-twins using HRT (Table 2). Also, nicotinamide nucleotide transhydrogenase, also a component of the energy transfer system in the integral mitochondrial membrane, was included in the top-ranked gene subset of this biological process. The mean expression value of 'cofactor catabolic process' explained 26% of the relative proportion of muscle ( $P = 0.004$ , Fig. 1B) and 20% of jumping height ( $P = 0.016$ , Fig. 2B). This observation appeared not to be



**Fig. 2** The relationships between muscle performance measured as jumping height and normalized mean expression values of enriched biological processes titled 'cell matrix adhesion' (A), 'cofactor catabolic process' (B) and 'response to nutrient' (C).

attributable to differences in the number of mitochondria, because the mitochondrial copy number was similar within the muscle tissue of both the users and the non-users ( $P = 0.21$ , Table 1). Moreover, SDH staining of the cryosections revealed no significant differences in the oxidative capacity per muscle cross section between the users and the non-users (fibers with low oxidative capacity:  $50.9 \pm 9.5\%$  vs.  $47.4 \pm 13.4\%$  and high oxidative capacity:  $37.3 \pm 9.6\%$  vs.  $40.2 \pm 12.4\%$ , respectively).

## Response to nutrient down-regulated in the co-twins using HRT

Long-term use of HRT was associated with the down-regulation of a gene set entitled 'response to nutrient'. One of the genes in the top-ranked gene subset was gene encoding peroxisome proliferator-activated receptor gamma (*PPARG*), a nuclear receptor known to play a requisite and sufficient role in the regulation of adipocyte differentiation and to have significant contribution to the whole-body glucose homeostasis and insulin sensitivity (Tontonoz *et al.*, 1994; Barroso *et al.*, 1999). Also, endosulfine alpha (*ENSA*), a gene encoding a protein suggested to regulate ATP-sensitive potassium ( $K_{ATP}$ ) channels, which possess a key role in the control of insulin release (Heron *et al.*, 1998), was identified in this category. The mean expression of this pathway was inversely correlated with both muscle mass and performance explaining 18% of the variation in relative proportion of muscle within thigh ( $P = 0.007$ , Fig. 1C) and 19% in jumping height ( $R = -0.433$ ,  $P = 0.023$ , Fig. 2C). According to serum analysis, the HRT users had on average four percent lower blood glucose ( $P = 0.041$ , Table 1), while no significant differences in HbA1c was observed ( $P > 0.05$ ).

## Association between use of HRT and body anthropometry and muscle properties

The co-twins using HRT had on average 10% lower body fat percent compared to their sisters not on HRT ( $P = 0.026$ , Table 1). Relative muscle area within thigh was on average 11% higher ( $P = 0.013$ ) and relative fat area 7% lower ( $P = 0.013$ ) in the HRT users in comparison with the non-users. An indication of better muscle power came from the data on vertical jumping height showing 21% better ability to jump within HRT users than their co-twins ( $P = 0.016$ ), while better mobility ( $P = 0.074$ ) – measured as MWS – within HRT users was observed as well, although the result did not reach statistical significance.

## Discussion

Our genetically identical female twin pairs, in which one co-twin was a long-term user of HRT, represent a powerful model to

investigate the effects of estrogen-based HRT independent of genetic factors and other familial factors, which may confound studies in individuals; within-pair comparisons also greatly reduce variability. Here, we explored global gene expression profiles in skeletal muscle tissue and identified pathways possibly underlying the observed differences in muscle phenotypes, especially muscle composition and performance, of these twins after long-term hormonal treatment. Our results suggest that HRT exerts only moderate, but still relevant, changes in muscle transcriptome. The most notable changes were found in the expression of genes related to organization of cytoskeleton, cell–environment interactions, energy metabolism and responses to nutrition, which in their turn also explained a substantial fraction of the observed variation in muscle mass and performance (Fig. 3). Overall, the transcription profiles suggested that rather than up-regulating gene expression, HRT appeared to down-regulate transcriptional activity. On the other hand, as a result of cross-sectional nature of our data, we may speculate that the processes being 'down-regulated' in the co-twins using HRT are actually preserved near or at the premenopausal levels, while they would be 'up-regulated' in the non-users as a result of aging and postmenopausal changes. Similar observation was also evident in our previous, longitudinal study (Pöllänen *et al.*, 2007).

