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Reeta Kangas

Aging and MicroRNA Messaging

Associations with Systemic Estrogen Levels and Physical Performance

JYVÄSKYLÄN YLIOPISTO

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“Little drops of water… make the mighty ocean.”
Julia Abigail Fletcher Carney

ABSTRACT

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Finnish summary

This thesis took an innovative approach, which involved methodological development, to investigate microRNAs as candidate molecules and as genome-wide regulators of age, menopausal status and physical condition in both sexes. The focus was on potential serum microRNAs indicative of age, pre- or postmenopausal status, with or without estrogen-based hormone replacement therapy (HRT), and long-term physical training. MicroRNA associations with adiposity and inflammatory markers were also analyzed.

Three different study designs were used: 1) the SAWEs study comprised healthy postmenopausal monozygotic twin sisters (n=11, 54-62 years) with discordance for HRT and premenopausal women (n=30, 20-40 years) without hormonal contraceptives; 2) the miRBody study comprised non-related postmenopausal women (54-62 years) either on (n=16) or not on HRT (n=17); and 3) the ATHLAS study comprised male masters sprinters in a 10-year follow-up design (n=49, baseline age 40-80 years) and in a cross-sectional design (n=67, 18-90 years). For the explorative microRNA discovery, we set up next generation sequencing method for serum exosome microRNAs, exomiRs, and utilized arrays for freely circulating serum and adipose tissue microRNAs. Selected microRNAs were further validated. MiR-21-5p and -146a-5p were analyzed in both sexes, owing to their hypothesized role in aging, inflammation and exercise response. Sex steroids, inflammatory markers and fat/glucose metabolites were measured.

Profiled microRNA patterns in serum and adipose tissue differed by age and HRT use vs. non-use. Validations revealed age associations for five, and HRT associations for three microRNAs in serum. In addition, seven adipose tissue microRNAs were higher with higher ages. MiR-146a-5p was negatively associated with HRT use in both serum and muscle. In the sprinters, a negative association with age was detected for serum miR-146a-5p and non-linear associations with specific power and speed capacity measures in 10 years for miR-146a-5p and -21-5p.

The depth of the methodologies and human designs used, enabled novel discoveries regarding aging, 17β-estradiol (E_2), and physiological (health) status-associated microRNAs. Of them, exomiR-27b-3p and -148a-5p, indicative of higher age, lower serum E_2 and poorer adiposity and inflammatory status in women, were the most promising candidates for early markers of worsening metabolism with declining systemic E_2. Moreover, serum miR-21-5p was indicative of more inflamed and fatty phenotype in women, whereas miR-146a-5p appeared indicative of a healthier phenotype in both sexes. Overall, this research underlines the potential for using serum microRNAs, together with other traditional markers, as tools in evaluating the physiological status of an individual, thus contributing new information to the health and aging field. Keywords: microRNA, serum, aging, estrogen, hormone replacement therapy, menopause, physical performance

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following four original articles, referred to in the text by their roman numerals.

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II. Kangas R, Morsiani C, Pizza G, Lanzarini C, Aukee P, Kaprio J, Sipilä S, Franceschi C, Kovanen V, Laakkonen EK, Capri M. 2017. Menopausal status associates with specific microRNA and target expressions in subcutaneous adipose tissue. Submitted manuscript.

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IV. Kangas R, Törmäkangas T, Heinonen A, Alen M, Suominen H, Kovanen V, Laakkonen EK, Korhonen MT. 2017. Declining physical performance associates with serum FasL, miR-21, and miR-146a in aging sprinters. Biomed Research International. Published online January 3. DOI: 10.1155/2017/8468469

ABBREVIATIONS

AGO	argonaute
AKT1	serine/threonine-protein kinase B 1, v-akt murine thymoma viral oncogene 1
APOE	apolipoprotein E
BCL-2	B cell lymphoma 2
BMI	body mass index
BRAF	serine/threonine-protein kinase B-raf
CCND1	cyclin D1
cDNA	complementary DNA
CMJ	countermovement jump
CREB	cAMP responsive element binding protein
DGCR8	DiGeorge syndrome critical region 8
E_1	estrone
E_2	17β-estradiol
E_3	estriol
ESR1	estrogen receptor 1; ERα
ESR2	estrogen receptor 2; ERβ
exomiR	exosome-packed microRNA
FasL	Fas-ligand
FDR	false discovery rate
FGF21	fibroblast growth factor 21
FOXO1A	forkhead box O1A
FOXO3A	forkhead box O3A
FSH	follicle-stimulating hormone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDM	gestational diabetes mellitus
GEE	generalized estimated equation
Gluc	glucose
GPER	G-protein coupled estrogen receptor; GPER30
HIIT	high-intensity interval training
HDL	high density lipoprotein
HIV	human immunodeficiency virus
hMSCs-Ad	human adipose-derived mesenchymal stem cells
HRT	hormone replacement therapy
hsCRP	high sensitivity C-reactive protein
IGF1R	insulin growth factor 1 receptor
IL-6	interleukin 6
Ins	insulin
LBM	lean body mass
LDL	low density lipoprotein
MCF7	Michigan Cancer Foundation-7 (human breast adenocarcinoma cell line)

MHCI/II	major histocompatibility complex I or II
mRNA	messenger ribonucleic acid
MVB	multivesicular bodies
NIH	National Institute of Health
NGS	next generation sequencing
PDCD4	programmed cell death protein 4
Pol II	polymerase II
PPAR-γ	peroxisome proliferator-activated receptor gamma
Prdm16	PR domain zinc finger protein 16
PTEN	phosphatase and tensin homolog
RISC	RNA-inducing silencing complex
RT-qPCR	real time quantitative polymerase chain reaction
SHBG	sex hormone-binding globulin
SVCs	stromal vascular cells
T	total testosterone
TNFα	tumor necrosis factor alpha
TriGly	triglyceride
WBC	white blood cell
WHI	Women's Health Initiative
WHO	World Health Organization

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1 INTRODUCTION

Why do we age, when does aging begin and could it be slowed, stopped or even reversed? These have been among the most intriguing questions for centuries. The modern biological aging theories can be roughly divided in to two categories: 1) programmed aging (Cornelius 1972, Davidovic et al. 2010, van Heemst 2010) and 2) error/damage-induced aging (Harman 1956, Bjorksten & Tenhu 1990, Brys, Vanfleteren & Braeckman 2007). The basic idea of the programmed aging theory is biological timing: development and growth are followed by aging, a process driven by upfront programmed changes in gene expression patterns. In the error/damage-induced aging theory, the driving force, cumulative damage, arises mainly from the effects of environmental factors on molecular mechanisms.

Aging is a natural non-pathological phenomenon that affects everyone. Commonly, age-associated processes are catabolic, leading to the weakening of an individual. These gradual detrimental processes are regulated by cell and molecular mechanisms occurring at the whole-organismal level, affecting defense mechanisms, physical functioning and cognition. One of the major factors limiting overall physical functioning with aging is weakened muscular properties including declining skeletal muscle force, mass and infiltration of fat into muscle. This complex phenomenon, referred to as sarcopenia, is caused by intramuscular, hormonal and neural changes leading to adverse outcomes. In women, the prevalence of sarcopenia clearly peaks after the menopausal transition, which is characterized by various hormonal changes, including a dramatic decrease in circulating estrogen levels (Phillips et al. 1993, Calmels et al. 1995, Samson et al. 2000). The negative effects of postmenopausal sarcopenia can be attenuated by estrogen-based hormone replacement therapy (HRT) (Phillips et al. 1993, Sipilä et al. 2001). These findings indicate that the systemic sex steroid hormones play a crucial role in defining the properties of skeletal muscle and physical performance capacity. In comparison to women, the aging-associated changes in the skeletal muscle of men are less sudden like the changes in the systemic hormonal milieu; thus they occur gradually. In addition to weakening of the musculature, aging, together with hormonal changes, affects overall body

metabolism, as has been detected in various tissues (reviewed e.g., by Faulds et al. 2012, Hevener, Clegg & Mauvais-Jarvis 2015, and Van Pelt, Gavin & Kohrt 2015). There are a few mechanisms which have been shown to counteract detrimental aging-related processes; these mechanisms include e.g., physical exercise, a healthy diet and western medicine, including HRT (Sorensen et al. 2001, Lanza et al. 2008, Erickson et al. 2011, Moreau et al. 2013, reviewed by Anton et al. 2016).

Today, one of the most intriguing questions in research on aging, and all the phenomena related to that progressive process, is how everything is orchestrated. We know that genetic components are to a fair degree responsible for how we age (Christensen, Johnson & Vaupel 2006). However, the traditional idea of how our gene codes are transcribed into messenger RNA and further translated and folded into functional proteins has been revolutionized over the past few decades by epigenetics. Epigenetic changes are heritable modifications which affect the gene expression of a cell without changing the DNA sequence (Holliday 1994). They occur naturally but can also be induced by environmental factors, age or disease (van Dongen et al. 2016). At least two different types of epigenetic changes have been identified, including DNA methylation or other DNA modifications and histone modification. In addition, if not counted as epigenetic mechanisms themselves, non-coding RNAs strongly interact with epigenetics. They affect the epigenetic machinery by regulating the gene expression of the enzymes crucial for DNA methylation and histone modifications and in turn the expression of non-coding RNAs is also controlled by these modifications (reviewed by Iorio, Piovan & Croce 2010).

MicroRNAs are a class of small non-coding RNAs regulating gene expression by binding to specific mRNA regions and blocking protein translation and potentially leading to degradation of corresponding mRNA (Valencia-Sanchez et al. 2006, Selbach et al. 2008, Friedman et al. 2009). They often have an influence on the cells in which they are produced or on the distant tissues to which they are being delivered via various transporting mechanisms in body fluids (Valadi et al. 2007, Vickers et al. 2011, Arroyo et al. 2011). MicroRNAs are specific, sensitive and transportable molecules that have been shown to have broad functional effects in different cell types from brain cells to skin cells, making them promising candidates as messengers and regulators in aging-related phenomena (reviewed by Lawson et al. 2016). As they are sensitive genetic regulators, it is important to understand the external and internal factors that affect microRNAs themselves.

The discrepancy between chronological and biological age in many individuals is one of the promising phenomena in aging research. As yet, we cannot precisely identify the triggering point for aging. Bearing this in mind, studies focusing on molecular-level events, need to be initiated rather early during the lifespan. Therefore, data for this thesis were gathered from women and men of different ages. The data on women focus on the systemic hormonal differences induced by age or the use of HRT. The data on men focus on the lifelong effects of physical exercise. This thesis combines a literature review

with a critical summary of four original research articles on microRNA messaging and its interaction with steroid hormones, physical exercise and aging.

2 LITERATURE REVIEW

2.1 Aging

According to the World Health Organization (WHO), the global average life expectancy for people born in 2015 is 71.4 years (73.8 years for women and 69.1 years for men), of which healthy life expectancy is 63.1 years. For Finland, average life expectancy (year of birth 2015) is 81.1 years (for women 83.8 years and for men 78.3 years), and healthy life expectancy 10 years less. These statistics draw attention to the aspect of aging that raises the most concerns: the number of years spent suffering from diseases and functional limitations. Higher life expectancy, and more crucially, longer time spent in in a state of morbidity or disability, has created a progressive need for prevention, early diagnostics and improved treatment for these age-associated conditions. This thesis seeks to contribute some useful perspectives on these issues.

The human lifespan has been shown to be in part genetically controlled. Twin studies have estimated that approximately 25 % of the lifespan is explained by heritability, and have further emphasized that the genetic effect becomes especially crucial after the age of 60 years (Christensen, Johnson & Vaupel 2006). According to Martin et al. (1997), as many as 7 000 genes potentially affect regulation of the lifespan. To date, however, only a few genes have been shown to be linked with longevity; these include the apolipoprotein E gene (APOE), which participates in cholesterol packaging and lipid transportation (Schachter et al. 1994, Christensen, Johnson & Vaupel 2006) and the forkhead box O3A (FOXO3A) gene, which functions in the insulin–IGF1 signaling pathway (Willcox et al. 2008, Flachsbart et al. 2009). Recent studies have emphasized that in expectant mothers external stressors occurring already during fetal development affect the subsequent development and lifespan of the next generation (Barker et al. 1993, Perera & Herbstman 2011, Grandjean et al. 2015).

In general, aging begins when growth no longer takes place and maturity has peaked. Hence, some aging phenomena can, with differences between

individuals, already be detected during the early thirties. Visible features typical of progressive aging are changes in stamina, strength and sensory perception, commonly leading to adverse outcomes in overall functioning. The biggest metabolic changes affect body composition, insulin resistance and the regulation of signaling pathways including sex steroids and growth factors (Barzilai et al. 2012, Bartke, List & Kopchick 2016, Griffin et al. 2016, Stroustrup et al. 2016). The underlying causes of age-associated changes and conditions are complex, as they are driven by genetics together with environment and occur at the tissue, cell and molecular levels. The detrimental processes linked with aging are broadly characterized by nine hallmarks: cellular senescence, stem cell exhaustion, genomic instability, telomere shortening, mitochodrial dysfunction, loss in protein homeostasis, changes in nutrient sensing, epigenetic alterations and changes in intercellular signaling (reviewed by Lopez-Otin et al. 2013).

It is a challenging task to distinguish the effects of primary biological aging and its gradual detrimental processes from the effects of secondary aging as the latter involves many conditions and diseases, and is strongly affected by lack of exercise and other unhealthy lifestyle choices, and by the surrounding environment. Processes linked to secondary aging, however, are often preventable.

2.1.1 Menopause

Female aging is characterized by relatively rapid hormonal changes leading to menopause, while male hormonal changes are much more subtle and gradual, and thus take place over a longer time period. Mean menopausal age in Western countries is 51 years, with variability linked to race and ethnicity (National Institutes of Health, NIA, www.nia.nih.gov) (reviewed by Baber et al. 2016). Signature for the menopause is the termination of the reproductive period, caused by declining function of the ovaries leading to decreased amounts of circulating ovarian hormones. In clinical terms, menopause is described retrospectively as amenorrhea for one year followed by the final menstrual period (reviewed by Baber et al. 2016). The menopausal transition shows high interindividual variability, but takes about 4.5 years on average, beginning from the late premenopausal phase and continuing via the peri- to postmenopausal state (Woods & Mitchell 2004). The perimenopause is typically initiated at least two years before the actual menopause and is characterized by hormonal fluctuations (Soules et al. 2001).

Female sex steroids can be divided in to three types of estrogens: 1) estrone (E_1), 2) 17β-estradiol (E_2) and 3) estriol (E_3). The dominant circulating estrogen during the reproductive years is E_2, which is mainly produced from cholesterol in the granulosa cells in the ovaries. During the menopausal transition, as ovarian E_2 production ceases, E_1, which is synthetized in adipose tissue and adrenal glands, becomes the principal systemic estrogen. E_3 in turn, is mainly produced in the placenta during pregnancy and has a relatively weak role among unpregnant women and during menopause (Ryan 1959). Other

menopause-related hormonal changes occur in the systemic levels of progesterone, showing a decrement, and in the levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both of which increase notably. The levels of certain androgens change also with age in women; however, the most notable decline occurs prior to menopause (reviewed by Davis 2002).

In addition to its crucial functions in reproductive tissues, ovarian E_2 has many protective effects in various other tissue types, including bone, skeletal muscle, brain and the cardiovascular system (Väänänen & Härkönen 1996, Kuiper et al. 1997, Mendelsohn & Karas 1999, Boland et al. 2008, Pompili, Arnone & Gasbarri 2012). The lack of systemic E_2 leads, therefore, to problems and risks related to metabolism, properties of the musculature and bone as well as cardiovascular and psychological health. Owing to ovarian E_2 depletion, the menopause is associated with the redistribution of body fats, increasing central adiposity (Tremollieres, Pouilles & Ribot 1996). This so called "male phenotype" and overall increment of fat depots are recognized as risk factors for metabolic complications such as metabolic syndrome, cardiovascular disease, hypertension and Alzheimer's disease (reviewed by Rocha & Libby 2009, reviewed by Jayaraman & Pike 2014). One of the reasons for the several pathologies induced by the increase in central adiposity is the contribution of adipose tissue to the inflammatory state. Adipose tissue is an active immunological organ known to have a high abundance of macrophages and to secrete several cytokines, often referred to as adipokines (Hotamisligil et al. 1995, Fried, Bunkin & Greenberg 1998, Weisberg et al. 2003, reviewed by Trayhurn & Wood 2004). Late-life health status is determined by the condition of the immune system. Aging is often accompanied with a three- to fourfold increase in the systemic inflammatory state, called "inflammaging" (Franceschi et al. 2007), which is typically measured by classical inflammatory molecules such as high sensitivity C-reactive protein (hsCRP), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) (Krabbe, Pedersen & Bruunsgaard 2004, Bruunsgaard 2006) from the circulation. This chronic phenomenon has been linked to various age-associated conditions affecting endocrine (e.g., metabolic disease, insulin resistance), neurological (dementia, depression), pulmonary (chronic obstructive pulmonary disease), musculoskeletal (osteoporosis, arthritis, sarcopenia) and circulatory (atheroschlerosis, heart failure) functions (Khansari, Shakiba & Mahmoudi 2009). As already described, aging is a multifactorial process, strongly interacting with hormonal changes during menopause which in turn has further effects on the whole body metabolism and development of different pathologies.

HRT is commonly used to treat menopausal symptoms, including vasomotor effects and urogenital atrophy and to offer protection from age-associated conditions such as osteoporosis, cardiovascular disease, neurological disorders and abdominal fat accumulation (see intensive clinical review Baber et al. 2016). The first form of HRT (Premarin) was made available in the early 1940s. However, HRT usage became controversial after a trial published by Women's Health Initiative (WHI) in 2002 (Rossouw et al. 2002). The WHI re-

ported that the use of HRT increases the risks for breast cancer and deep vein thrombosis, while having the opposite effects on colorectal cancer and osteoporosis. The release of the WHI study significantly decreased the use of HRT in several countries, especially among women at risk age (Zbuk & Anand 2012, Jewett et al. 2014). Since the WHI study, a great number of studies have demonstrated negative, neutral, positive and controversial effects of HRT. Among the unbeneficial effects, cancer risks have been emphasized (Bernstein 2006, Canfell et al. 2008, Lambe et al. 2010) whereas protective effects have been reported on musculoskeletal properties, coronary heart disease, diabetes and dementia, especially if the start of the treatment has been timed to coincide with the perimenopausal or early postmenopausal years, thereby creating no gap between natural E_2 and supplemented E_2 levels (Farish et al. 1989, Cauley et al. 2003, Ronkainen et al. 2009, Szmuilowicz, Stuenkel & Seely 2009, Sherwin 2009, Mikkola et al. 2011, Tella & Gallagher 2014, Hodis et al. 2016). The WHI study has been strongly criticized due to the high average age of the participants when they started the HRT supplementation (Klaiber, Vogel & Rako 2005, Tan, Harman & Naftolin 2009). In 2007, a reanalysis of the WHI study concluded that the risks and benefits of HRT are tied to the age, at which the supplementation of HRT is initiated (Rossouw et al. 2007), pointing to the importance of the appropriate window of opportunity. Quite recently, at the Annual Meeting of The North American Menopause Society (NAMS, 2016), it was acknowledged, based on several recent studies, that for women under the age of 60 years the benefits of HRT might outweigh the possible risks. A recent restudy by Levine et al. (2016) demonstrated that menopause accelerates the epigenetic aging of blood leukocytes. In their study, both earlier menopausal transition and longer time since menopause, predicted higher biological age, measured with an epigenetic clock (DNA methylation), emphasizing the role of epigenetic regulation in the menopausal transition and women's aging. A recent study by Sillanpää et al. (2017) on postmenopausal monozygotic twin sisters discordant for the use of HRT (the same twins as used in this thesis) showed that leukocyte or skeletal muscle telomere length did not differ between the co-twins. However, the authors demonstrated that telomere length in different cell types has diverse association patterns with body composition measures.

As seen, menopause has global physiological effects. HRT treatments, however, owing to their diverse effects depending on the cell or tissue type and the timing, content and amount of the treatment prescribed, have shown conflicting effects.

2.1.2 Physical performance

Aging is often associated with physical inactivity, which is a multifactorial phenomenon, leading to broad adverse physiological outcomes. The benefits of lifelong activity, although well recognized, are, however, relatively rarely achieved. Involvement in continuous physical activity has been shown to maintain physical, mental, cardiovascular and metabolic health and even increase life expectancy (Katzmarzyk, Janssen & Ardern 2003, reviewed by Frankel, Bean & Fron-

tera 2006, Lanza et al. 2008, Erickson et al. 2011, Moore et al. 2012). It has also been shown to have a beneficial influence on inflammatory status (Vieira et al. 2009, Beavers, Brinkley & Nicklas 2010). While the detrimental effects of sedentariness are evident, they can fortunately, be counteracted by increasing the daily level of physical activity (DiPietro 2001).

The human body adapts well to changes in physical loading, a capacity which is maintained into old age. Declining physical performance is a natural consequence of physical inactivity and a typical characteristic of advanced age. Masters athletes are athletes who continue actively training and competing after 35 years of age. These individuals exhibit strenuous activity levels even at advanced ages, and therefore, form an ideal population with which to investigate primary biological aging without the confounding factors caused by a sedentary life style (reviewed by Hawkins, Wiswell & Marcell 2003, reviewed by Harridge & Lazarus 2017). It is reasonable to state, that masters athletes represent the highest physical potential that can be reached among middle-aged and older people. During recent decades, masters athletes' results and levels of physical performance in general have improved owing to improved training programs, nutrition strategies and equipment (Louis et al. 2012, Piacentini et al. 2013, Brisswalter & Louis 2014, Pugliese et al. 2015). However, even the most competitive masters athletes eventually confront decline in their physical performance as a result of primary physiological aging (Shephard et al. 1995).

There are a few individual major reasons behind the age-associated decline in healthy masters athletes: genetics, sex and the type and duration of the training performed both throughout the lifespan and currently. Whether endurance, strength or power training has the most beneficial effects on function and performance in older age, is being widely researched. Endurance training has been shown to have beneficial effects on the cardiovascular system and relative strength (Spina, Turner & Ehsani 1998, DeSouza et al. 2000, Giallauria et al. 2005, Fujimoto et al. 2010). However, aerobic capacity declines over the lifespan, accelerating notably after age 70 (Fleg et al. 2005). In untrained people, aging is associated with decline in maximal and, particularly, explosive strength and power along with changes in skeletal muscle fiber type properties: there is a selective atrophy of fast-contracting type II fibers, the faster myosin heavy chains have been shown to be replaced by slower ones and the shortening velocities of muscle fibers have been shown to decrease (Larsson et al. 2001). Strength and power types of training are considered to be the modalities that can counteract these age-related changes in the muscle structural and functional properties. While the rate of the age-related decline has been shown to be similar in both trained an non-trained individuals, studies have shown that size of fast muscle fibers and considerably better muscle function at older ages can be preserved with strength and power types of training (Klitgaard et al. 1990, Slade et al. 2002, Pearson et al. 2002, Aagaard et al. 2007). However, since the highly-trained athletes' physical functioning is maintained at a level corresponding to that of non-athletes 25-30 years younger, at the point at which deterioration starts they are naturally in better shape (Klitgaard et al. 1990, Pear-

son et al. 2002, Korhonen et al. 2006). Sprinting performance requires complex coordination from various muscle groups as well as the whole neuromuscular system. Speed and force production properties, typically well-developed among sprinters, are among the properties that decline with advanced age, a situation that for the elderly has functional and clinical implications. Therefore, it can be assumed that the versatile sprint type of training, including e.g. running, jumps and maximal and explosive strength training, could be beneficial for the ability to cope independently during the later years of life. A study by Korhonen et al. (2003), comprising world level female and male masters sprinters, demonstrated that 100 m sprinting performance declined progressively with age in both sexes, with the greatest change occurring approximately after ages 65 to 70. Despite the motivation and active training of masters athletes, decline in their performance with increasing age is eventually evident.

The effects of exercise and of physiological adaptation to lifelong training are ultimately delivered via signaling processes at the cellular and molecular levels (reviewed by Neufer et al. 2015). While central neural mechanisms and neuroendocrine responses influencing respiration, blood flow, fuel supply and thermoregulation are relatively well-characterized, the regulatory functions related to the molecules coordinating tissue cross-talk remain unknown. Therefore, research is currently focusing more and more on the mechanism and signaling in and between different tissues. A study by Robinson et al. (2017), performed with different aged non-athletes, showed that a 12-week high-intensity interval training (HIIT) program increased the abundance of gene transcripts in skeletal muscle more efficiently, especially in older humans, than other training modalities, including resistance training and combined training. HIIT was also shown to reverse some of the age-related proteome changes linked to mitochondrial function. This study focused on the diverse effects of different exercise modalities, in this case among non-athletes. Lately, the epigenetic bridges between age, training and physical performance have also received attention. Epigenetic regulation is likely to be one of the mechanisms delivering the intercellular effects and adaptation patterns induced by exercise (McGee et al. 2009, reviewed by Sapp et al. 2017). Clinical aspects of exercise have also received attention. A recent study by Safdar et al. (2016) demonstrated that endurance exercise releases exosome-packed "exerkines" that have potential for use as exercise-induced therapeutics for metabolic conditions. These exerkines include plenty of small molecules with potential to affect and regulate many biological processes.

2.2 MicroRNAs

As stated earlier, microRNAs are closely connected with epigenetics. There is increasing evidence that aging is associated with changes in microRNA expression in various tissues and body fluids (Hackl et al. 2010, Lanceta et al. 2010, Weilner et al. 2013). In addition, associations of microRNA regulation with sex

steroids (Kuokkanen et al. 2010, Waltering et al. 2011, reviewed by Klinge 2012, reviewed by Sharma & Eghbali 2014) and physical performance have been strongly proposed (reviewed by Sapp et al. 2017).

2.2.1 MicroRNA biogenesis and regulation

Originally, microRNAs were detected in a nematode called *Caenorhabditis elegans* (Lee, Feinbaum & Ambros 1993). MicroRNAs are 22-26 nt-long RNA molecules produced from stem-loop transcripts into mature single-strand post-transcriptional regulators. Commonly, microRNAs are thought to be negative regulators of their target gene expression. MicroRNAs are localized in every cell type. Their biogenesis is initiated in the nucleus and further processed in the cytoplasm. Both temporal and spatial factors affect microRNA biogenesis, which is a complex process regulated at multiple levels (FIGURE 1).

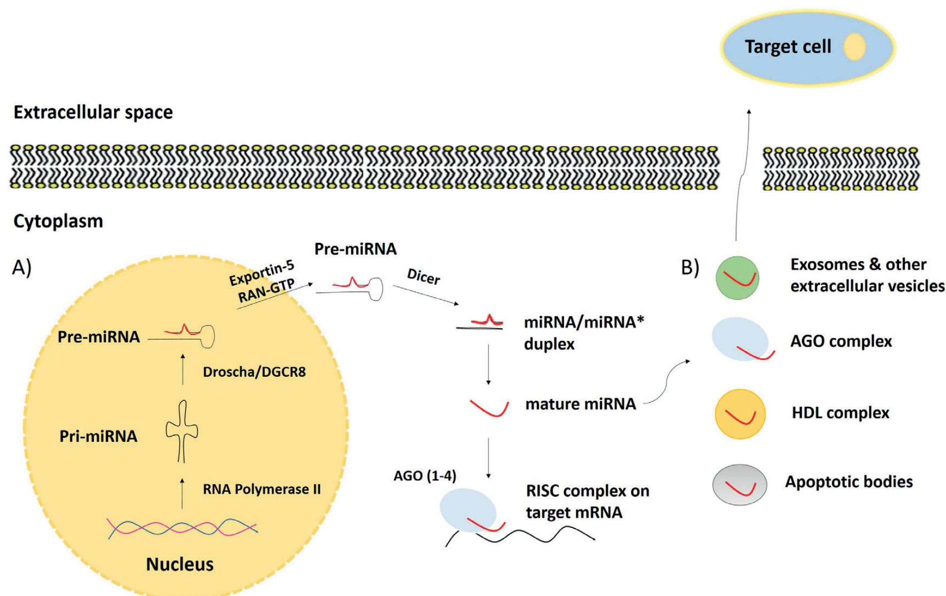


FIGURE 1 MicroRNA biosynthesis and packaging. A) The biosynthesis of microRNAs and their transportation from nucleus to cytosol. B) The options for microRNA packaging.

In general, microRNAs are highly conserved structures (Lagos-Quintana et al. 2003, Lim et al. 2003a, Lim et al. 2003b). However, the genomic location of microRNA sequences varies among species. In humans, these regions are most commonly located in the introns of coding or non-coding transcripts (Rodriguez et al. 2004). Typically, various microRNA loci are found very close to each other, forming microRNA clusters which are co-transcribed (Lee et al. 2002). Subsequently, more specific individual regulation of microRNAs occurs at the post-transcriptional level. RNA polymerase II (Pol II) is responsible for mi-

croRNA transcription in the nucleus (Cai, Hagedorn & Cullen 2004, Lee et al. 2004, Davis-Dusenbery & Hata 2010). After transcription, stem-loop-like pri-microRNAs (70 nt) are further processed into shorter hairpin pre-microRNAs (FIGURE 1). This is initiated with the microprocessor complex involving two enzymes belonging to RNase III endonucleases, DROSCHA and its cofactor DGCR8 (Lee et al. 2003, Denli et al. 2004). DGCR8 recognizes the junction between the single- and double-stranded region (SD junction) and the stem of the pri-microRNA structure (33 bp). Subsequently DROSCHA cleaves the small stem (11 bp) forming a pre-microRNA (65-70 nt) (Han et al. 2006). After being processed by DROSCHA and DGCR8, the pre-microRNAs are transported from the nucleus into the cytosol by Exportin-5 - RanGTP-binding -complex (Lund et al. 2004). In the cytoplasm, DICER cleaves the pre-microRNAs, forming a small miRNA-miRNA -duplex (Bernstein et al. 2001). The duplex is subsequently incorporated with stabilizing Argonaute (AGO) protein (1-4), to form a pre-RNA-induced silencing complex (pre-RISC) (Winter & Diederichs 2011). AGO consists of two major domains: PAZ and PIWI. PAZ is responsible for binding to the guide strand of the miRNA-miRNA duplex whereas PIWI possesses RNAse H activity, later used in target mRNA cleavage (Tahbaz et al. 2004). After separating the less abundant passenger microRNA strand (miRNA*) from the miRNA-miRNA duplex, the guide microRNA strand (~22nt) and AGO (1-4) form a mature RISC (Huntzinger & Izaurralde 2011). The transcriptional and post-translational control of the most important enzymes in microRNA biogenesis, including Pol II, microprocessor proteins and DICER, crucially affect the steps of the overall process (Ha & Kim 2014). Although microRNAs are sometimes considered epigenetic regulators, the biogenesis of microRNAs themselves is also affected by epigenetics, adding to the complexity of microRNA regulation (Bhat, Jarmolowski & Szweykowska-Kulinska 2016). In addition, microRNAs are also under hormonal regulation, and are thus sensitive to, e.g., estrogens and estrogen receptors (ERs) (Klinge 2009).

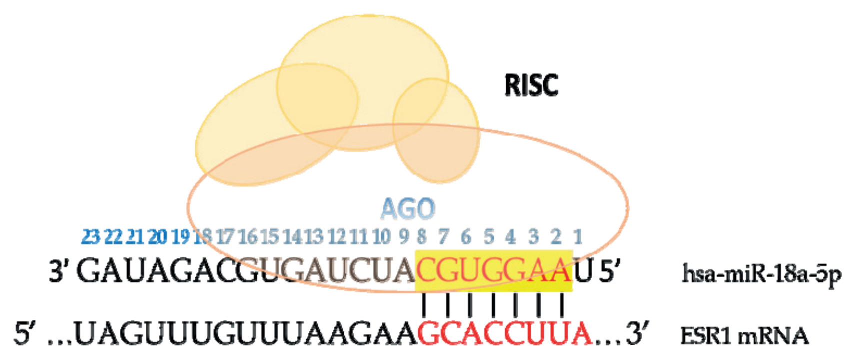


FIGURE 2 Sequence-specific binding of microRNA hsa-miR-18a-5p to its target mRNA ESR1 3′-UTR region. Seed region is highlighted in yellow.

Mechanistically, regulation by microRNAs is based on translational repression or messenger RNA (mRNA) degradation caused by the specific binding of the mature RISC to the 3´-UTR region of the target mRNA (Figure 2). The guide microRNA in the RISC includes the so called "seed region" at its 5`tail (residues 2-7), which leads the RISC to occupy the right position on the target mRNA (Doench & Sharp 2004). In general, complementary base pairing leads to target mRNA degradation by AGO RNAse H activity. If only partial pairing occurs, the mRNA is silenced. One of the main reasons for mRNA silencing is the recruitment of several distal factors by the RISC complex, including TRNC6 which is responsible for destabilizing the target mRNA. Functionally, both mechanisms lead to translational repression (Valencia-Sanchez et al. 2006).

To date, 2 588 mature microRNAs have been identified (mirbase.org) (Kozomara & Griffiths-Jones 2014). It has been estimated that roughly 60 % of genes are regulated by microRNAs (Selbach et al. 2008, Friedman et al. 2009), making them one of the largest families of post-transcriptional regulators. MicroRNAs often function in a combinatorial fashion, meaning that several microRNAs target and fine-tune the expression of one target mRNA. In addition, one single microRNA can have many different target mRNAs (Lim et al. 2005, Krek et al. 2005). This all comes down to base-pair interactions. MicroRNAs either affect the cells they are being produced in or are transported by various mechanisms into the target tissues via body fluids. When transported, microRNAs can be attached to AGO molecules (Arroyo et al. 2011, Turchinovich et al. 2011) or localized in apoptotic vesicles (Zernecke et al. 2009), in exosome-packaged vesicles (Valadi et al. 2007) or in HDL particles (Vickers et al. 2011) (FIGURE 1).

2.2.2 Circulating microRNAs

Although some microRNAs are passively leaked from cells into body fluids followed by cell damage or senescence (reviewed by Bronze-da-Rocha 2014), active cellular crosstalk via microRNA messaging is evident (Hergenreider et al. 2012). Owing to the stability of microRNAs and minimal invasiveness of the sampling procedures, body fluids are promising platforms for the detection of microRNAs and changes in microRNA levels due to intra- or extracellular stimuli. Up to 90 % of the circulating microRNAs have been suggested to be membrane-free microRNAs, which are most commonly attached to Ago2 proteins (Arroyo et al. 2011, Turchinovich et al. 2011). However, the functionality of these systemic membrane-free microRNAs in intercellular signaling remains unclear.

As mentioned, exosome trafficking is one of the mechanisms for microRNA delivery from one cell to another (Valadi et al. 2007). Exosomes are small bilayer vesicles (20-100 nm in diameter) inside the multivesicular bodies (MVB) budded from late endosomes. When fused with the plasma membrane, MVBs release the exosomes into extracellular space (Heijnen et al. 1999). Exosomes are known to contain bioactive lipids, proteins and RNAs including microRNAs (Valadi et al. 2007, Record et al. 2014, Braicu et al. 2015) (FIGURE 3).

In addition, exosomes display several surface and transmembrane proteins, rendering their interaction with the recipient cells highly specific. These include, e.g., targeting and adhesion molecules such as integrins and tetraspanins (CD9, CD63, CD81, CD82) and antigen-presenting molecules (MHC class I or II molecules) (Escola et al. 1998, Zitvogel et al. 1998, Thery, Zitvogel & Amigorena 2002). Both the surface proteins as well as exosome cargo depend on the cell of origin.