Only one biological process – 'regulation of anatomical structure morphogenesis' – was significantly up-regulated within co-twins using HRT. The top-ranked gene subset of this enriched process included genes encoding proteins such as MAPT, a stabilizer of the microtubule network and a player important in the generation and maintenance of neurites. Overexpression of MAPT has been documented to inhibit kinesin-dependent transportation of peroxisomes into neurites predisposing the cells to oxidative stress (Stamer *et al.*, 2002). A link between MAPT and muscle has been reported as well. More specifically, transgenic mice expressing human MAPT with a common mutation, namely P301L, were observed to exhibit skeletal muscle with neurogenic atrophy (Lewis *et al.*, 2000). The link between the up-regulation of *MAPT* mRNA and the preserved muscle composition in the HRT users remains, however, enigmatic. Also, CDC42 effector proteins (CDC42EP) 1 and 4, interacting with CDC42 to regulate the organization and assembly of the actin

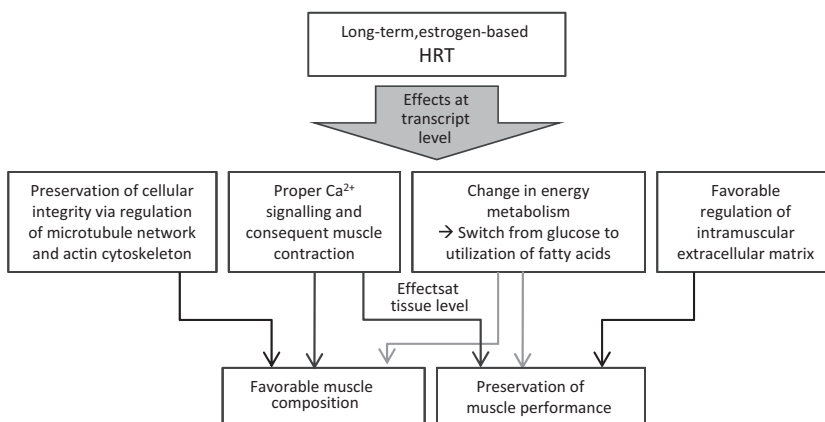


Fig. 3 Summary of the main findings.

cytoskeleton (Bishop & Hall, 2000), were included in the gene list responsible for the enrichment. Intriguingly, inhibition of CDC42 by exogenous expression of a dominant negative form has been documented to result in a block in myogenesis (Takano *et al.*, 1998; Luo *et al.*, 1994), although contradictory results have also been published (Gallo *et al.*, 1999; Meriane *et al.*, 2000). Nonetheless, CDC42 is undoubtedly an important agent in the milieu of skeletal muscle and, in our study design, is included in the biological process, whose mean expression explains 19% of the variation observed in relative proportion of muscle within thigh. Our result concerning the link between HRT and cell structure is supported by some studies such as that by Kublickiene and colleagues documenting that estrogen-based HRT may preserve the morphological integrity of endothelial cells by regulatory actions on cytoskeleton (Kublickiene *et al.*, 2008). Moreover, exposure of endothelial cells to physiological levels of estradiol has been reported to result in rapid remodeling of actin cytoskeleton (Simoncini *et al.*, 2006).

Aside from being a major protein reservoir, skeletal muscle specializes in generating force and producing movement. The stabilizing structures of muscle tissue, located in contractile filaments, between sarcomeres and next to sarcolemma, hold the filaments together and enable appropriate function of this tissue and form an essential player in force transmission. With age, muscle stem cells are demonstrated to switch from myogenic fate to fibrotic one further resulting in impaired muscle regeneration and enhanced fibrotic response (Brack *et al.*, 2007). Our results implying that genes related to 'cell matrix adhesion' are down-regulated in the sisters using HRT may actually indicate that the respective process is up-regulated in the nonusers and maintained close to premenopausal levels in the HRT users. This notion would explain the significant inverse association observed between the mean expression of 'cell matrix adhesion' and jumping height. Speculatively, the higher expression of the genes related to the regulation of extracellular matrix and cell-matrix interactions in skeletal muscle in the non-users could impede proper force transmission through affecting the amount and quality of the matrix components in the interstitial space of muscle tissue. This scheme with impaired muscle composition is further supported by our recent paper reporting that relative proportion of fat within thigh is on average 7% lower among the HRT users compared to the non-users in this same study sample (Ronkainen *et al.*, 2009). Moreover, among the genes of top-ranked gene subset was, for example, FBLN-5, an organizer of elastic fiber assembly. A truncated form of FBLN-5, unable to carry its role in elastogenesis, is reported to accumulate with age (Hirai *et al.*, 2007). Thereupon, higher expression of this gene in the nonusers may represent an indication to compensate for the aging-induced accumulation of the truncated form upon increased production of Extracellular Matrix components.

'Cofactor catabolic process' was one of the down-regulated pathways among co-twins using HRT. The pathway included, for example, the four subunits of the SDH complex functioning in the mitochondrial respiratory chain in the inner membrane of

mitochondria (Chen *et al.*, 2009). We found no differences in the mitochondrial copy number or in the oxidative capacity of the muscle per cross section. The down-regulated expression of the components of the complex II of mitochondria cannot, however, be underestimated (Chen *et al.*, 2009). Interestingly, aside from the down-regulated genes along this pathway, pyruvate dehydrogenase kinase isoenzyme 4 (PDK4), known to be expressed primarily in skeletal muscle and heart (Bowker-Kinley *et al.*, 1998), had the second highest FC (mean HRT user/non-user, 2.4) of all the probes in the array. PDK4 functions as an inactivator of the pyruvate dehydrogenase complex (PDC), a pivotal metabolic switch for fuel selection, thereby leading to the utilization of fatty acids instead of glucose and contributing to the overall control of aerobic oxidation of carbohydrate fuels. Importantly, PDK4 has been suggested to contribute to the regulation of the adaptive response or long-term control of the activity of PDC (Randle, 1986; Priestman *et al.*, 1992). In human muscle cell culture, insulin reverses the up-regulation of *PDK4* caused by glucose deprivation and fatty acid supplementation (Abbot *et al.*, 2005). The link between the down-regulation of the components of SDH complex and up-regulation of PDK4 could imply an overall decrease in glucose oxidation and possible preference for the utilization of fatty acids as an energy source after long-term use of HRT. In our study population, this switch in metabolic status of muscle tissue resulted in both higher relative proportion of muscle tissue within thigh and better muscle performance. Our hypothesis is supported by a previous study in which the decline in fat-free mass was the best single predictor of the decline in basal fat oxidation in humans (Calles-Escandon *et al.*, 1995). Moreover, another study with rats reported that aging was associated with a decreased ability of muscle to oxidize fatty acids, a fact that was suggested to explain the accumulation of triglycerides in muscle, which again is a possible contributor to several metabolic disorders such as insulin resistance (Tucker & Turcotte, 2002).