Exosomes have been suggested to be well protected carrier systems for functional RNAs, such as microRNAs, as RNases cannot reach the exosome cargo (Cheng et al. 2014). During the last few decades, there has been a research emphasis on the functional role of exosome trafficking in cell-cell signaling, as presented in the extensive review by Lawson et al. (2016). However, this role is somewhat controversial, as exosomes have been shown to deliver important messages that help maintain basic biological processes as well as proliferative messages favoring tumor progression and metastases (reviewed by Braicu et al. 2015). The characteristics of exosomes, especially their specificity in targeting different cell types, is causing exosomes to be regarded as clinically promising tools. Zitvogel et al. (1998) was the first to show that exosomes derived from dendritic cells of mice can induce T-cell mediated immune response. Ten years later, Skog et al. (2008) demonstrated that patients with glioblastoma had microRNA content inside exosomes that resembled the tumor tissue itself. In addition, the exosome content of these patients differed from that of healthy individuals, an observation pointing to the important role of exosome microRNAs in both health and diseases. Quite recently, many studies have investigated the content of circulating exosome microRNA in connection with various malignancies and conditions of illhealth (Nedaeinia et al. 2017, Nuzhat et al. 2017, Hubal et al. 2017).

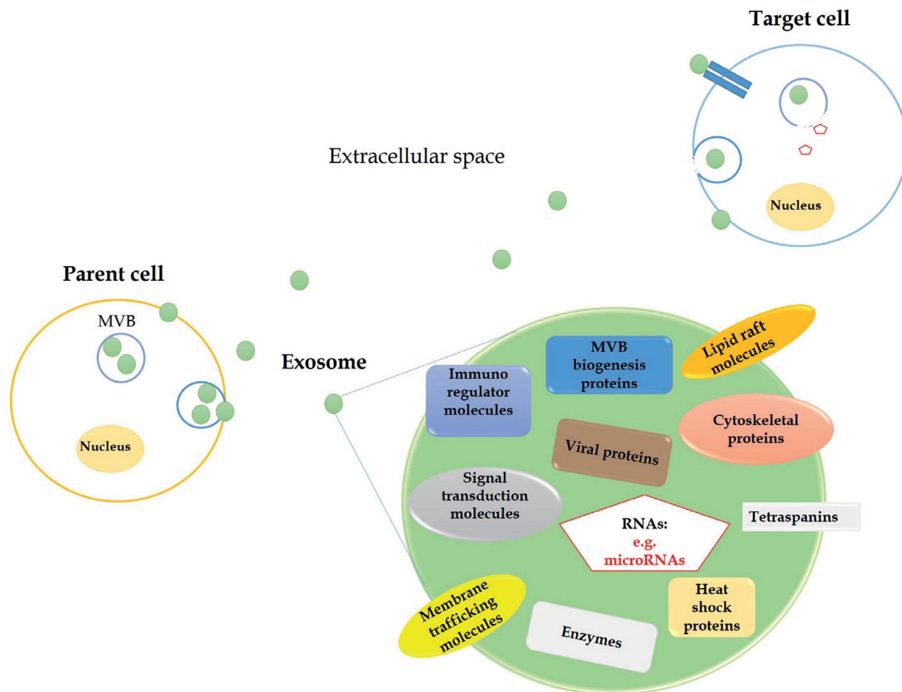


FIGURE 3 An illustration of exosome cargo and exosome trafficking between parent and target cells.

2.2.3 Adipose and skeletal muscle tissue microRNAs

Several studies have shown altered expression of microRNAs in various tissues and cell types caused by age or cellular senescence (Hackl et al. 2010, Marasa et al. 2010, Bai et al. 2011, Shin et al. 2011, Rippe et al. 2012, Kim et al. 2012). As mentioned earlier, tissue specificity has a crucial role in microRNA expression patterns, not forgetting the extracellular stimuli coming from other cell types and the environment. Sex differences also have an influence on microRNA expressions, which is due to various physiological mechanisms including hormonal discrepancies and differences in body composition between men and women (Sharma & Eghbali 2014, Ameling et al. 2015).

Adipose tissue is one of the major endocrine organs that function not only in energy homeostasis and lipid metabolism, but also in reproduction and immune response. Several microRNAs regulating the development, proliferation and differentiation of adipose cells have been identified (Karbiener et al. 2009, Tang et al. 2009, Keller et al. 2011, Kang et al. 2013, Chen et al. 2014, Chuang et al. 2015, Guan et al. 2015). The expression pattern of microRNAs has been shown to differ between preadipocytes and mature differentiated adipocytes (Shi et al. 2016). In their study, Shi et al (2016) compared their own microarray results to meta-analyses performed by others, and found 42 microRNAs that were differentially expressed in mature adipose cells compared to human adi-

pose-derived mesenchymal stem cells (hMSCs-Ad) or human stromal vascular cells (SVCs).

Associations with aged adipose tissue and microRNA regulation have also been demonstrated. The overall expression of microRNAs in white adipose tissue have been shown to decrease with age owing to the decline in the amounts of DICER enzyme, which takes part in microRNA biosynthesis (Mori et al. 2012). Replicative senescence of hMSCs-Ad cells has been shown to be induced by miR-486-5p (Kim et al. 2012). In addition, the dual role of microRNAs in the pathogenesis of aging-related metabolic conditions, such as obesity has also been recognized. Several studies have shown that microRNAs can function either by enhancing or suppressing adipogenesis, depending on the target mRNAs (Lin et al. 2009, Shi et al. 2015).

Adipose tissue has been shown to be one of the major contributors of circulating microRNAs, especially exosome-packed microRNAs (Thomou et al. 2017). Thomou et al. (2017) demonstrated that the adipose tissue-derived circulating exomiR profile depends on the fat depot they are being released from and that they have potential in affecting whole-body metabolism by regulating the gene expression of Fibroblast growth factor 21 (FGF21) in the liver. With aging, the body fat distribution changes, rendering adipose tissue a potential contributor to the changing expression of circulating microRNAs in aging. Moreover, a recent study by Nunez Lopez et al. (2016) showed that serum carries an abundance of diabetes-related microRNAs that could possibly be used as prediabetic indicators in early prognostics. These microRNAs are likely derived from inflammatory cells or adipose cells, both of which are affected by aging.

Skeletal muscle tissue is the largest of the tissues in human body, constituting up to 50 % of body mass, and thus, playing a huge role in energy metabolism. It is sensitive to insulin, which adds to its important function in the whole organism metabolism, including in the development of age-associated metabolic disorders. As an adaptive tissue, skeletal muscle is affected and modulated by either use or disuse of the tissue. Some of the stimulus to and from skeletal muscle tissue is delivered via microRNA regulation, in other words, by producing, releasing and up-taking specific microRNAs. Several studies have demonstrated that microRNAs affect skeletal muscle mass, composition and function by regulating processes such as differentiation, proliferation and senescence (Wang 2013, Hu et al. 2014, Hudson et al. 2014, Kovanda, Rezen & Rogelj 2014, Guess et al. 2015, Soriano-Arroquia et al. 2016). Skeletal muscle, together with cardiac muscle tissue, have been shown to contain muscle specific microRNAs, referred as myomiRs, including miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486 and miR-499 (reviewed by McCarthy 2011). In addition to specific microRNAs, skeletal muscle tissue expresses many other microRNAs that regulate various processes. Skeletal muscle microRNAs are responsive to external stimuli such as physical exercise or steroid hormones (Olivieri et al. 2014a, Nielsen et al. 2014b). A monozygotic twin study performed by Olivieri et al. (2014a) demonstrated that the use of postmenopausal HRT decreases the expression of miR-223 and miR-182, both targeting insu-

lin growth factor 1 receptor (IGF1R), forkhead box O1A (FOXO1A) and FOXO3A, in skeletal muscle tissue, leading to activation of the insulin signaling pathway. This study emphasized the interaction and benefits of higher systemic E_2 levels, microRNA regulation and insulin sensitivity in skeletal muscle tissue.

2.2.4 Challenges in microRNA detection

A reliable method of microRNA detection is of importance when investigating the function of microRNAs and their relation to various biological processes and diseases. The demand for more sensitive, specific and affordable microRNA detection methods has increased during recent decades with the perception of the significant regulatory function of microRNAs in health and disease. While this has led to the continuous development of new methods, standardizing the protocols is lagging slightly behind, hindering comparability between studies. The traditional microRNA detection methods include RT-qPCR (Chen et al. 2005, Salone & Rederstorff 2015, Varkonyi-Gasic 2017), microarrays (Li & Ruan 2009) and northern blotting (Valoczi et al. 2004, Varallyay, Burgyan & Havelda 2008, Koscianska et al. 2011), all three having their pros and cons. The first two are techniques based on qPCR amplification with specific microRNA probes. RT-qPCR is normally used for single microRNA detection, whereas microarrays are meant for profiling a larger set of microRNAs. The detection range in both methods is broad; however, the relatively costly equipment and materials needed impose limits on their use, especially for microarrays. RT-qPCR has been used as a "gold standard" for microRNA detection and, typically, results obtained with other methods are being confirmed with it. Northern blotting allows both microRNA quantity and size detection. However, it is a rather complex and time-consuming method that calls for radiolabeling (Koscianska et al. 2011). It also demands a relatively large amount of RNA, which can be a limiting factor. In addition to these traditional methods, Next Generation Sequencing (NGS) is being broadly utilized to obtain high-throughput microRNA detection. In general, NGS technology detects all the sequences in a sample and enables the detection of novel microRNAs as well as possible modifications in microRNA entities (Landgraf et al. 2007). Despite its sensitivity, the method is, however, vulnerable to possible biases resulting from RNA ligation and qPCR amplification. In addition, the method is relatively expensive and requires a professional data analyst to interpret the results.

The detection of microRNAs from body fluids is more challenging than their detection from tissue or cell samples. This can be explained by the weaker abundance of microRNAs in liquid biopsies as well as the lack of endogenous controls which the traditional normalization methods are often based on. In addition to the already "approved" detection procedures, the traditional methods are constantly being improved, owing to more sensitive reagents or devices, and several new methods have been developed. RT-qPCR detection has taken new forms such as Droplet digital PCR (ddPCR), in which the reaction mixture is dispersed into small microdroplets which are better able to tolerate for inhibitors affecting microRNA amplification (Pinheiro et al. 2012, Dingle et al. 2013).

This method has also been shown to work well in the detection of circulating microRNAs (Hindson et al. 2013, Miotto et al. 2014). Some of the novel detection methods are based on nanoparticle-derived probes (Hwang et al. 2010), isothermal amplification (Li et al. 2011) and electrochemical methods (Li et al. 2014a), all three focusing on combining multi-step signal amplification with a sensitive detector to obtain more accurate results.

2.3 Aging and microRNAs

2.3.1 Circulating microRNAs and aging

Owing to the invasiveness of tissue biopsy sampling, liquid biopsies offer more practical options for detecting microRNAs and changes in their abundance in regulation related to aging and other conditions. The basic idea behind the usefulness of liquid biopsies is that the changes in microRNA expression in different tissues are potentially reflected in the blood circulation and other body fluids such as saliva, urine and breast milk (Skog et al. 2008, Kosaka et al. 2010, Michael et al. 2010, Mall et al. 2013). MicroRNAs have even been shown to cross the blood-brain barrier if carried inside the exosomes (Lakhal & Wood 2011).

MicroRNA arrays and NGS techniques offer promising platforms for scanning the overall profiles of microRNAs. To date, age-related microRNA patterns in the human circulation have been shown in a few studies (TABLE 1). A genome-wide array study focusing on human longevity by Elsharawy et al. (2012) demonstrated that the level of 64 microRNAs was lower and that of 16 microRNAs higher in the circulation of centenarians and nonagenarians (mean age 96.4 years) compared with younger controls (mean age 45.9 years). Further RT-qPCR validation on selected microRNAs confirmed lower levels for five of these microRNAs and higher levels for three with advanced age. An array study by Olivieri et al. (2012) demonstrated that plasma microRNA content is significantly different among young, older and the oldest old people (centenarians). Furthermore, a sequencing study by Noren Hooten et al. (2013) showed that people in their 60s have significantly lower levels of several circulating microRNAs compared to 30-year-old individuals. Zhang et al. (2015), using NGS, demonstrated that the level of 10 circulating microRNAs differed significantly by age among healthy Chinese aged from 22 to 70 years. Further validation by RT-qPCR confirmed that eight of the 10 microRNAs were associated with age. A relatively large population-based cohort study by Ameling et al. (2015) revealed that specific circulating microRNA levels differed owing to age; however, BMI, sex and particularly blood cells also contributed to the microRNA levels. In addition to human studies, several microRNA profiling experiments have been performed on model organisms, such as nematodes and mice, showing age-associated changes in microRNAs present in the circulation (Ibanez-Ventoso et al. 2006, Victoria et al. 2015).

TABLE 1 Age-related changes in circulating microRNAs.

Organism	Source	Detection method	MicroRNA	Change with higher age	Ref.
Human	blood	Array + RT-qPCR	**miR-106a-5p, miR-126-3p, miR-20a-5p, miR-144-5p, miR-18a-5p**	↓	(El-Sharawy et al. 2012)
			miR-30d-5p, miR-320d, miR-320b	↑	
Human	plasma	Array	miR-15b-5p, miR-16-5p, miR-301a-3p, miR-17-5p, miR-18a-5p, miR-320a, miR-20a-5p, miR-20b-5p, miR-361-5p, miR-25-3p, miR-26a-5p, miR-26b-5p, miR-423-5p, miR-29-3p, miR-451a, miR-29c-3p, miR-92a-3p, miR-486-3p, miR-93-5p, miR-106a-5p, miR-541-3p, miR-106b-5p, miR-128-3p, miR-532-5p, miR-132-3p, miR-146b-5p, miR-576-3p, miR-140-5p, miR-142-3p, miR-625-5p, miR-145-5p, miR-148a-3p, miR-652-3p, miR-155-5p, miR-185-5p, Let-7b-5p, Let-7g-5p, Let-7c-5p, Let-7d-5p, miR-191-5p, miR-192-5p, miR-193a-5p, miR-199a-3p, miR-222-3p, miR-224-5p, miR-200d, miR-200c-3p, miR-212-3p, miR-425, miR-579-3p	↓	(Olivieri et al. 2012)
			miR-19b-3p, **miR-21-5p**, miR-30c-5p, miR-126-3p, miR-186-5p, miR-328-3p, miR-331-3p, miR-335-5p, miR-339-3p, miR-376a-3p, miR-484, miR-590-5p	↑	
Human	plasma	RT-qPCR	**miR-126-3p**	↑	(Olivieri et al. 2014b)
Human	serum	NGS + RT-qPCR	**miR-151a-3p**, miR-151-5p, **miR-181a-3p**, miR-181-5p, **miR-1248**, miR-21-3p, miR-21-5p, miR-3607-5p, miR-3607-3p	↓	(Noren Hooten et al. 2013)
Human	serum	NGS + RT-qPCR	**miR-29b-3p, miR-106b-5p, miR-130b-3p, miR-142-5p, miR-340-3p**	↓	(Zhang et al. 2015)
			miR-92a-3p, miR-222-3p, miR-375	↑	
Human	plasma	Array	miR-93-5p	↓	(Ameling et al. 2015)
			let-7a-5p, miR-126-3p, miR-142-3p, miR-21-5p, miR-30b-5p, miR-30c-5p	↑	
Human	blood	Array, NGS	miR-1248, miR-93-3p, miR-1262 miR-34a-5p, miR-145-5p	↑	(Meder et al. 2014)

RT-qPCR validated results are bolded. ↑ increase, ↓ decrease

Commonly the pathology of a disease is initiated prior to detection of the actual symptoms. Therefore, early detection, and subsequently early treatment, are crucial to avoid more serious disease outcomes. The search for circulating aging and age-related disease biomarkers has been in the spotlight over the past decades, the goal being to find tools to increase the life expectancy, vitality and function of aging individuals. Research attention has recently focused on the age-related diseases and circulating microRNAs. A recent study by Cao et al. (2017) demonstrated that patients with Parkinson's disease have higher levels of serum exosome-packed miR-195 and miR-24 and lower levels of miR-19b compared to age- and sex-matched control groups. Circulating microRNAs have also been linked to osteoporotic pathologies (Kocijan et al. 2016) and the development of Alzheimer's disease (Xie et al. 2017). These studies indicate the potential usefulness of systemic microRNAs in age-related disease diagnostics and therapeutics.

2.3.2 Estrogen regulation and microRNAs

The effects of estrogens are delivered via ERs in a tissue-specific manner. These receptors are divided into nuclear receptors, including ERα (Jensen 1962) and ERβ (Kuiper et al. 1996), and membrane receptors, including splice variants of ERα and ERβ and G-protein-coupled estrogen receptor (GPER) (Maggiolini & Picard 2010), all these receptors having distinct roles from each other. It has been demonstrated that estrogens and ERs affect microRNA expression either by regulating the molecules affecting microRNA biosynthesis or later phases of microRNA function (Adams, Claffey & White 2009, Cheng et al. 2009, Ferraro et al. 2012, Nassa et al. 2014). On the other hand, ERs themselves are targeted by several different microRNAs (Adams, Furneaux & White 2007, Pandey & Picard 2009, Castellano et al. 2009, Leivonen et al. 2009, Zhao et al. 2011, Klinge 2012, Eichelser et al. 2014, Liu et al. 2014, Newie et al. 2014, Zhu et al. 2015). This interaction forms an interesting loop between estrogen levels, their receptors and specific microRNAs, thus providing more complex and alternate regulation patterns.