Intriguingly, one of the down-regulated genes in the pathway 'response to nutrient' encoded PPARG, which plays a critical role as a transcriptional regulation of both adipogenic and lipogenic programs (Spiegelman, 1998). PPARG is also suggested to represent a molecular link between fatty acids and insulin sensitivity (Way *et al.*, 2001; Evans *et al.*, 2004; Tontonoz & Spiegelman, 2008). The inhibition of the expression of PDK4 has been postulated to represent a mechanism by which PPARG agonists enhance glucose metabolism in muscle. In this context, the down-regulation of PPARG and up-regulation of PDK4 with a parallel down-regulation of the components of mitochondrial respiratory chain may, again, pose an indication of lowered utilization of glucose in skeletal muscle after estrogen-based HRT, while exploitation of fatty acids as energy source would be enhanced. Interestingly, also ENSA and stanniocalcin 1 (STC1) were included in the top-ranked gene subset of this down-regulated pathway concerning responses to nutrients. Previously, ENSA has been shown to block  $K_{ATP}$  channels, possibly triggering insulin secretion via membrane depolarization, activating voltage-gated  $Ca^{2+}$  entry and finally increasing the levels of

intracellular  $\text{Ca}^{2+}$  (Heron *et al.*, 1998). Therefore, ENSA can be considered to play a key role in coupling cell metabolism to electrical activity. Also, inclusion of STC1, a secreted glycoprotein hormone and a regulator of calcium and phosphate homeostasis (Madsen *et al.*, 1998), in this pathway may perhaps suggest the impact of long-term HRT on calcium homeostasis in muscle. STC was originally identified in fish, in which it is secreted as a response to a rise in serum calcium and prevents hypercalcemia (Wagner *et al.*, 1989). The high mean expression of this pathway together with poor relative proportion of muscle and muscle performance in the non-users may indicate that calcium signaling is boosted at transcript level but is not translated into improved function of this tissue, while the users would have a satisfactory calcium signaling and consequently better muscle properties.

This study has some limitations. The data are based on a limited sample size. However, despite the rather small amount of participants, the MZ co-twin controlled study represents perhaps one of the best case-control study designs available in humans owing to the complete or at least close match for genetic background, age and gender as well as for intrauterine and childhood environment. Furthermore, the number of participants in the present study design with discordant MZ twins is comparable to the number of participants in previous reports (e.g., Laustiola *et al.*, 1988; Bouchard *et al.*, 1990; Pietiläinen *et al.*, 2008; Leskinen *et al.*, 2009) and has sufficient power to detect clinically relevant differences. The participants used different preparations currently prescribed in clinical practice. A subgroup analysis for the pairs with the users of estrogen-only ( $n = 5$  pairs) and estrogen plus progestogen treatments ( $n = 6$  pairs) is not included as the sample sizes would then perhaps be too small for statistical testing. The lack of a fixed HRT preparation reflects the real-life nature of the current design. Furthermore, a Randomized controlled trial with a comparable duration of HRT may not be possible to conduct. The HRT users had no discordance in daily energy intake, physical activity, smoking or medication, thereby allowing the discordance to be mostly attributable to use of HRT.

Nowadays, several articles report results from microarrays without separate confirmation of gene expression by quantitative PCR (qPCR) (e.g., Zahn *et al.*, 2006; Pietiläinen *et al.*, 2008; Pöllänen *et al.*, 2010; Stephens *et al.*, 2010). This appears justified as several previous studies have shown that qPCR nicely reproduces the results from microarrays (e.g., Roth *et al.*, 2002; Rome *et al.*, 2003; Welle *et al.*, 2003; Zambon *et al.*, 2003; Welle *et al.*, 2004; Melov *et al.*, 2007; Pöllänen *et al.*, 2007; Thalacker-Mercer *et al.*, 2007). This series of literature suggests that the quality and reproducibility of the microarray experiments are well established, and additional validation with qPCR may perhaps not be necessary anymore.

Several studies have been conducted during the last years to evaluate the possible risks and benefits of HRT. The risks involve, for instance, increased incidence of stroke (Anderson *et al.*, 2004; Rossouw *et al.*, 2002) and venous thromboembolism (Anderson *et al.*, 2004; Rossouw *et al.*, 2002; Canonico *et al.*,

2008; Sare *et al.*, 2008), whereas the benefits include the improvement in vasomotor symptoms (MacLennan *et al.*, 2001; Notelovitz *et al.*, 2000; Stearns *et al.*, 2002, 2002), as well as protection against both colon cancer (Nanda *et al.*, 1999; Grodstein *et al.*, 1999) and osteoporosis (Torgerson & Bell-Syer, 2001; Wells *et al.*, 2002). Despite the major role of skeletal muscle in overall health, the effects of HRT on muscle tissue are frequently neglected. This study sheds a light on the differences in global gene expression profiles in muscle samples that emerged after long-term use of estrogen-based HRT. Our real-life example with female twin pairs discordant for HRT offers an excellent opportunity to carefully dissect the changes in transcriptome with an advantage of total control of variation in genetic background. Because women typically use HRT for many years, investigating the effects of long-term administration rather than solely short-term exposure is of immense importance. In summary, we found that rather than exerting massive changes, long-term HRT creates subtle, but pertinent, differences in muscle transcript profiles. The better muscle composition and performance among the HRT users appeared to be orchestrated by improved regulatory actions on cytoskeleton, preservation of muscle quality via regulation of intramuscular extracellular matrix and a switch from glucose-oriented metabolism to utilization of fatty acids.

## Experimental procedures

### Study design

This study is part of a larger research project, 'Sarcopenia – Skeletal Muscle Adaptation to Postmenopausal Hypogonadism and Effects of Hormone Replacement Therapy and Physical Activity in Older Women: a Genetic and Molecular Biological Study on Estrogen-related Pathways' (SAWEs), which was set up to investigate the effects of HRT on muscle properties at molecular level in a genetically controlled design. The recruitment process has been described elsewhere (Ronkainen *et al.*, 2009). Shortly, out of 537 MZ female twin pairs from selected age-groups of the population-based Finnish Twin Cohort (Kaprio & Koskenvuo, 2002), who were invited to self-select themselves as being discordant for the use of HRT, a total of 16 pairs responded to the invitation, met the inclusion criteria and were further invited to the laboratory examinations. Subjects with any contraindications for participation in the measurements (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) and for biopsy sampling (hemorrhagic diseases or use of warfarin) were excluded. The zygosity of the twins was verified at the Paternity Testing laboratory (National Public Health Institute, Helsinki, Finland) using DNA extracted from a venous blood sample with a battery of ten highly polymorphic gene markers. Fifteen of the 16 pairs participating in the measurements were confirmed to be MZ



pairs, whereas one twin pair turned out to be dizygotic and was excluded from the present analyses.

Of the HRT users, five women used estradiol-only preparations (the amount of the estrogenic agent in the preparation 1–2 mg), whereas six were taking a combined treatment including estrogenic (1–2 mg) and progestogenic compounds. Four pairs, in which the user administered a tibolone preparation, were excluded from the present study, because tibolone-containing therapy was found to have differential effects on muscle properties at phenotype level compared to estrogen-based treatments (Ronkainen *et al.*, 2009) and the focus in the present study was set especially on estrogen-based therapies. Mean duration of HRT usage in the eleven included pairs was  $7.3 \pm 3.7$  years (range, 2–16 years). There were no differences between the users and the nonusers in the time since the last menstrual periods ( $7.7 \text{ years} \pm 3.7$  vs.  $7.1 \pm 3.1$ ,  $P = 0.31$ ). All the laboratory measurements and data analysis were carried out blind to HRT status.

The 15 twin pairs forming the entire study group were previously reported not to differ in physical activity, daily energy intake, use of medication or smoking behavior (Ronkainen *et al.*, 2009). Reanalyzing the data within the eleven pairs that are included in this study resulted in the same observation; no differences within these background variables were found.

The Ethics Committee of the Central Finland Health Care District approved the study, and the study was conducted according to the guidelines as laid down by the World Medical Association in the Declaration of Helsinki (2000). Written informed consent was provided by the participants before the measurements.