The associations between estrogen signaling and microRNAs have been especially extensively studied in breast cancer tissue, owing to its high abundance of ERs (Bhat-Nakshatri et al. 2009, Klinge 2009, Di Leva et al. 2010, Di Leva et al. 2013). A microarray study by Klinge et al. (2009) with ERα-positive MCF-7 breast cancer cells demonstrated that E_2 treatment altered the expression of 38 microRNAs, of which 29 were upregulated and 9 downregulated. Mir-21 is one of the main microRNAs studied in the breast cancer field (Iorio et al. 2005, Si et al. 2007) and it has also been shown to be sensitive to estrogen in breast cancer cells (Wickramasinghe et al. 2009, Selcuklu et al. 2012). According to Selcuklu et al. (2012) E_2 downregulated miR-21, which led to upregulation of its target Jagged-1 (JAG1), whereas Wickramasinghe et al. (2009) showed downregulation of miR-21 by E_2 and further upregulation of its targets, Pdcd4, PTEN and Bcl-2, in MCF7 cells. Both studies demonstrated the functionality of mi-

croRNA-target interaction and the role of E_2-sensitive miR-21 in regulating the cell fate of cancer cells.

Other tissues, including brain, bone, skeletal muscle and adipose tissue also possess ERs, making them responsive to estrogens and, potentially, to microRNA regulation via estrogen signaling (Anderson, Peck & Clark 1973, Eriksen et al. 1988, Pedersen et al. 1996, Lemoine et al. 2003, Wiik et al. 2003, Pöllänen et al. 2011, Park et al. 2017). A study by Rao et al. (2013) demonstrated that estrogen and age have notable effects on the microRNAs regulating memory and stress responses in the brain of female rats, emphasizing the role of E_2 in neuronal functioning. In bone tissue, estrogen has been shown to maintain the bone remodeling balance by regulating FasL/Fas signaling via specific microRNAs i.e., miR-181a (Shao et al. 2015) and miR-21 (Sugatani & Hruska 2013). Both studies demonstrated that estrogen deficiency leads to increased expression of miR-181a (in bone marrow-derived mesenchymal cells) or miR-21 (in primary mouse bone marrow-derived monocyte/macrophage precursors), downregulating their target FasL, which further suppresses osteoclast apoptosis. A study by Olivieri et al. (2014a) demonstrated the associations of specific microRNAs in skeletal muscle tissue with serum E_2 levels. Interaction between serum E_2 levels, ERs and specific microRNAs has also been proposed in omental adipose tissue of women with gestational diabetes mellitus (GDM) (Shi et al. 2014). In their study, Shi et al. (2014) showed that estrogen-induced insulin resistance in GDM patients is in part due to miR-222 and its target ERα interaction. Clearly, interactions between E_2, ERs and microRNAs exist; however, the more specific knowledge on their interaction in health and disease and in different tissues needs more investigation.

2.3.3 Physical exercise and microRNAs

Depending on its type, duration and intensity, physical exercise changes microRNA expression in tissues adaptive to exercise. Furthermore, physical condition, training background of the individual, age and sex also have an influence on microRNA responses. Physical exercise functions as an external stressor that has been shown to create microRNA responses in skeletal muscle tissue (McCarthy & Esser 2007, Drummond et al. 2008, Nielsen et al. 2010, Rivas et al. 2014, Fyfe et al. 2016, Ogasawara et al. 2016), endothelial cells (Riedel et al. 2015), cardiac muscle tissue (Fernandes et al. 2011, Souza et al. 2015) and inflammatory cells (Radom-Aizik et al. 2010). Muscle-specific microRNA responses have mostly focused on the effects of endurance and resistance exercises. Nielsen et al. (2010) reported that an acute bout of exercise, prior to 12 weeks endurance training, resulted in increment of myomiRs 1 and 133a in the skeletal muscle of healthy male non-athletes, whereas an acute bout after the training period did not have a similar effect. In addition, the basal levels of the four studied myomiRs (miR-1, -133a, -133b and -206) decreased after 12 weeks of training. This study suggests that, in non-athletes, the adaptation to acute response by specific myomiRs occurred owing to the relatively short endurance training program. A study by Ogasawara et al. (2016) found that the skeletal muscle microRNA pro-

file changed differently among untrained healthy low and high responder males both following an acute bout of resistance exercise and after 12 weeks of resistance training. This study suggests that specific microRNAs mediate skeletal muscle adaptation to resistance training depending on the responder type. Interestingly, a study by Rivas et al. (2014) demonstrated that adaptation to resistance exercise in young and older men created a differential response in skeletal muscle microRNAs. In their study, an acute bout of exercise changed the expression of 21 microRNAs in healthy young men but none in the older men. One of the microRNAs was miR-126, which regulates pathways related to growth, development and myogenic differentiation. The study suggests that declining skeletal muscle plasticity in aging is controlled by dysregulated microRNA-delivered adaptation.

The microRNA responses to acute exercise and adaptations to regular exercise training are possibly mediated between different tissues and organs via the circulation or other body fluids (reviewed by Sapp et al. 2017). It is expectable that the microRNA responses detected in the circulation also differ between non-athletes and athletes and between different exercise modalities. To study the microRNA responses indicative of long-term training adaptations, it is important that the research design includes athletes with a regular training background (TABLE 2). A study by Uhlemann et al. (2014) on healthy middle-aged male and female non-athletes suggested that increased circulating miR-126 could be indicative of endothelial cell lysis immediately after marathon performance, whereas miR-133a could be indicative of muscle damage after eccentric resistance training. Baggish (2014), in a study on marathon runners in their 50s, showed similar upregulated responses in plasma miR-126-3p and miR-133a-3p expression profiles after marathon. Mooren et al. (2014) also showed a similar acute response for miR-133a-3p in male endurance masters runners after marathon. These findings indicate that the acute response in the expression profiles of the two microRNAs after marathon performance does not differ between middle-aged or older endurance athletes and healthy non-athletes. However, another study by Baggish et al. (2011) demonstrated that immediately after exhaustive cycling exercise and chronic rowing training, the levels of miR-133a-3p remained unchanged in young competitive male rowers. In relation to the above-mentioned studies, it can be speculated whether the stability or even decrement in circulating miR-133a-3p levels are indications of training adaptation or of an age-association in specific groups of young competitive athletes. Cui et al. (2015), who also studied muscle-enriched microRNAs, reported that plasma miR-1, -133a, miR-133b and miR-206 were acutely upregulated after both high-intensity interval exercise and vigorous-intensity continuous exercise in young men habituated to endurance-type exercises for at least two years (more than 3 times/wk). The results of this study when considered together with the results of the other studies on miR-133a, indicate that habituated endurance exercisers do not reach the same level of adaptation in the miR-133a response as the more intensively trained competitive rowers in the study by Baggish (2011), suggesting sensitive adaptation thresholds for miR-133a in men

with different training backgrounds. In addition to muscle-enriched microRNAs, Baggish et al. (2011) were the first to show that in young male athletes, an acute bout of cycling exercise and/or a longer training period changes the levels of specific plasma microRNAs related to angiogenesis (miR-20a, miR-222) and inflammation (miR-146a, miR-21). Baggish reported that miR-146a-5p levels increased after an acute bout of endurance exercise before and after 90 days rowing training as well as at rest after the training period, whereas miR-21-5p increased acutely only before and at rest after the training. Another study by Sawada et al. (2013) on recreationally active healthy males reported that serum miR-146a-5p levels decreased after three days of resistance exercise while miR-21-5p levels remained unchanged. The results obtained in young trained men by Nielsen et al. (2014a) showing that plasma miR-21-5p levels decrease after 12-weeks of cycling measured at rest also conflict with Baggish's findings on training adaptations. A study by Bye et al. (2013) on healthy men and women showed that serum miR-21-5p levels were higher in men with low VO_{2max} than in men with high VO_{2max}. Similar differences were not detected in women, which emphasizes the sex-differences in miR-21-5p response. In addition, Banzet et al. (2013) reported that muscle-enriched microRNA response to eccentric and concentric exercise modes differed from each other depending on whether they were measured from the plasma immediately after training or during the early recovery phase (up to 6 h) in recreationally active men.

The above-mentioned studies support the possible utilization of circulating microRNAs in evaluating adaptation to exercise and training modes, while also indicating the importance of the type of training, training background, timing of measurements and sex of the individual. However, most studies have focused on the microRNA response to an acute or relatively short period of training, hence, creating an information gap and need for studies on the effects of long-term training and the possible interaction of the aging process and systematic training.

TABLE 2 The exercise/training responses of selected circulating microRNAs, enriched in endothelium, muscle or cardiac tissue, among young and masters athletes.

Age	Detection method	microRNA	Type of exercise	Response	Ref
> 35 yrs	RT-qPCR	miR-21-5p (p)	-max cycling -90-days rowing	↑ acute ↑ at rest	(Baggish et al. 2011)
	Array/RT-qPCR		-12-weeks cycling	↓ at rest	(Nielsen et al. 2014a)
	RT-qPCR		-none: endurance vs. resistance	↑ at rest	(Wardle et al. 2015)
	RT-qPCR	miR-146a-5p (p)	-max cycling -90-days rowing -max cycling after 90-days rowing	↑ acute ↑ at rest ↑ acute	(Baggish et al. 2011)
	Array/RT-qPCR		-cycling 60 min	↓ acute	(Nielsen et al. 2014a)
	RT-qPCR		-none: endurance vs. resistance	↑ at rest	(Wardle et al. 2015)
	RT-qPCR	miR-221-3p (p)	-max cycling -90-days rowing	↑ acute ↑ at rest	(Baggish et al. 2011)
	Array/RT-qPCR		-cycling 60 min	↓ acute	(Nielsen et al. 2014a)
	RT-qPCR		-none: endurance vs. resistance	↑ at rest	(Wardle et al. 2015)
	RT-qPCR	miR-222-3p (p)	-max cycling -90-days rowing -max cycling after 90-days rowing	↑ acute ↑ at rest ↑ acute	(Baggish et al. 2011)
	RT-qPCR		-none: endurance vs. resistance	↑ at rest	(Wardle et al. 201
	Array	miR-140-5p (p) miR-3620-3p (b)	-6-day training, endurance vs. strength	↑	(Hecksteden et al. 2016)
	Array	miR-513b-5p (p) miR-650 (b)	-6-day training, endurance vs. strength	↓	(Hecksteden et al. 2016)
	RT-qPCR	miR-20a-5p (p)	-90-days rowing	↑ at rest	(Baggish et al. 2011)
	Array/RT-qPCR	miR-148a-3p (p)	-12-weeks cycling	↓ at rest	(Nielsen et al. 2014a)
< 35 yrs	RT-qPCR RT-qPCR	miR-1-3p (p)	- marathon - marathon	↑ acute ↑acute+24h	(Baggish et al. 2014) (Mooren et al. 2014)
	RT-qPCR	miR-21-5p (p)	-marathon	-	(Mooren et al. 2014)
	RT-qPCR	miR-126-3p (p)	-marathon	↑ acute	(Baggish et al. 2014)
	RT-qPCR RT-qPCR	miR-133a-3p (p)	- marathon - marathon	↑ acute ↑acute+24h	(Baggish et al. 2014) (Mooren et al. 2014)
	RT-qPCR	miR-134-5p (p)	-marathon	↑ acute	(Baggish et al. 2014)
	RT-qPCR	miR-146a-5p (p)	-marathon	↑ acute	(Baggish et al. 2014)
	RT-qPCR	miR-206 (p)	-marathon	↑acute+24h	(Mooren et al. 2014)
	RT-qPCR RT-qPCR	miR-208-3p (p)	-marathon -marathon	↑ acute ↑ acute	(Baggish et al. 2014) (Mooren et al. 2014)
	RT-qPCR RT-qPCR	miR-499-5p (p)	-marathon -marathon	↑ acute ↑ acute	(Baggish et al. 2014) (Mooren et al. 2014)

p: plasma, b: blood, ↑ increase, ↓ decrease, - no change

3 AIMS OF THE STUDY

The aim of this study was to determine the associations of age, menopausal status with or without postmenopausal HRT, or lifelong physical training with systemic microRNA levels. Further, the interaction of the studied age-associated microRNAs with health-related variables or with specific physical performance measures were assessed. Specific microRNA expressions were analyzed from adipose and skeletal muscle tissue and blood leukocytes.

The specific aims of this thesis were:

1. To investigate whether age or serum E_2 levels are associated with circulating microRNA levels and/or microRNA expressions in different tissues.
2. To find associations between microRNA patterns and markers indicative of adiposity and inflammation.
3. To investigate the potential of specific serum microRNAs as markers of physical performance over time.

4 METHODS

4.1 Study designs and participants

This thesis is based on three human study designs : 1) the **SAWEs study,** which investigated pairs of postmenopausal monozygotic (MZ) twin sisters discordant for HRT (SAWEs MZ twins) and non-related premenopausal women with natural hormonal status (SAWEs Pre-women); 2) the **miRBody study**, which investigated postmenopausal unrelated women either on HRT or not (miRBody Post-women); and 3) the **ATHLAS study,** which investigated male masters athletes longitudinally over a 10-year period and masters and young male athletes cross-sectionally. The roman numerals I, II, III and IV shown in brackets below refer to the articles in which study design was used. The study set-ups are defined in detail in the original articles, and hence only a short description is given here:

1) **a. The SAWEs MZ twins (I, II, III)** studied for this thesis form part of the larger research project **S**arcopenia and Skeletal Muscle **A**daptation to Postmenopausal Hypogonadism: Effects of Physical Activity and Hormone Replacement Therapy in Older **W**omen – a Genetic and Molecular Biology Study on Physical Activity and **E**strogen- related Pathways (SAWEs; FIGURE 4A). The participants comprise healthy postmenopausal MZ twin sister pairs, aged 54 to 62 years (n=15 pairs), discordant for hormone replacement therapy (HRT) i.e. one sister was a long-term HRT user, while her co-twin had never used HRT treatment (Ronkainen et al. 2009). The subjects were recruited from the Finnish twin cohort (Kaprio & Koskenvuo 2002). The SAWEs study was carried out in 2007 and started with mailed pre-questionnaires and phone interviews. The volunteer participants, matching the inclusion criteria, underwent a medical examination by a physician, a set of physical performance tests and body and muscle composition measurements. Structured questionnaires were used to define health status and life-

style factors. In addition to these measurements, muscle and adipose tissue biopsies and blood samples were taken. The subset of samples used in the present thesis comprised 11 twin pairs, in which the sister on HRT was using either an E_2-only (1-2 mg, n=5) or E_2 (1-2 mg) + progesterone (n=6) -based treatment. Nine women were taking these HRT preparations as pills, one E_2-only user as a gel and another as a patch. Ten twin pairs were included in article I, five twin pairs in article II and all 11 twin pairs in article III.

b. The SAWEs Pre-women (I, II, III) for the present thesis were randomly selected from 56 healthy premenopausal women aged 29-40 years. Originally, the SAWEs Pre-women were recruited from this age-cohort living in the Jyväskylä area, as described by Pöllänen et al. (2011). They were required to fulfill the same inclusion criteria and undergo the same measurements as the SAWEs MZ twins (FIGURE 4A). These women were not and had not been using estrogen or progesterone -based hormonal contraceptives during the preceding five years and all had a regular menstrual cycle. The subset of the sample used in this thesis comprised 30 premenopausal women. Fifteen women were included in article I, 20 in article II and 8 in article III.

2) **The miRBody Post-women (II)** form part of the **Mi**cro**R**NAs and **Body** composition (miRBody) study, which investigates unrelated postmenopausal women (n= 33, 54 to 62 years old) scheduled for non-malignant gynecological surgery (incontinence, myomas, hysterectomy, bleeding disorders, prolapse) in 2015 and 2016 at the University Hospital of Tampere or Central Finland Healthcare District Hospital of Jyväskylä, Finland (FIGURE 4B). Half of the participants were HRT users (n= 16, mean duration of HRT use 6.6±6.3 years) using an E_2-only (5) or E_2 + progesterone -based (11) preparations and the other half was not receiving treatment (n= 17). A medical questionnaire, slightly modified from that used in the SAWEs study, was filled in and blood samples collected prior to surgery. During surgery, a small abdominal subcutaneous adipose tissue specimen was collected from the patient.

3) **The athletes (IV)** used in the thesis form part of a larger research project, the Athlete Aging Study (ATHLAS) comprising 25 male sprinters aged 16 to 34 years and 83 male masters sprinters aged 40 to 85 years. The subset of masters sprinters used in this thesis participated in the research in 2002 (Korhonen, 2009) and in the follow-up in 2012 (n=49), whereas the younger sprinters only participated in 2012 (n=18). The same measurements, biopsies and blood samplings were repeated in both years. The participants were recruited from among the members of Finnish athletic organizations. The sprinters had a long-term background in sprint-training and national or international success in 100-400 m sprinting events. The main focus of the study was on character-

izing skeletal muscle properties and defining the physical performance of the athletes. In addition to the measurements, muscle biopsies and blood samples were taken from the subjects. Subjects over the age 55 submitted to a medical examination (FIGURE 4C). For the present thesis, the athletes were subdivided into four age groups based on their age in 2012 A) 28.1±6.1 years (n=18), B) 59.4±5.3 years (n=16), C) 74.5±3.8 years (n=18) and D) 82.9±3.4 years (n=15).

More detailed information on the recruitment and exclusion criteria for each study are shown in FIGURE 4.