### Serum analyses

Fasting blood samples were taken from the antecubital vein in a supine position between 7 AM and 9 AM. Sera were stored at  $-70^\circ\text{C}$  immediately after sampling. The serum levels of glucose and HbA1c were derived from blood count (Sysmex KX-21N; Roche Diagnostics Inc., Espoo, Finland). Sex hormone-binding globulin (SHBG) concentrations was measured with solid-phase, chemiluminescent immunometric assays (Immulite 1000; Diagnostic Products, Los Angeles, CA, USA). The interassay coefficient of variation (CV) was 8.4% for 32.4 nM (SHBG). Serum  $E_2$  levels were determined in duplicate by extraction Radioimmunoassay as previously described (Ankarberg-Lindgren & Norjavaara, 2008). Interassay CV was 19% at 6 pM and < 14% for concentrations of 12 pM and above. Serum testosterone was measured as previously described (Turpeinen *et al.*, 2008). Limit of quantification was 70 pM, and interassay CV was 5.2% for the concentration 4.7 nM.  $E_2$  and testosterone were utilized together with SHBG in calculating the respective free hormone levels according to previously presented methods (Vermeulen *et al.*, 1999; Bjornerem *et al.*, 2004). Estrone ( $E_1$ ) was determined as a dansyl derivative by LC-MS/MS with an API 4000 mass spectrometer as previously described (Nelson *et al.*, 2004). Interassay CV was 7.8% for the concentration of 200 pM, while the limit of quantification was 10 pM.

### Muscle biopsy sampling

Muscle biopsies were obtained from the mid-part of the *vastus lateralis* defined as a midpoint between the greater trochanter and the lateral joint line of the knee. Following the removal of all visible residues of fat and connective tissue, the biopsy samples for RNA and DNA analysis (on average 40 mg) were snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use. The biopsies for immunohistochemical analysis were oriented vertically and mounted in O.C.T. embedding medium (Tissue-Tek<sup>®</sup>, Sakura Finetek Europe B.V.) followed by snap-freezing in isopentane ( $-160^\circ\text{C}$ ) precooled in liquid nitrogen. Adjacent transverse cross sections (10  $\mu\text{m}$ ) were cut with a cryomicrotome. Traditional hematoxylin–eosin staining provided no evidence of any pathology in muscle tissue in any participants.

### RNA and DNA extraction and cRNA synthesis

Frozen muscle biopsies were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) utilizing FastPrep FP120 apparatus (MP Biomedicals, Illkrich, France). Total RNA and DNA fractions were extracted according to the manufacturer's guidelines and used in microarray hybridizations and in determining mitochondrial copy number, respectively. For microarray analysis, the RNA/cRNA concentrations were checked with Nanodrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and RNA/cRNA quality using Bio-Rad's Experion electrophoresis station (Bio-Rad Laboratories, Hercules, CA, USA) both before and after amplifications. The amplification of total RNA (200 ng) was performed with Ambion's Illumina RNA TotalPrep Amplification kit (Ambion, Austin, TX, USA). The reaction was performed overnight, during which the RNA was biotinylated.

### Microarray hybridizations

Each sample was hybridized to Illumina's Sentrix<sup>®</sup> Human-WG6 V3 Expression BeadChip (cat no BD-101-0603; Illumina, San Diego, CA, USA) containing probes for 48 803 transcripts. The hybridizations were performed by the Finnish DNA Microarray Center at Turku Center for Biotechnology according to Illumina<sup>®</sup> Whole-Genome Gene Expression Direct Hybridization protocol (revision A). Hybridization was detected with 1  $\mu\text{L mL}^{-1}$  Cyanine3-streptavidine (cat no PA43001, GE Healthcare Biosciences, Waukesha, WI, USA). The chips were scanned with Illumina BeadArray Reader. The numerical results were extracted with Bead Studio 3.1.3.0 (module v. 3.3.7, Illumina, San Diego, USA) without any normalization or background subtraction. Generation of cRNA, hybridizations of the arrays and quality control of the raw data were performed by the Finnish DNA Microarray Center at Turku Center for Biotechnology. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE21421 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21421>). The MIAME guidelines

were followed during array data generation, preprocessing and analysis.