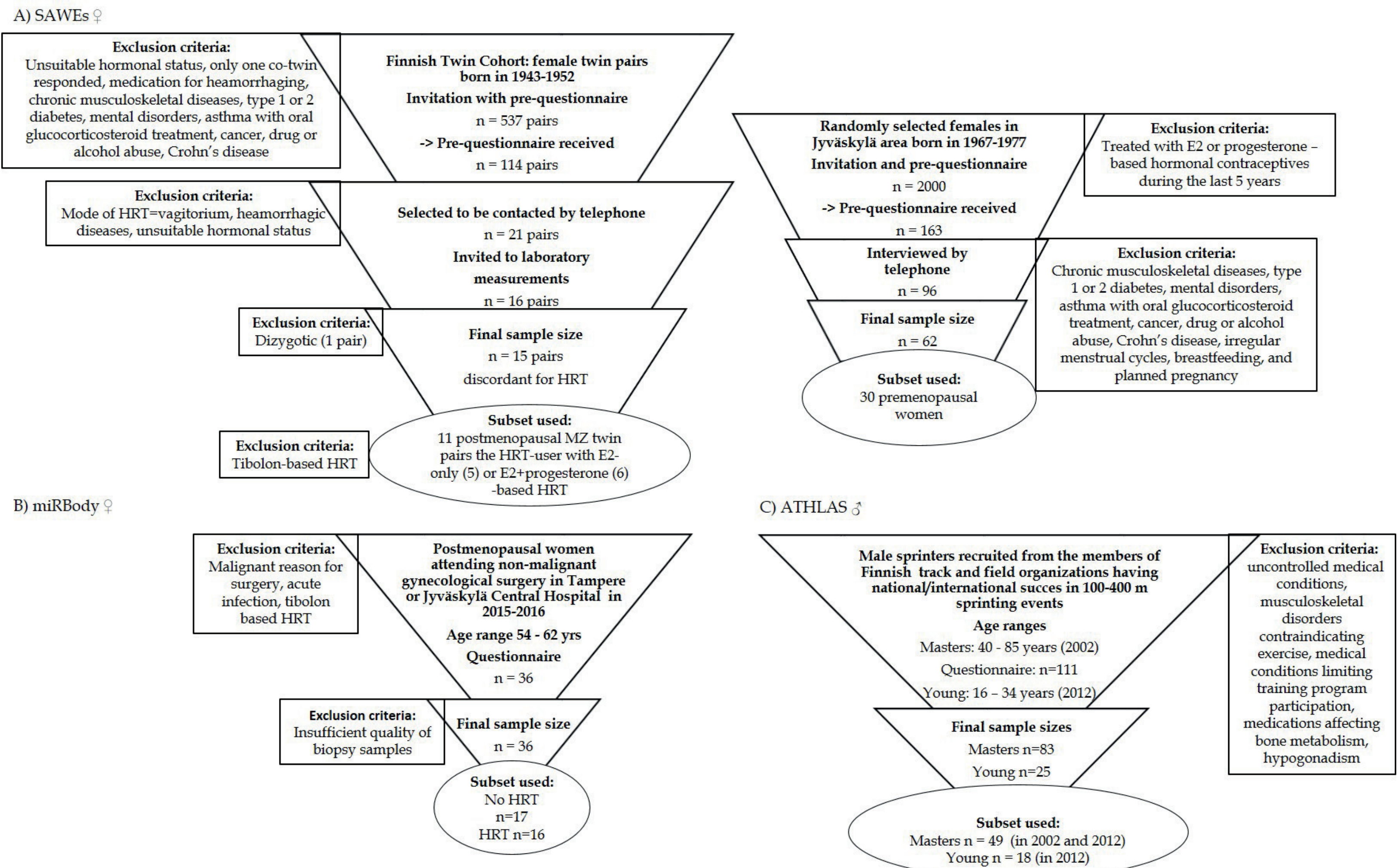


FIGURE 4 Recruitment protocol for A) SAWEs, B) miRBody and C) ATHLAS studies used in the thesis.

4.2 Ethics

All the studies in the present thesis have been conducted in accordance with the Declaration of Helsinki and have been approved by the following Ethical committees. SAWEs study: Central Finland Hospital District (E0606/06); miRBody study: Central Finland Healthcare District (1U2015); ATHLAS study: Ethics Committee of the University of Jyväskylä (in 2002) and Central Finland Healthcare District (in 2012). A written informed consent, including permission for the use of the gathered data for research purposes, was provided by the study subjects.

4.3 Measurements

4.3.1 Body anthropometrics

Body mass index (BMI) was calculated, using the standard procedure, from body mass and height, i.e., by dividing mass (kg) by the square of height (m). Total body fat and lean mass measurements were performed with multifrequency bioelectrical impedance analyzer (InBody 720, Biospace, Seoul, Korea) (I, II, III) and by bioelectrical impedance (Spectrum II, RJL System, Detroit, MI) (IV).

4.3.2 Physical activity and performance

The self-reported physical activity levels in the SAWEs and miRBody studies (I, II, III) were assessed with questionnaires using a slightly modified six-point Grimby scale (Grimby 1986) which was reclassified into three activity levels: sedentary (no activities, except at most light walking ≤ 2 times/week), moderately active (walking or other light exercise at least 3 times/week, but no other more intensive activities) and active (moderate or vigorous exercise at least 3 times/week).

The participants in the ATHLAS study (IV) took part in similar two-day measurements in 2002 and 2012 at the University of Jyväskylä. Physical activity and detailed training status/background, including training frequency (times/wk), sprint training (h/wk) and other training (h/wk), of the athletes were assessed with a self-reported questionnaire. On the first measurement day, the participants performed physical performance tests and measurements as described in more detail earlier (Korhonen et al. 2006, Cristea et al. 2008, Korhonen et al. 2009, Korhonen et al. 2012). The physical performance measures used in this thesis included 1) a maximal 60-m sprint on an indoor running track with spiked running shoes, 2) explosive force production of lower limbs by a countermovement jump (CMJ), 3) isometric unilateral knee flexion force, and 4) isometric upper limb force in bench press (David Fitness and Medical

Ltd, Outokumpu, Finland). In each test, the best performance of 2 or 3 trials was used for the analyses. Due to personal injuries in a few cases, certain physical tests were not performed. The ability to participate in physically demanding measurements was assessed individually ad hoc by the study physician.

4.3.3 Collection of biological samples

4.3.3.1 Blood sampling

Blood samples were collected from the antecubital vein after overnight fast in a supine (SAWEs; I, II, II) or sitting (miRBody; II, ATHLAS; IV) position to obtain whole blood (leukocytes) and serum. The samples for serum analysis were allowed to clot for 30 mins at room temperature followed by serum separation by centrifugation at 4 000 rpm. Serum samples were stored at -80°C in 0.5 ml aliquots until further analyzed. Serum samples for the SAWEs study were taken on the same day as muscle biopsies. If the muscle biopsy day did not occur during the follicular phase among the premenopausal women, an additional blood sample representing the lowest E_2 concentrations, was obtained during the first five days of the menstrual cycle. Blood samples for the miRBody study (II) were collected prior to surgery. Depending on the time of surgery, full overnight fasting could not always be guaranteed. In the ATHLAS study (IV) blood samples were collected on the morning following the performance measures.

4.3.3.2 Adipose and muscle tissue biopsies

Adipose tissue samples were collected from abdominal subcutaneous adipose tissue. The biopsies for the SAWEs study (II) were obtained by the study physician from beneath the navel with needle-aspiration method (12 G) (Ahtiainen et al. 2012). Possible blood traces were cleaned with 0.9 % NaCl and samples were snap frozen in liquid nitrogen and stored at -80°C until further analyzed. The 0.5 cm x 0.5 cm samples for the miRBody study (II) were obtained during gynecological surgery from the proximity of the navel and stored in All Protect Reagent (Qiagen) at -20°C until further analyzed.

Skeletal muscle tissue samples for the SAWEs study (III) were obtained from the mid-part of the *m. vastus lateralis* by needle biopsy with suction (Ronkainen et al. 2010). Blood traces were removed, and samples were snap frozen in liquid nitrogen and stored at -80°C until further analyzed.

4.3.4 Biochemical measurements

4.3.4.1 Hormone measurements

Serum 17β-estradiol (E_2) levels were measured using an extraction radioimmunoassay (SAWEs study, I, II, III) as described previously (Ankarberg-Lindgren & Norjavaara 2008). Serum E_1 levels were measured with liquid chromatography-tandem mass spectrophotometry (LC-MS/MS) on an API 4000

mass spectrometer as described previously (Nelson et al. 2004). Serum total testosterone (T), dihydrotestosterone (DHT) and androsterone (A) were measured using the LC-MS/MS method (Turpeinen, Hämäläinen & Stenman 2008, Pöllänen et al. 2011) (III). Solid-phase chemiluminescent immunometric assays (Immulite 1000, Diagnostic Products, Los Angeles, CA, USA) were used for the E_2 measurements in the miRBody study and for the serum follicle stimulating hormone (FSH), luteinizing hormone (LH) and sex hormone-binding globulin (SHGB) measurements (SAWEs study, miRBody study, I, II, III). Solid-phase chemiluminescent immunometric assays (Immulite 2000 XPi, Diagnostic Products, Los Angeles, CA, USA) were used for the serum E_2, T and SHBG measurements in the ATHLAS study (IV).

4.3.4.2 Inflammatory markers

Serum hsCRP, interleukin 1B (IL-1B), interleukin 6 (IL-6), interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α) were measured using solid-phase, chemiluminescent immunometric assays (Immulite 1000, Diagnostic Products, Los Angeles, CA, USA) (I, II, III, IV). Serum IL-6 receptors IL-6R and soluble glycoprotein 130 (sgp130) and monocyte chemoattractant protein-1 (MCP-1) concentrations were measured using the Quantikine® ELISA Immunoassay (R&D Systems, Minneapolis, MN, USA) (III) according to the manufacturer’s protocol.

4.3.4.3 Apoptotic markers

Serum Fas-ligand (FasL) concentration was measured using the Quantikine® ELISA Immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol (III, IV). Cell-free DNA (cfDNA) was measured from plasma samples with a Quant-iTTM DNA High-Sensitivity Assay kit and a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA) as described previously (Jylhävä et al. 2011) (III).

4.3.5 MicroRNA, mRNA and protein expression

4.3.5.1 RNA extraction

Serum: The total RNA from serum samples was extracted from 100 µl of serum with a Total RNA Purification kit (Norgen Biotek Corp, Thorold, Canada) (II, III, IV) according to the manufacturer’s protocol. Additionally, spike-in cel-miR-39 (20-25 fmol, Qiagen, Hilden, Germany) was added to the serum samples at the lysis step.

Serum exosomes: Serum exosomes (I) were isolated from 450 µl of serum sample by using Exoquick Exosome Precipitation Solution according to the manufacturer’s protocol (#EXOQ5A-1, System Biosciences). Exosome extraction was followed by RNA extraction with Trisure reagent (Bioline) according to the manufacturer’s instructions with slight modifications. Briefly, 7 µl of synthetic

cel-miR-39 miR mimic (1,6 x 108 copies/µl, Qiagen cat. no 219610) was added to each sample to serve as a spike-in control. Chloroform was used for the phase separation and 1 µl of nuclease-free glycogen (Glycogen RNA Grade, 20 mg/ml, Fermentas) for enhancing the RNA precipitation.

Adipose tissue: Total RNA from adipose tissue samples (~20 mg pieces) was extracted with a mirvana MicroRNA isolation kit (Ambion, by Life Technologies, NY, USA) according to the manufacturer's protocol (II). For the mRNA target analyses, RNA was treated with DNase using a TURBO DNA-free Kit (Ambion, by Life Technologies).

Muscle tissue: Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer´s instructions (III).

Leukocytes: Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer´s instructions (III).

4.3.5.2 microRNA and mRNA expression by RT-qPCR

The RT-qPCR method was optimized for each tissue and measurement. The primers used are listed in TABLE 3.

For the microRNA analyses of serum samples (II, III, IV), RNA was transcribed into cDNA (V_{tot}=5-10 µl) with a Taqman Reverse Transcription Kit (incubation: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min) followed by qPCR (V_{tot}= 5-25 µl) with a Taqman Universal Mastermix II NO Ung (Applied Biosystems, Foster City, CA) using Taqman MicroRNA Assays. Incubation was performed at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. (Applied Biosystems, ABI 7300 or iCycler, Biorad).

cDNA-synthesis for serum exosome microRNAs (I) was performed using miScript II RT kit (Qiagen) followed by RT-qPCR with a miScript SYBR Green PCR kit and miScript Primer Assays according to the manufacturer's protocol (TABLE 3). Added synthetic spike-in microRNA levels were measured in each serum analysis to control the efficiency of the RNA extraction procedures. Normalization (ΔCt) was performed using spike-in values (I, II), human miR-17 values (III) or the corresponding value of a reference sample of an average study athlete (IV).

For the microRNA analyses of adipose tissue (II) 10 ng of sample RNA was transcribed into cDNA with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, by Life Technologies) followed by RT-qPCR with TaqMan MicroRNA Assays according to the manufacturer's protocol. Incubation was performed at 95°C for 20 s, 40 cycles of 95°C for 1 s and 60 °C for 20 s. The RNU44 value of each sample was used for normalization (ΔCt). For the mRNA analyses, the total RNA of the adipose tissue samples was reverse-transcribed into cDNA with an iScript cDNA Synthesis Kit (BioRad, Hercules, California, U.S.A.). The expression of *braf, esr1, bcl2, ccnd1* and *akt1*, were analyzed with RT-qPCR using specific primers (iTaq Universal SYBR Green Supermix, BioRad) (TABLE 3). Data were normalized to GAPDH expression level (ΔCt).

For the microRNA analyses of the muscle and leukocyte samples (III), total RNA was transcribed into cDNA (Vtot=10 µl) with a Taqman Reverse Transcription Kit (incubation: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min) followed by RT-qPCR (Vtot= 10 µl) with a Taqman Universal Mastermix II NO Ung (Applied Biosystems, Foster City, CA) using Taqman MicroRNA Assays. Incubation was performed at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min (Applied Biosystems, ABI 7300). The RNU44 value of each sample was used for normalization (ΔCt).

4.3.5.3 Microarray profiling

Microarray profiling was performed for a subset of serum and adipose tissue samples of the HRT-MZ twin pairs and Pre-women (II). TaqMan human MicroRNA Array A (Applied Biosystems, by Life Technologies, NY, USA) containing 377 of the most common human microRNA assays, was used for the screening. RNA was transcribed into cDNA with a mixture of looped primers (MegaPlex kit, Applied Biosystems by Life Technologies) according to the manufacturer's protocol. Pre-amplification was performed using 3 µl of input RNA for the adipose tissue and 6 µl for the serum (PreAmp Kit, Life Technologies). The profiling was performed using an Applied Biosystems 7900 HT real-time PCR instrument. Normalization was performed using the median of all microRNAs in one sample. Fold changes were calculated with $2^{-\Delta\Delta Ct}$. MicroRNAs having Ct value less than 30 (adipose tissue) and 31 (serum) were included in the analysis.

4.3.5.4 Next generation sequencing (NGS)

NGS was performed on the exosome microRNAs extracted from the serum samples of postmenopausal MZ twins and premenopausal women in the SAWEs study (I).

The small RNA libraries were prepared using a TruSeq Small RNA Sample Preparation Kit (Illumina, USA) with multiplexing adapters. Following the TruSeq Small RNA Sample Preparation Kit user guide (Rev. E), the total RNA, including the small RNA fractions, was sequentially ligated to the 5' and 3' adaptors before convertion to cDNA by reverse transcription. PCR, using primers containing unique six base index sequences distinguishing different samples from one another, was used for cDNAs amplification. Finally, the samples were subjected to 6 % (w/v) non-denaturing polyacrylamide gel electrophoresis (PAGE). cDNA library fragments between 145 and 160 bp, corresponding to the microRNA libraries, were excised from the gel, purified and eluted. The final microRNA library pellet was air-dried and resuspended in 10 µl nuclease-free water. Quantity of the libraries was measured with Qubit fluorometer. MicroRNA library pools (8-16 samples in a single pool) were loaded to a MiSeq V3 Flow Cell (Illumina, USA) in 12 pM concentrations. To increase signal integrity, 10 % of PhiX was spiked in the library pool. MicroRNA libraries were sequenced with a MiSeq Reagent Kit V3 with 150 cycles using 36 bp reads with single-end chemistry.

4.3.5.5 Protein expression

The primary and secondary antibodies used are listed in TABLE 3. Proteins from the subcutaneous adipose tissue (II) were lysed with Pierce RIPA Buffer (#89900, Thermo Scientific) including a Halt protease and phosphatase inhibitor cocktail (1x), EDTA 0.5M (1x) and Pepstatin A (1x). 50 µl of the mix was used per 10 mg of adipose tissue sample. TissueLyzer was used to homogenize the samples (20 Hz for 2 min). Samples were centrifuged for 20 mins at 12 000 g at +4°C and supernatant with no lipid remains was collected. Protein concentration was measured with a Pierce™ BCA Protein Assay Kit (#23225, Thermo Scientific) according to the manufacturer's instructions with slight modifications (10 µl of 1/6 diluted sample into each well).

For the western blot, 16 µg of sample was pipetted into each well of the gel (Criterion Precast TGX, 4-20%, #567-1094, Bio-Rad). Run time was 45 min at 200 V. The samples were blotted on nitrocellulose membrane for 2 hours at 400 mA +4°C, after which the membrane was dyed with Ponceau and imaged (BioRad Universal Hood II, Biorad Laboratories, CA). Blocking was performed for 1 h on a swing with commercial Blocking Buffer (#1168-9670, Licor). The primary antibodies from Cell Signaling were diluted 1:1 000 (B-Raf, BCL-2, CCND1, AKT1) and from the Santa Cruz Technologies 1:200 (ESRα) and 1:40 000 (GAPDH) to blocking buffer and PBS-0.1% Tween 20 mix (1:2). Primary antibody was let to affect overnight at +4°C. Anti-Rabbit Odyssey IRDye anti-rabbit IgG (H+L) (1 mg/ml 926-32213) was used as a secondary antibody (1:20 000) for 1h covered on a swing. The imaging and quantitation was performed with Odyssey CLx (LI-COR) Image Studio Ver 2.0.38. All samples were on the same gel and were normalized to Ponceau dye (marker at 60 kDa).

TABLE 3 Primers and antibodies used in microRNA, RNA and protein measurements.

	Original article	Name	Code/sequence	Detection method	
micro-RNA primer	I	hsa-miR-148a-3p	MS00003556	SYBR Green	
	I	hsa-miR-27b-3p	MS00031668	SYBR Green	
	I	hsa-miR-126-5p	MS00006636	SYBR Green	
	I	hsa-miR-106b-3p	MS00003402	SYBR Green	
	I	hsa-miR-28-3p	MS00009254	SYBR Green	
	I	hsa-miR-30a-5p	MS00007350	SYBR Green	
	I	cel-miR-39	MS00019789	SYBR Green	
	I	hsa-miR-21-5p	MS00011487	SYBR Green	
	II	hsa-miR-16-5p	000391	Taqman	
	II	hsa-miR-18a-5p	002422	Taqman	
	II	hsa-miR-19a-3p	000395	Taqman	
	II	hsa-miR-223-3p	002295	Taqman	
	II	hsa-miR-363-3p	001271	Taqman	
	II	hsa-miR-451a	001141	Taqman	
	II	hsa-miR-486-5p	001278	Taqman	
	III, IV	hsa-miR-21-5p	000397	Taqman	
	III, IV	hsa-miR-146a-5p	000468	Taqman	
	III	hsa-miR-17	0002308	Taqman	
	II, III	RNU44	0001094	Taqman	
	III	cel-miR-39	’UCACCGGGUGU-AAAUCAGCUUG'	Taqman	
	II, IV	cel-miR-39	000200	Taqman	
RNA primer	II	GAPDH	qHsaCED0038674	SYBR Green	
	II	BRAF	qHsaCID0023755	SYBR Green	
	II	ESR1	qHsaCED0033920	SYBR Green	
	II	CCND1	qHsaCID0013833	SYBR Green	
	II	AKT1	qHsaCID0011338	SYBR Green	
	II	BCL-2	qHsaCED0004655	SYBR Green	
Protein antibody		1°	Catalog No.	2°	Catalog No.
	II	GAPDH	Santa Cruz Biotechnology sc-32233	Anti-Rabbit	Licor 926-32213
	II	BRAF	Cell Signaling 14815	Anti-Rabbit	Licor 926-32213
	II	ESRα	Santa Crux Biotechnology sc-543	Anti-Rabbit	Licor 926-32213
	II	CCDN1	Cell Signaling 2922	Anti-Rabbit	Licor 926-32213
	II	AKT1	Cell Signaling 2938S	Anti-Rabbit	Licor 926-32213
	II	BCL-2	Cell Signaling 2872	Anti-Rabbit	Licor 926-32213

4.3.6 Statistics

In general, Pearson's correlation coefficient was used for parametric variables and Spearman's correlation coefficient for non-parametric variables (I, II, III, IV). Paired samples T-test was used for parametric variables and Wilcoxon signed rank test for non-parametric variables in the twin analyses and longitudinal comparison analyses. For unrelated samples, Individual sample T-test was used for parametric variables and the Mann Whitney coefficient for non-parametric variables. Normality was based on the Shapiro-Wilk test. In the prediction analyses (IV), used for the athletes' physical performance and serum biomarkers, a Generalized Estimating Equations (GEE) model was created to predict the possible age-related associations. A p-value of less than 0.05 was considered significant in all analyses. When corrected with multiple testing, significant p values are presented as FDR values. Data analyses and visualizations were carried out using IBM SPSS Statistics (version 23.0, Chicago, IL) and R Studio (R Studio Team 2015, Boston, MA).

4.3.7 Bioinformatics

A set of bioinformatic tools were assessed in analyzing the NGS results (I). Briefly, the quality of the raw reads obtained from the Illumina sequencing was assessed with FastQC and the reads trimmed using cutadapt, based on the FastQC 'Overrepresented sequences' module output, and using a minimum read length filter of nine bases. Trimmed read files were analyzed with miRDeep2, a comprehensive computational tool for microRNA analysis and discovery which uses a probabilistic model of microRNA biogenesis to score the compatibility of the position and frequency of sequenced RNA with the secondary structure of the microRNA precursor (Friedlander et al. 2012). Mapping to the reference genome (hg38) was performed using bowtie (version 1.0.1) and miRbase version 18 was used for retrieving microRNA information. Differential expression analysis was performed on miRDeep2 output data utilizing a custom R script adapted from the differential expression module in the CAP-miRSEQ tool (Friedlander et al. 2012, Sun et al. 2014). The tools employed in different steps rely on various programming languages. To simplify the workflow, a set of R functions was created to carry out quality control, trimming, miRDeep analysis and assessment of differential expression in a pipeline-like fashion run from one wrapper script. In addition, a Poisson-normal regression model was created to analyze the related participants (unpublished manuscript). Briefly, read counts were modeled as Poisson-distributed variables with over distribution modeled through a normally distributed random variable. The advantage of this model over, e.g., the negative-binomial model is that it allows flexible modeling of dependency between related subjects through the random effect correlation matrix. The model was applied to each microRNA using MPlus version 7 and the FDR approach was used to adjust for multiple testing. All other statistical computations were done in R software (versions 3.1.3 – 3.3.1). For the Ingenuity Pathway Analyses (IPA), comparisons with fold

change values greater than 1.9 were included in the analysis and an activation *Z*-score greater than two was considered significant (II).

5 RESULTS

5.1 Characteristics of the participants

5.1.1 Anthropometrics and body composition

TABLE 4 shows the general characteristics of the participants, including body anthropometrics, physical activity levels, training frequencies and physical performance measures from the different data sets used in this thesis (I, II, III, IV). Comparisons between the studied groups are marked in the table. There were no significant differences between the SAWEs participants in the body composition measures, except for height, which was lower among the postmenopausal No HRT women compared to premenopausal women (TABLE 4). A slightly unhealthier trend in the measured variables was detected with increasing age and the absence of HRT treatment (see fat mass, LBM, hsCRP). Similar results were obtained for the postmenopausal miRBody women whether on HRT or not. The youngest sprinters in the ATHLAS study differed significantly from both the oldest and second oldest groups of masters athletes in height, weight and LBM, the younger being taller, heavier and having higher LBM ($p<0.001$, $p=0.001$, $p<0.001$, respectively) ($p<0.001$, $p=0.002$, $p<0.001$, respectively). BMI was significantly higher in the youngest masters athletes compared to the oldest group ($p=0.025$). No differences in body fat mass were found between any of the age groups.

TABLE 4 Body anthropometrics, physical activity, training frequencies and physical performance measures of the SAWEs, miRBody and ATHLAS participants.

	SAWEs (I, II, III) ♀			miRBody (II) ♀		ATHLAS (IV) ♂			
	Pre n=30	**No HRT** n=11	**HRT** n=11	**No HRT** n=17	**HRT** n=16	**Young** A (n=18)	**Masters** B (n=16)	C (n=18)	D (n=15)
Age (years)	33.4±3.6	57.6±1.9*	57.6±1.9*	58.6±3.1*	57.7±3.0*	28.1±6.1	59.4±5.3^{a,}	74.5±3.8^{a,b}	82.9±3.4^{a,b,c}
Height (cm)	165.5±4.9	161.8±4.4*	162.4±4.7	165.6±4.6	163.6±5.2	180.1±5.0	178.2±8.1	172.8±4.7^{a,b}	173.7±6.0^{a,b}
Weight (kg)	73.1±13.9	73.6±15.3	67.6±8.7	78.3±8.4	72.9±14.1	78.3±6.8	80.2±9.7	71.0±6.2^{a,b}	72.1±10^{a,b}
BMI	26.6±5.0	28.2±6.5	25.7±3.8	28.6±3.2	27.0±4.9	24.2±2.6	25.3±2.7	23.8±2.0	23.3±1.7^{b}
LBM (kg)	45.8±4.0	43.7±4.3	43.5±3.3	-	-	67.2±5.7	66.3±7.0	60.2±4.1^{a,b}	58.3±4.8^{a,b}
Fat (kg)	24.1±10.9	27.3±11.8	21.4±6.9	-	-	11.2±4.1	13.9±6.1	10.8±3.7	11.1±3.6
Physical activity									
Sedentary	-	-	-	3	2	-	-	-	-
Mod. active	14	3	5	9	8	-	-	-	-
Active	16	8	6	5	5	18	16	18	15
Training frequency times/wk	-	-	-	-	-	6.8±2.3 (n=15)	3.6±1.6^{a} (n=14)	3.5±1.3^{a}	3.1±1.1^{a} (n=14)
Sprint training h/wk	-	-	-	-	-	7.0±5.5	2.6±1.7^{a} (n=14)	3.2±3.2^{a}	2.7±2.3^{a} (n=14)
Other training h/wk	-	-	-	-	-	2.5±4.2	0.4±0.6^{a} (n=14)	1.3±2.0	2.0±2.5^{b} (n=14)
Sprint 60 m (s)^{NP}	-	-	-	-	-	7.52±0.36 (n=12)	8.43±0.63^{a} (n=13)	9.29±0.61^{a,b} (n=13)	10.81±1.30^{a,b,c} (n=10)
CMJ (cm)	-	-	-	-	-	45.7±11.8 (n=15)	32.0±4.2^{a} (n=12)	25.8±5.0^{a,b} (n=12)	18.9±4.7^{a,b,c} (n=11)
Isometric knee flexion (N)	-	-	-	-	-	326±86 (n=17)	253±57^{a} (n=15)	227±34^{a} (n=14)	190±49^{a,b,c} (n=12)
Isometric bench press (N)	-	-	-	-	-	1307±347 (n=17)	945±159^{a} (n=14)	717±175^{a,b} (n=13)	565±126^{a,b,c} (n=11)

Values are presented as mean ± SD. *$p < 0.05$ compared to group Pre of SAWEs. $^{\#}p < 0.05$ compared to No HRT group of the same study. ^{a} $p < 0.05$ compared to ATHLAS group A, ^{b} $p < 0.05$ compared to ATHLAS group B, ^{c} $p < 0.05$ compared to ATHLAS group C. All ATHLAS values are from the cross-sectional design (year 2012). ^{NP} Non-parametric variable. Independent-samples T test was used for parametric variables and Mann Whitney test for non-parametric variables. Paired samples T test (parametric) and Wilcoxon Signed ranked test (non-parametric) were used for MZ twin comparisons. BMI: body mass index, LBM: lean body mass, CMJ: countermovement jump.

5.1.2 Physical activity/performance

Physical activity level of the SAWEs (I, III) and miRBody (II) women was assessed with a modified Grimby scale dividing the participants into sedentary, moderately active or active groups. The premenopausal SAWEs participants were considered either moderately active or active. The postmenopausal SAWEs co-twins did not differ from each other in their physical activity level, all belonging to either the moderately active or active groups. The miRBody participants were slightly less active than the SAWEs participants as few women were considered sedentary; however, the HRT users and non-users did not differ from each other, equally representing all three physical activity levels (TABLE 4).

Measured by the Grimby scale, all the ATHLAS sprinters (IV) could be considered as active owing to their regular training programs and annual competitions. Self-reported training frequency (times/wk) and weekly sprint-training hours were significantly higher in the young athletes (A) than those in the other age groups (B, C, D, $p<0.05$). Other training hours were significantly lower in the youngest masters athletes (B) than in the young athletes (A) or the oldest masters athletes (D) ($p<0.05$). Of the physical performance measures 60-m sprint time, CMJ height and isometric bench press strength were significantly progressively lower with age ($p<0.05$). Isometric knee flexion strength, however, did not differ between age groups B and C. The questionnaire data gathered from the athletes indicated that overall training frequency, and the number of hours per week of sprint training and other training decreased among the masters athletes over the 10-year study period ($p<0.001$, $p<0.001$, $p<0.01$, respectively). When all the masters athletes were included in the analysis, a decrement in specific physical performance measures was detected in 60-m sprint time, CMJ height and isometric bench press force ($p<0.001$ for all), but not in isometric knee flexion force. The results remained significant even when the athletes were grouped into three age groups.

5.1.3 Hormonal status

E_2, T, SHBG and FSH levels were measured from serum. Clinical reference values for the serum hormones of premenopausal women (follicular phase) and postmenopausal women are: E_2; 73-1285 pmol/l (Pre), < 218 pmol/l (Post), T; 2.95 nmol/l (Pre and Post), SHBG; 17.4-52.1 nmol/l (Pre and Post) and FSH; 1.3-9.9 IU/l (Pre), 19.3-100.6 IU/l (Post) (Laposata, Weitz & Yoo 2014). Compared to these reference values, the serum hormone levels of the SAWEs and miRBody women were inside the ranges, except for SHBG, which was slightly higher, especially in the postmenopausal women (TABLE 5). Among the SAWEs subjects, serum E_2 levels were significantly lower in postmenopausal age than premenopausal age (Pre vs. No HRT $p<0.001$, Pre vs. HRT $p=0.001$), and lowest among the postmenopausal No HRT women compared to their twin sisters ($p=0.003$). The unrelated women in the miRBody study showed similar results for the postmenopausal HRT and No HRT groups ($p=0.003$). However,

their E_2 levels were significantly higher than those of the SAWEs participants (HRT: p=0.026, No HRT: p<0.001); this is most probably due to the different method of E_2 analysis used in these two study set-ups. The premenopausal women in the SAWEs study had significantly higher E_2 levels than the post-menopausal No HRT women in the miRBody study (p<0.001); however, no significant differences were detected between the SAWEs premenopausal women and the postmenopausal miRBody women using HRT. No differences in E_2 levels were detected between any of the athlete groups. According to a study published in *Clinical Endocrinology* (Leifke et al. 2000), the average serum reference E_2 values for healthy non-obese men (BMI 24.6±2.5) based on age were as follows: 20-29 years: 102.9 pmol/l; 30-39 years: 94.17 pmol/l; 40-49 years: 90.76 pmol/l; 50-59 years: 81.02 pmol/l; 60-69 years: 78.82 pmol/l; and 70-80 years: 80.37 pmol/l. Compared to these values, the levels of the ATHLAS sprinters were slightly higher although relatively wide variation was observed among this sample of athletes.

Serum T levels among the premenopausal women were significantly higher than those in both postmenopausal groups (Pre vs. No HRT p<0.001, Pre vs. HRT p<0.001). Serum T was not measured in the miRBody subjects. Among the athletes, serum T levels were significantly higher in the youngest group than in the second youngest and the oldest masters athletes (p=0.003, p=0.046, respectively). The average reference serum T-values for healthy non-obese men are as follows (Leifke et al. 2000): 20-29 years: 22.46 nmol/l; 30-39 years: 20.45 nmol/l; 40-49 years: 18.22 nmol/l; 50-59 years: 16.57 nmol/l; 60-69 years: 14.20 nmol/l; and 70-80 years: 13.65 nmol/l. Compared to these reference values, the T levels of the studied sprinters were slightly lower in the youngest athletes but slightly higher in the oldest masters sprinters.

There were no significant differences in serum SHBG levels between the SAWEs groups. SHBG levels were not measured in the miRBody study. Among the male sprinters, SHBG levels increased with age with significantly higher values in the oldest masters sprinters than in all the other age-groups (starting from the youngest: p<0.001, p<0.001, p=0.009, respectively). The reference SHBG values for healthy non-obese men are as follows (Leifke et al. 2000): 20-29 years: 45.54 nmol/l; 30-39 years: 46.20 nmol/l; 40-49 years: 51.92 nmol/l; 50-59 years: 55.34 nmol/l; 60-69 years: 74.21 nmol/l; and 70-80 years: 83.11 nmol/l. Compared to these reference values, the values of the studied athletes seemed to be lower throughout the age range.

Serum FSH levels differed significantly between the pre- and postmenopausal women, with higher values in all the postmenopausal groups (SAWEs: Pre vs. No HRT p<0.001, Pre vs. HRT p<0.001, miRBody: Pre vs. No HRT p<0.001, Pre vs. HRT p=0.001).

TABLE 5 Serum hormone and hsCRP levels in SAWEs, miRBody and ATHLAS studies.

	SAWEs (I, II, III) ♀			miRBody (II) ♀		ATHLAS (IV) ♂			
	Pre n=30	**No HRT** n=11	**HRT** n=11	**No HRT** n=16	**HRT** n=17	**Young** A; n=18	**Masters** B; n=16	C; n=18	D; n=15
$\mathbf{E_2}^{NP}$ (pmol/L)	382±278	33.3±27.4*	173±203*#	118±48.3*	284±183#	140±60.2	131±31.3	119±53.4	171±169
T (nmol/L)	1.3±0.5	0.6±0.3*	0.7±0.3*	-	-	19.8±3.9	15.3±4.2[a]	16.5±5.7	14.1±8.1[a]
SHBG(nmol/L)	53.4±18.7	45.9±12.6	70.5±30.1	-	-	36.7±8.4	42.9±12.0	51.8±18.5[a]	69.8±20.2[a,b,c]
FSH (IU/L)	5.7±2.3	82.7±31.2*	56.1±29.8*	29.3±7.1*	32.9±25.4*	-	-	-	-
hsCRP^{NP} (mg/L)	1.2±1.4	1.4±1.0	1.1±0.9	2.4±2.3 (n=15)	3.5±3.9 (n=15)	1.6±5.2	1.7±3.3[a]	2.3±6.0[a]	1.3±1.2[a]

Values are presented as mean ± SD. *$p < 0.05$ compared to the group Pre of SAWEs. #$p < 0.05$ compared to No HRT group of the SAWEs. [a] $p < 0.05$ compared to ATHLAS group A, [b] $p < 0.05$ compared to ATHLAS group B, [c] $p < 0.05$ compared to ATHLAS group C. All ATHLAS values are from the cross-sectional design (year 2012). ^{NP} Non-parametric variable. Independent-samples T test was used for parametric variables and Mann Whitney test for non-parametric variables. Paired samples T test (parametric) and Wilcoxon Signed ranked test (non-parametric) were used for the MZ twin comparisons. E_2: 17β-estradiol, T: testosterone, SHBG: sex hormone-binding globulin, FSH: follicle stimulating hormone, hsCRP: high sensitivity C-reactive protein. Different hormone measurements were used in different studies (see methods 4.3.4.1/2).