### Microarray data preprocessing and enrichment analysis

The overall objective in microarray data mining was to identify significantly up- and down-regulated biological pathway compositions rather than sporadic information concerning significantly regulated individual genes. Initial data analyses for microarray data were performed utilizing R software (<http://www.R-project.org>) together with Bioconductor development software (<http://www.bioconductor.org>). The raw data of each chip were first-quantile-normalized utilizing the affy package for R (Gautier *et al.*, 2004). Hierarchical clustering was carried out in estimating the quality of the data. Pairwise analyses of the gene expression data were conducted utilizing limma package with a model of Bayesian shrinkage (Smyth, 2004). The data were ranked according to co-twin FC, which is a base-two logarithm resulting from dividing the expression value of each HRT user with the expression value of the respective sister (the non-user) to correct for the identical genetic background.

Enrichment for functionally related genes across a spectra of 825 gene sets of Gene Ontology biological processes (C5: BP, <http://www.broadinstitute.org/gsea/msigdb/collections.jsp>) was tested using Gene Set Enrichment analysis (GSEA; Version 2.0) (Subramanian *et al.*, 2005). Gene set enrichment analysis on a preranked gene list was performed. Two preranked gene lists were constructed: a list with up-regulated genes in the co-twins using HRT and a list including down-regulated genes in the co-twins using HRT each sorted according to *P* value.

Five thousand permutation cycles were carried out, and gene sets with at least seven and no more than 250 genes were taken into account in each analysis. This was because gene set classifications are organized in a tree-like manner so that gene sets become progressively larger and less descriptive while moving up the tree. Hence, to focus on the biologically most meaningful pathway collections, a cutoff was set to a maximum of 250 genes as the largest gene list to be documented. For the genes with multiple probes on the chip, the probe with the highest rank in the preranked list of genes (i.e., the smallest *P* value) was chosen to represent a given gene. Each analysis was carried out at least three times. A gene set with FDR < 0.150 was considered as significantly enriched.

The normalized mean expression values were calculated for the most important biological processes and correlated with the phenotype data. These mean expressions were calculated from quantile-normalized data by including the data from genes in the leading edge subset (top-ranked gene subset) of each biological process in the analyses. 'Standardized values' were calculated for these quantile-normalized expression values of each gene set for each individual utilizing SPSS software. 'The normalized mean expression value' for each biological process used in correlation analyses is the mean of these standardized values of the top-ranked genes in a given biological process.

### Mitochondrial copy number

Mitochondrial copy number was determined essentially as previously described (Pietiläinen *et al.*, 2008). Shortly, ABI 7300 Real-Time PCR System was used to quantify the amount of mitochondrial DNA. The mtDNA copy number was calculated based on the simultaneous amplification of the mitochondrial cytochrome b (Cytb, mtDNA, forward: GCCTGCCTG ATCCTCCAAAT, reverse: AAGGTAGCG GATGATTAGCC, probe: FAM – CAC-CAGACG CCTCAACCGCT T – TAMRA) and the nuclear amyloid protein beta precursor protein (APP, nuclear DNA, forward: TGTGTGCTC TCCCAGGTCTA, reverse: CAGTTCTGG ATGGTCACTGG, probe: VIC – CCCTGAACT GCAGATCACCAA-TGTGGTAG – TAMRA) and expressed as the ratio of mtDNA to nuclear DNA converted to percentage in each specimen. Cloned plasmids containing the human APP or CytB gene (kind gifts from Professor Anu Suomalainen) were utilized in constructing standard curves. Standards were included in each run and used at concentrations of  $10^3$ – $10^7$  copies. Quantification was performed in the exponential amplification phase.