5.1.4 Inflammatory status

Serum hsCRP levels were used to evaluate the overall inflammatory status of the study subjects. The clinical reference value for hsCRP is < 3.0 mg/l (Casas et al. 2008). No significant differences between the pre- and postmenopausal women were detected (TABLE 5). MiRBody women showed slightly higher hsCRP values than those in the SAWEs groups, but the differences were not statistically significant. The hsCRP values found for the ATHLAS subjects were different between the youngest athletes and the other three groups, being significantly lower in the youngest than in the second and third youngest group (p=0.046, p=0.002 respectively) but higher in the youngest than oldest masters athletes (p=0.002). The longitudinal hsCRP measurements of individual masters athletes showed no significant differences over the 10-year period.

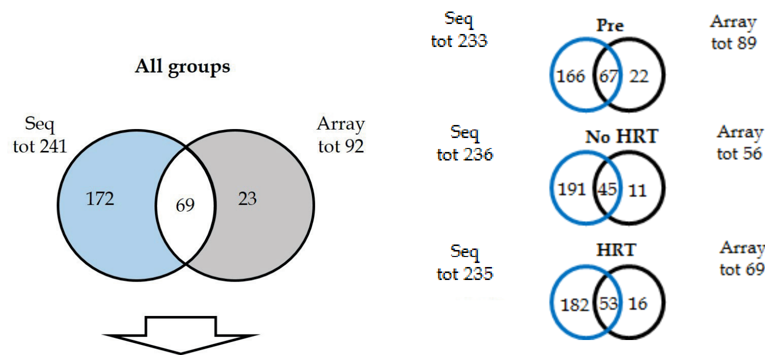
5.2 MicroRNA expression

5.2.1 Serum microRNAs

5.2.1.1 Profile of exomiRs vs. freely circulating microRNAs

Articles I and II focused on the explorative profiling of the serum microRNA contents in women of different ages and with different hormonal status. The first article (I) explored the microRNA content inside the circulating exosomes (exomiRs, shown *in italics* in the text) and the second (II) explored the freely circulating microRNAs, consisting the total circulating microRNA pool. The exomiR content was sequenced whereas a microRNA array card was used to characterize the freely circulating microRNAs. By sequencing the exomiR content (I), 241 different microRNAs were detected (9.3 % of all the 2 588 mature microRNAs listed in miRbase). Of the 241 microRNAs, 10 were differentially expressed between the premenopausal women and postmenopausal non-users as well as HRT users, while 14 differed between the postmenopausal co-twins (FDR<0.05). In the microRNA array profiling (II), 92 (24.4 %), of the 377 listed microRNAs were detected in the serum. Of these, 23 showed a differential expression pattern between the premenopausal women and postmenopausal non-users, 8 between the premenopausal women and postmenopausal HRT users and 27 between the postmenopausal co-twins (Fold Change ≥ 1.9 or ≤ -1.9). The comparisons of the microRNA contents obtained by these two methods are presented in FIGURE 5. Altogether, 69 common microRNAs, 172 sequencing-specific exomiRs and 23 array-specific serum microRNAs were detected when the microRNAs detected in all the studied groups were included in the analysis. A list of all the microRNAs mentioned is included in FIGURE 5. Comparison of the results across the different groups, revealed that the total number of sequencing-specific microRNAs remained almost unchanged; however, the total

number of array-specific microRNAs decreased with age and absence of HRT. Sequencing enables detection of all the microRNAs expressed in the exosomes whereas the array-based method is limited to the 377 known microRNAs present on the array card and by the detection thresholds set by the data analyst. Therefore, the differences in sensitivity between the two methods needs to be acknowledged when interpreting the comparisons.



Exclusively in Seq			Common microRNAs	Exclusively in Array
hsa-miR-16-2-3p	hsa-miR-378c	hsa-miR-320c	hsa-miR-148a-3p*	hsa-let-7d
hsa-miR-30a-5p*	hsa-miR-421	hsa-miR-148a-5p	hsa-miR-28-3p*	hsa-let-7e
hsa-miR-500a-3p	hsa-miR-210-3p	hsa-miR-93-3p	hsa-miR-106b-5p*	hsa-miR-20b-5p
hsa-miR-144-5p	hsa-miR-23b-3p	hsa-miR-30e-3p	hsa-miR-375	hsa-miR-106a-5p
hsa-miR-32-5p	hsa-miR-769-5p	hsa-miR-188-5p	hsa-miR-26a-5p	hsa-miR-140-5p
hsa-miR-125a-5p	hsa-miR-361-3p	hsa-miR-98-3p	hsa-miR-16-5p*	hsa-miR-185-5p
hsa-miR-126-5p*	hsa-miR-342-5p	hsa-miR-181c-3p	hsa-miR-93-5p	hsa-miR-195-5p
hsa-miR-363-3p*	hsa-miR-424-3p	hsa-miR-576-5p	hsa-miR-30c-5p	hsa-miR-323-3p
hsa-let-7i-5p	hsa-miR-3158-3p	hsa-miR-483-3p	hsa-miR-532-5p	hsa-miR-331-3p
hsa-miR-15a-5p	hsa-miR-141-3p	hsa-miR-7706	hsa-miR-451a*	hsa-let-7b
hsa-miR-106b-3p	hsa-miR-409-3p	hsa-miR-1468-5p	hsa-miR-30b-5p	hsa-miR-193a-5p
hsa-miR-151a-3p	hsa-miR-98-5p	hsa-miR-22-5p	hsa-miR-27b-3p*	hsa-miR-193b-3p
hsa-miR-378a-3p	hsa-miR-1307-3p	hsa-miR-146b-3p	hsa-miR-146a-5p*	hsa-let-7c
hsa-miR-941	hsa-miR-3605-3p	hsa-miR-548h-5p	hsa-miR-484	hsa-miR-365a-3p
hsa-miR-142-5p	hsa-miR-335-5p	hsa-miR-548x-5p	hsa-miR-126-3p	hsa-miR-376a-3p
hsa-miR-21-3p	hsa-miR-654-3p	hsa-miR-548g-5p	hsa-miR-425-5p	hsa-miR-485-3p
hsa-miR-652-3p	hsa-miR-33b-5p	hsa-miR-548aj-5p	hsa-miR-130b-3p	hsa-let-7g
hsa-let-7g-5p	hsa-miR-1285-3p	hsa-miR-548ar-5p	hsa-miR-92a-3p	hsa-miR-590-5p
hsa-miR-4732-3p	hsa-miR-181a-3p	hsa-miR-582-3p	hsa-miR-660-5p	hsa-let-7a
hsa-miR-151a-5p	hsa-miR-183-5p	hsa-miR-548f-5p	hsa-miR-27a-3p	hsa-miR-518f-3p
hsa-miR-181b-5p	hsa-miR-942-5p	hsa-let-7a-3p	hsa-miR-191-5p	hsa-miR-618
hsa-miR-101-3p	hsa-miR-130b-5p	hsa-miR-424-5p	hsa-miR-25-3p	hsa-miR-130a
hsa-miR-3615	hsa-let-7e-5p	hsa-miR-889-3p	hsa-miR-99b-5p	hsa-miR-483-5p
hsa-let-7b-5p	hsa-miR-26b-3p	hsa-miR-454-5p	hsa-miR-320a	
hsa-miR-128-3p	hsa-let-7b-3p	hsa-miR-148b-5p	hsa-miR-192-5p	
hsa-miR-744-5p	hsa-miR-550a-3p	hsa-miR-3688-3p	hsa-miR-29a-3p	
hsa-miR-1307-5p	hsa-miR-18a-3p	hsa-miR-671-3p	hsa-miR-222-3p	
hsa-miR-151b	hsa-miR-361-5p	hsa-miR-204-5p	hsa-miR-99a-5p	
hsa-miR-140-3p	hsa-miR-339-5p	hsa-miR-431-5p	hsa-miR-181a-5p	
hsa-miR-486-3p	hsa-miR-497-5p	hsa-miR-874-3p	hsa-miR-486-5p*	
hsa-miR-30e-5p	hsa-miR-17-3p	hsa-miR-125b-2-3p	hsa-miR-19b-3p	
hsa-miR-130a-3p	hsa-miR-6511a-3p	hsa-miR-4508	hsa-miR-186-5p	
hsa-miR-10b-5p	hsa-miR-450b-5p	hsa-miR-4446-3p	hsa-miR-342-3p	
hsa-miR-423-3p	hsa-miR-133a-3p	hsa-miR-550a-3-5p	hsa-miR-345-5p	
hsa-miR-22-3p	hsa-miR-877-5p	hsa-miR-150-3p	hsa-miR-423-5p	
hsa-let-7d-3p	hsa-let-7i-3p	hsa-miR-30d-3p	hsa-miR-142-3p	
hsa-miR-181c-5p	hsa-miR-223-5p	hsa-miR-550a-5p	hsa-miR-199a-3p	
hsa-miR-144-3p	hsa-miR-381-3p	hsa-miR-1306-5p	hsa-miR-103a-3p	
hsa-miR-23a-3p	hsa-miR-205-5p	hsa-miR-4732-5p	hsa-miR-223-3p*	
hsa-miR-100-5p	hsa-miR-410-3p	hsa-miR-374a-3p	hsa-miR-221-3p	
hsa-miR-107	hsa-miR-501-3p	hsa-miR-338-5p	hsa-miR-21-5p*	
hsa-miR-340-5p	hsa-let-7c-5p	hsa-miR-29b-3p	hsa-miR-143-3p	
hsa-miR-199b-3p	hsa-miR-132-3p	hsa-miR-3173-5p	hsa-miR-150-5p	
hsa-miR-181a-2-3p	hsa-miR-199a-5p	hsa-miR-627-5p	hsa-miR-26b-5p	
hsa-miR-182-5p	hsa-miR-136-3p	hsa-miR-429	hsa-miR-328-3p	
hsa-miR-320b	hsa-miR-6511b-3p	hsa-miR-548av-3p	hsa-miR-215-5p	
hsa-miR-589-5p	hsa-miR-625-3p	hsa-miR-199b-5p	hsa-miR-17-5p	
hsa-miR-584-5p	hsa-miR-96-5p	hsa-miR-374b-5p	hsa-miR-15b-5p	
hsa-miR-30d-5p	hsa-miR-1260a	hsa-miR-362-3p	hsa-miR-20a-5p	
hsa-miR-10a-5p	hsa-miR-338-3p	hsa-miR-5010-5p	hsa-miR-19a-3p*	
hsa-miR-194-5p	hsa-miR-505-3p	hsa-miR-326	hsa-miR-339-3p	
hsa-let-7a-5p	hsa-miR-1180-3p	hsa-miR-30a-3p	hsa-miR-125b-5p	
hsa-let-7f-5p	hsa-miR-374a-5p	hsa-miR-4433b-3p	hsa-miR-324-3p	
hsa-miR-92b-3p	hsa-miR-4433b-5p	hsa-miR-378i	hsa-miR-148b-3p	
hsa-miR-29c-3p	hsa-miR-145-3p	hsa-miR-6741-3p	hsa-miR-197-3p	
hsa-let-7d-5p	hsa-miR-1260b	hsa-miR-24-2-5p	hsa-miR-24-3p	
hsa-miR-15b-3p	hsa-miR-1304-3p		hsa-miR-574-3p	
hsa-miR-502-3p	hsa-miR-411-5p		hsa-miR-155-5p	
			hsa-miR-152-3p	
			hsa-miR-139-5p	
			hsa-miR-18a-5p*	
			hsa-miR-146b-5p	
			hsa-miR-127-3p	
			hsa-miR-532-3p	
			hsa-miR-301a-3p	
			hsa-miR-28-5p	
			hsa-miR-145-5p	
			hsa-miR-885-5p	
			hsa-miR-122-5p	

* Indicates microRNAs analyzed in more details in the articles included in this thesis.

FIGURE 5

Comparison of the contents of sequenced serum exomiRs (Seq) and freely circulating microRNAs (Array) from the SAWEs study. Pre: premeno-pausal; No HRT: postmeno-pausal non-users; HRT: post-meno-pausal HRT.

5.2.1.2 MicroRNA expression, age and sex

The relative expressions of serum microRNAs obtained from the cross-sectional designs (I, III, IV) are presented in TABLE 6. The levels of serum exomiRs *miR-27b-3p*, *-148a-3p* and *-126-5p* were significantly lower in the premenopausal than postmenopausal women (Mann Whitney, p<0.05) (I). *MiR-28-3p* levels were significantly highest in the premenopausal women (Mann Whitney, p<0.05), whereas *miR-30a-5p* levels showed no difference between the two groups. In the cross-sectional designs (III, IV), serum miR-146a-5p levels seemed to be the highest among the premenopausal women compared to the postmenopausal women (Mann Whitney, p<0.05) and among the youngest male athletes (under 40 years of age) compared to the masters athletes (Mann Whitney, p<0.05), indicating a negative age-association in both sexes. miR-21-5p in turn, was significantly lower in the premenopausal than postmenopausal women (Mann Whitney, p<0.05), thus showing a positive age-association in women, whereas no significant differences or age-associations were detected in the male athletes. In addition, significant differences owing to HRT treatment were detected for *miR-106b-5p*, miR-21-5p and miR-146a-5p: the level of the first microRNA was higher and the levels of the latter two were lower in the postmenopausal HRT users than in their co-twins (p<0.05).

TABLE 6 Relative expression values of the validated serum exomiRs and freely circulating microRNAs among the SAWEs and ATHLAS subjects in the cross-sectional design.

SAWEs	Pre (n=8-10)	No HRT (n=5-10)	HRT (n=5-10)	Study
miR-27b-3p	0.51 (0.38-0.71)	1.44 (1.39-2.32)*	0.92 (0.92-1.40)*	I
miR-148a-3p	0.78 (0.62-0.87)	1.19 (1.07-1.29)*	1.07 (0.85-1.23)*	I
miR-126-5p	0.50 (0.42-0.52)	0.71 (0.60-0.82)*	0.63 (0.61-0.74)*	I
miR-28-3p	5.95 (4.84-7.82)	0.57 (0.54-0.82)*	0.72 (0.52-1.14)*	I
miR-30a-5p	1.07 (0.97-1.13)	0.98 (0.95-1.01)	1.04 (0.90-1.07)	I
miR-106b-5p	0.79 (0.71- 1.18)	0.98 (0.97-1.32)^	1.60 (1.48-1.89)*	I
miR-21-5p	0.79 (0.63-0.88)	2.20 (1.46-3.05)*^	1.54 (1.29-1.64)*	III
miR-146a-5p	0.82 (0.47-1.29)	0.44 (0.28-0.62)^	0.26 (0.24-0.38)*	III

Values are presented as median (IQR). *P < 0.05 when compared to Pre, ^P < 0.05 when compared to HRT. Serum exomiRs are shown *in italics*.

ATHLAS	A (n=18) 28.1±6.1 yrs	B (n=16) 59.4±5.3 yrs	C (n=18) 74.5±3.8 yrs	D (n=15) 82.9±3.4 yrs	All masters (n=49)	
miR-21-5p	1.81 (1.17-2.42)	1.64 (1.30-3.49)	1.37 (0.91-2.20)	1.50 (1.06-2.05)	1.54 (1.11-2.25)	IV
miR-146a-5p	5.82 (4.24-6.52)	1.65 (0.82-2.59)[#]	1.31 (0.85-2.22)[#]	1.34 (0.92-1.57)[#]	1.40 (0.87-2.13)[#]	IV

Values are presented as median (IQR). [#] P < 0.05 when compared to group A. The relative expression values of the different studies presented in this table are not comparable with each other.

MiR-21-5p and miR-146a-5p levels increased significantly among the masters athletes over the 10-year study period ($p<0.001$ and $p=0.005$, respectively) (FIGURE 6). When the athletes were grouped into three age-groups, the increment in miR-21-5p was significant for the youngest (40-56 to 50-66 years) and oldest masters athletes (69-80 to 79-90 years) ($p=0.007$ and $p=0.017$, respectively) while the increment in miR-146a-5p was significant only for the oldest masters athletes ($p=0.011$).

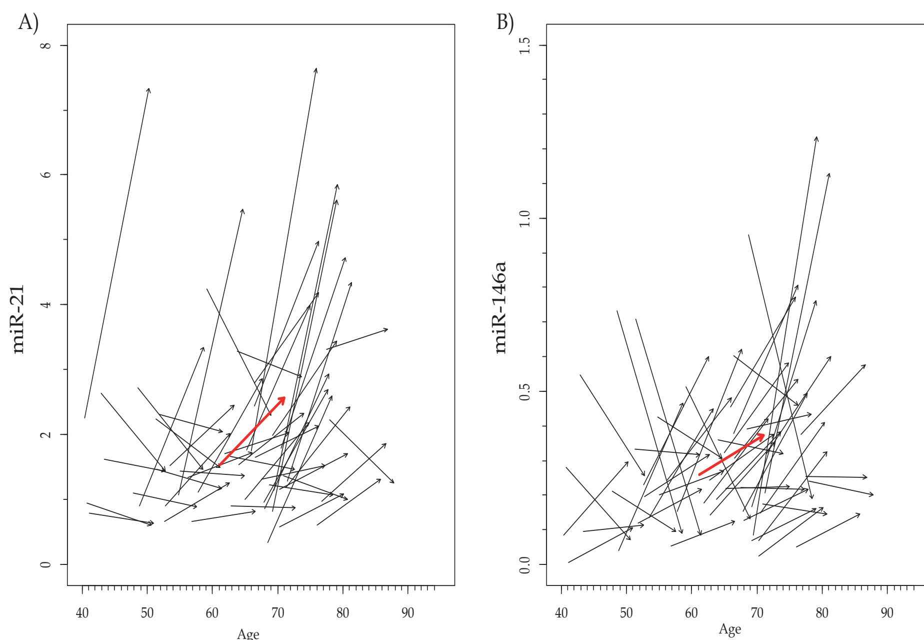


FIGURE 6 Change in serum miR-21-5p (A) and miR-146a-5p (B) expression over 10 years among the masters athletes. The red arrow indicates the average change in the athletes.