### Succinate dehydrogenase staining

The activity of SDH was used as a marker for oxidative capacity of muscle. SDH staining was performed as previously described (Pette & Tyler, 1983). The 8-bit images converted from the stained sections were processed and analyzed using ImageJ software (NIH). Intensity threshold separating areas with low and high oxidative capacity according to SDH activity was set manually and separately for all images. Finally, two intensity-scaled fractions representing low and high level of oxidative capacities were expressed as the percentage of the total measured area studied. Average percentage area fractions from the images (1–3 from each specimen) were analyzed. Five samples from the non-users and seven from the users were eligible for further analysis. These participants formed altogether four twin pairs. The data analysis was performed both between the groups (Mann–Whitney *U* test) and the within the four pairs (Wilcoxon's signed rank test).

### Body anthropometry and muscle phenotype

Body anthropometry was measured and muscle phenotyping was performed as previously described (Ronkainen *et al.*, 2009). BMI was calculated from weight and height measured by standard procedures. Shortly, body anthropometry including the percentage of body fat was determined between 07:00 and 10:00 hours after overnight fasting using a multifrequency bio-electrical impedance analyzer [InBody (720); Biospace, Seoul, Korea]. CT scans (Siemens Somatom Emotion scanner; Siemens AG, Erlangen, Germany) were obtained from the midpoint between the greater trochanter and the lateral joint line of the knee and relative proportions of muscle and fat analyzed using software developed at the University of Jyväskylä for cross-sectional CT image analysis (Geanie 2.1; Commit Ltd, Espoo,

Finland). Muscle power, i.e., the ability of the neuromuscular system to produce the greatest possible force as fast as possible, was assessed as the height that the participant was able to elevate her body's center of gravity during a vertical jump (vertical jumping height) on a contact mat. Maximal walking speed for 10 m was measured in a laboratory corridor utilizing photocells.

## Statistics

Owing to the relatively small number of observations, the statistical significance of the differences between the means of phenotypic variables in HRT users and non-users was tested with Wilcoxon's signed rank test. Data are shown as means and SDs unless otherwise stated. Percentage intrapair differences (IPD%) were calculated as follows: (HRT user – non-user):(non-user) × 100. In addition, the 95% confidence interval (CI) was calculated for the IPD%. In individual twins, Pearson correlation coefficient together with linear regression analysis corrected for clustered sampling was used in determining the relationships between gene expression and muscle phenotypes. Coefficient of determination ( $R^2$ ) is presented in the figures for the relationship between the given variables. The skewness and kurtosis were tested for the variables, which were not normally distributed, and considered to be acceptable. Thereby, parametric testing was performed for all correlations. The results from SDH staining were tested with both Mann–Whitney  $U$  test and Wilcoxon's signed rank test. The level of significance was set at  $P \leq 0.05$ . Data analyses were carried out with SPSS (version 15.0; SPSS, Chicago, IL, USA) and Stata (version 10; Stata Corporation, Texas, AZ, USA).

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## Author contributions

VK, SS, UK, PR and EP designed the study. JK was responsible for the Finnish Twin Cohort based on which the twin pairs were

recruited. PR, EP, MA, RP, JP and SS contributed to the acquisition of the data, and VK supervised the process. PR was mainly responsible for data analysis. All authors contributed to the interpretation of the data, critical revision of the manuscript and approving the final version of the manuscript, while PR drafted the article.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Table S1** The most significantly up-regulated sequences within the co-twins using HRT (nominal  $P \leq 0.001$  from the Empirical Bayes test statistics, limma, R/Bioconductor)

**Table S2** The most significantly down-regulated sequences within the co-twins using HRT (nominal  $P \leq 0.001$  from the Empirical Bayes test statistics, limma, R/Bioconductor)

**Table S3** Thirty genes with the highest fold change

**Table S4** Thirty genes with the lowest fold change (artificial cut off for HRT user/non-user  $\leq 0.85$ )

**Table S5** Genes responsible for the enrichment in the up-regulated and down-regulated pathways in the twins using HRT

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