5.2.1.3 Serum exomiRs and their associations with E_2, metabolic and inflammatory markers

The associations of sequenced serum exomiR clusters (I) with E_2, body composition characteristics and with metabolic and inflammatory markers are presented in FIGURE 7. A correlation heatmap analysis, based on 21 differentially expressed exomiRs, revealed two distinct exomiR clusters, one indicative of a healthier and the other of an unhealthier phenotype. Six of the microRNAs were significantly associated with serum E_2 levels (FDR<0.05). Of these microRNAs, *miR-106b-5p* (belonging to the healthier cluster), showed a positive association, whereas *miR-148a-5p, -27b-3p, -28-3p, -126-5p* and *-30a-5p* (belonging to the unhealthier cluster) showed negative associations with serum E_2. In addition, *miR-27b-3p* and *miR-148a-5p* were associated with weaker body composi-

tion characteristics, i.e., fat, glucose and inflammatory profile. A positive age association (p<0.05) was detected *for miR-148a-5p, -28-3p, -126-5p* and *-30a-5p*, all belonging to the "unhealthier" cluster.

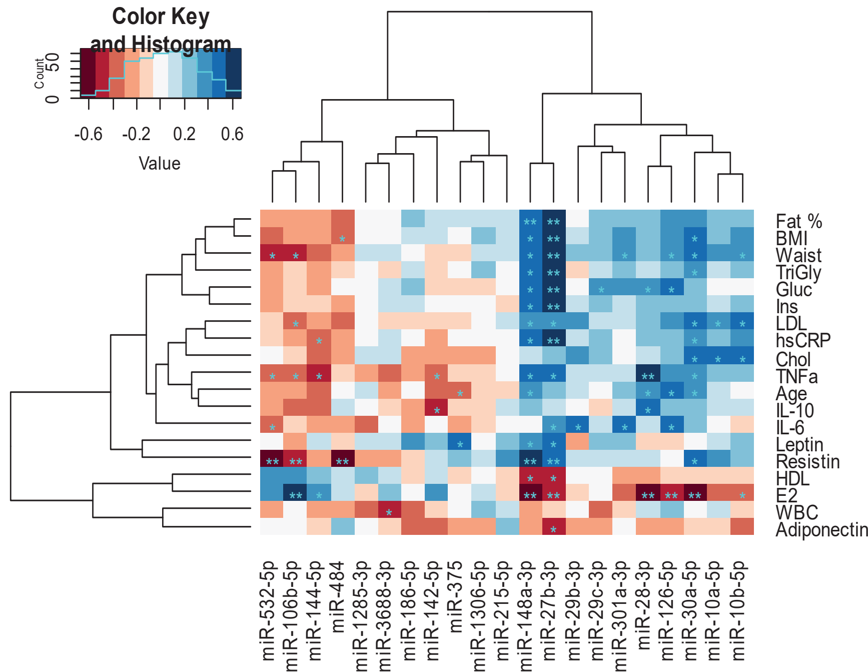


FIGURE 7 Clustered heatmap of sequenced exomiRs with differential expression pattern between SAWEs Pre, HRT and No HRT women. ** FDR<0.05, * p(nominal)<0.05. Figure is presented in Kangas et al. 2017 and reproduced with kind permission of Nature Publishing Group.

5.2.1.4 MiR-21-5p and miR-146a-5p associations with body adiposity markers and hsCRP

The associations of circulating miR-21-5p and miR-146a-5p with adiposity markers (BMI, Fat mass, LBM) and hsCRP were analyzed in the SAWEs (III) and ATHLAS (IV) study participants (FIGURE 8). In women, including both the pre- and postmenopausal women (n=30), miR-21-5p was positively associated with BMI, fat mass and hsCRP (FDR<0.05). No associations for male athletes in any age group were detected. MiR-146a-5p showed a positive association with LBM in both women and men (FDR<0.05) and a negative association with hsCRP in men (p<0.05).

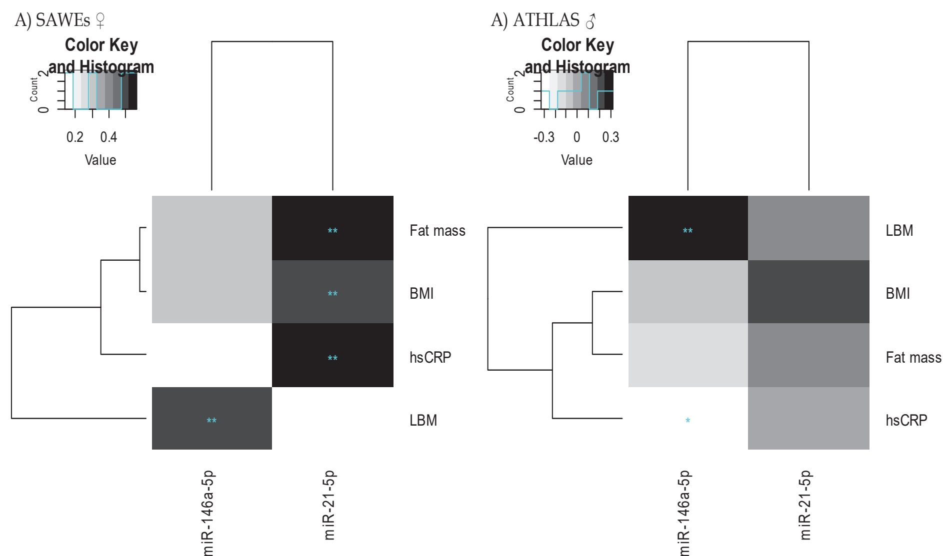


FIGURE 8 Associations of systemic miR-21-5p and miR-146a-5p levels with adiposity markers and hsCRP. A) represents the associations in the SAWEs study and B) the associations in the ATHLAS study. ** FDR<0.05, * p<0.05. Spearman correlation coefficient was used in the analyses.

5.2.1.5 MicroRNA expressions in adipose- and skeletal muscle tissue and blood leukocytes

Altogether, 203 microRNAs were detected by microRNA array in the subcutaneous adipose tissue extracted from the SAWEs pre- and postmenopausal women (II). Of these microRNAs, 10 had higher and six lower expression with higher age (Pre vs. Post No HRT), two had higher and 22 lower expression with higher age together with HRT (Pre vs. Post HRT) and one higher and 30 lower expression when comparing the HRT users to their non-user co-twins. RT-qPCR validation performed on the premenopausal women of the SAWEs study and on the postmenopausal women of miRBody study showed that miR-16-5p, -451a, -223-3p, -18a-5p, -19a-3p, -363-3p, and -486-5p expressions were significantly higher with higher age (p<0.001; p<0.001; p=0.003, p=0.003; p=0.006; p<0.001; p=0.002, respectively) and that miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-363-3p, and miR-486-5p were significantly higher with higher age combined with HRT (p=0.002; p=0.014; p=0.009; p=0.024; p=0.036; p=0.039, respectively). The validated microRNA expressions did not differ significantly between the postmenopausal non-users and HRT users unless the No HRT women with high serum E_2 levels were excluded from the analyses. After exclusion (values higher than 110 pmol/l excluded), miR-19a-3p expression was significantly higher in the adipose tissue of the postmenopausal No HRT women

than HRT users (p=0.043). A similar trend was detected for miR-18a-5p (p=0.094).

MiR-21-5p and miR-146a-5p relative expressions were measured from skeletal muscle tissue and blood leukocytes of the postmenopausal MZ twins by RT-qPCR (III). Overall, skeletal muscle showed a relatively high abundance of miR-21-5p compared to leukocytes, but no significant differences were detected between the postmenopausal co-twins. MiR-146a-5p expression in skeletal muscle tissue was relatively low but significantly higher in the postmenopausal No HRT women compared to their co-twins on HRT (p=0.012). Muscle miR-21-5p or leukocyte miR-21-5p and miR-146a-5p expressions did not differ significantly between co-twins.

5.2.1.6 Change in physical performance and serum miR-21-5p and miR-146a-5p levels

The decline in the physical performance measures among the masters athletes over the 10-year study period seemed to go in parallel with the increments in miR-21-5p and miR-146a-5p serum levels (IV) (FIGURE 9). MiR-21-5p showed a significant non-linear association with knee flexion (p=0.023, FIGURE 9A) and bench press strength (p=0.004, FIGURE 9B), whereas miR-146a-5p levels were associated with sprint time (p<0.001, FIGURE 9C), knee flexion (p<0.001, FIGURE 9D) and bench press strength (p<0.001, FIGURE 9E) when aging was included in the analyses as a continuous determinant.

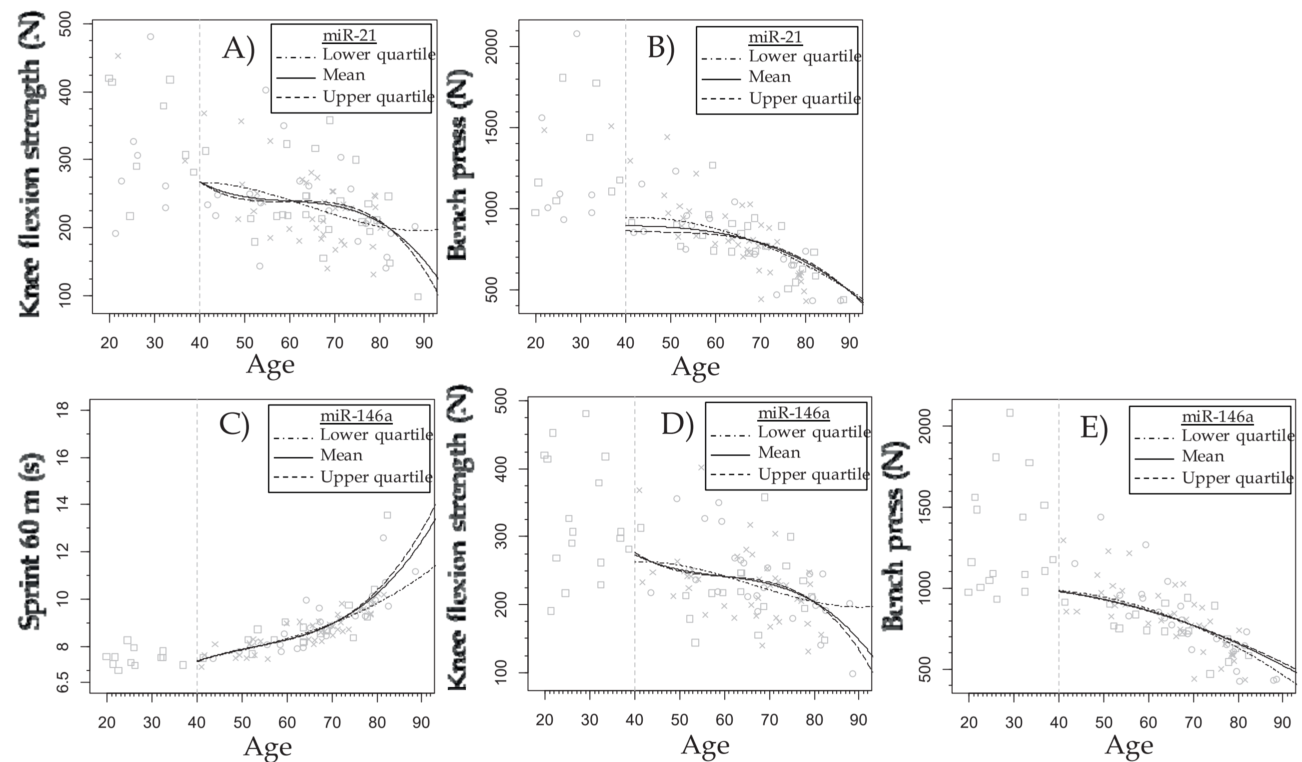


FIGURE 9 Prediction model of circulating microRNA levels and specific physical performance measures over time. A cross (X) indicates that the case is located within 0-37.5 %, circle (o) within 37.5-67.5 % and square (□) within 67.5-100 % of the cumulative share of the microRNA-distribution. Figure is presented in Kangas et al. 2017 and reproduced with kind permission by Hindawi.

6 DISCUSSION

This thesis investigated the associations of circulating microRNAs with age, pre- or postmenopausal status (with or without HRT) and lifelong physical training. The purpose was to find out whether age or external factors, i.e., postmenopausal estrogen replacement in women, or lifelong physical training in men, are linked with circulating or tissue microRNAs, and if these microRNAs are associated with markers of adiposity, inflammation and physical performance capacity.

The serum levels of several microRNAs were dependent on age in both women and men. Menopausal status (Pre vs. Post) and the age-gap between young and masters athletes (40 to 50 years) was associated with differences in serum microRNA levels. In addition, the use of HRT was associated with some of the measured microRNAs, indicating that serum E_2 levels partially influence microRNA patterns. The microRNAs studied were also associated with several adiposity markers, creating clusters of microRNAs linked with healthier or unhealthier physiological status in women. Associations with specific physical performance measures, indicative of power and speed capacity in male athletes, were also detected.

In addition to pure biological aging processes (Hackl et al. 2010, Lanceta et al. 2010, Weilner et al. 2013), microRNAs can be linked with several diseases and conditions related to older age (Lu et al. 2008, Haider et al. 2014, Hammond 2015, Fiorillo et al. 2015, Kocijan et al. 2016, Cao et al. 2017, Xie et al. 2017). Nevertheless, very little is known about sex differences in circulating microRNA levels. However, a very recent study by Bammert et al. (2017) revealed that circulating endothelial-derived miR-125a expression was lower and miR-34a expression higher in middle-aged men than middle-aged women. This finding indicates potential sex-related differences in microRNA regulation in cardiovascular disease in which the prevalence, which is known to differ between the sexes, is partly due to hormonal disparities (reviewed by Rosano, Spoletini & Vitale 2017). Altogether, more detailed knowledge of the microRNA action and delivery mechanisms inside and between different cells and tissues is crucial if microRNAs are to be used as prognostic, diagnostic and therapeutic tools.

In particular, exomiR trafficking via extracellular vesicles has been recognized as one of the tools with the most potential for increasing understanding of tissue cross-talk in various biological processes and pathological conditions (Cheng et al. 2014, Braicu et al. 2015). Thus, the novel information provided by this thesis, suggesting that exomiR content differs according to age and systemic E_2 status, opens the door to new ideas about menopause-associated changes in intercellular communication, thereby encouraging research on the topic.

This thesis introduces potential candidate microRNAs and microRNA clusters for further study as potential indicators of the physiological status or physical capacity of an aging individual. However, more research with different study populations and standard procedures is needed before these microRNAs can be established as useful tools in clinical practice and for following the performance and physical adaptation of an athlete or a non-athlete to training.

6.1 MicroRNAs as indicative of age and/or associated with systemic E_2 levels

The search for biomarkers of aging arises from the assumption that a certain marker could reflect biological aging status more accurately than age in years. The American Federation for Aging Research has described the characteristics of a proper biomarker for aging (Johnson 2006). The most prominent requirements for a potential biomarker are the following: to be able to predict the rate of aging better than chronological age and monitor primary aging excluding the contribution of diseases, to be suitable for continuous testing with minimal harm to the individual and to function in both humans and laboratory animals.

In all the original articles on which this thesis is based, some of the studied microRNAs were associated with age, thereby offering a starting point for evaluating the suitability of a specific microRNA as an indicator of biological age. Validated circulating exomiRs *miR-126-5p, -27b-3p, -148a-3p* and miR-21-5p levels were higher whereas miR-146a-5p and *-28a-3p* were lower with more advanced age in women. While previous studies have demonstrated associations of these microRNAs with age-related diseases, pure age associations have been little reported, except for miR-21 (TABLE 1) (Olivieri et al. 2012, reviewed by Olivieri et al. 2013). In the ATHLAS study, serum miR-146a-5p was negatively associated with age when both young and masters athletes were included in the analyses, but no significant correlations were detected if only masters athletes were included in the analyses. This finding indicates that the biggest change in serum miR-146a-5p levels occurs prior to age 40. Interestingly, in the follow-up design among the masters athletes, the most significant increase in the 10-year miR-146a-5p levels seemed to occur among the oldest masters athletes, indicating this phenomenon could be related to aging.

MicroRNAs are highly sensitive to internal and external signals. Hormonal fluctuations are one of the factors affecting microRNA expression, as also

shown in the present thesis (I, II, III). *MiR-126-5p, -27b-3p and -148a-3p* levels were higher in both postmenopausal groups (HRT or No HRT) compared to the premenopausal women and no differences were observed between the postmenopausal co-twins. These findings suggest a similar age-related association for these microRNAs irrespective of the use of HRT. In addition to the age difference (Pre vs. both Post groups), validated serum miR-21-5p levels were significantly higher in the postmenopausal No HRT women than in their co-twins on HRT. This finding suggests that the aging increment in serum miR-21-5p levels occurs in both postmenopausal groups, however, the increment being more gradual in HRT users compared to non-users. Interestingly, contrary to what was expected, the levels of miR-146a-5p and *miR-106b-5p*, showed greater similarity in the premenopausal women and postmenopausal No HRT women than in the premenopausal women and postmenopausal women on HRT. These results suggest that the estrogen sensitivity of miR-146a-5p and *miR-106-5p* differ owing to the origin of the circulating E_2, i.e., whether E_2 is natural or supplemented.

No age association for miR-148a-3p was detected in a recent study on plasma microRNAs by Ameling et al. (2015). However, the authors demonstrated that miR-148a-3p is associated with male phenotype and with BMI. Taken together with the results of Ameling et al. (2015), our NGS findings on *miR-148a-3p* show an interesting pattern as, in addition to a positive age association, we also found *miR-148a-3p* to have a positive association with body adiposity and negative association with serum E_2 levels. There is also an indication that mir-148a is associated with bone osteoclastogenesis. Cheng et al. (2013), using mice CD14+ peripheral blood mononuclear cells, demonstrated that silencing miR-148a results in an increment in bone mass. As mentioned, miR-148a-3p was positively associated with age and negatively with serum E_2 levels. This finding together with the results of Cheng's study (2013) suggest the potential involvement of miR-148a-3p in the regulation and messaging of osteoporosis, a condition strongly related to age and estrogen deficiency in females. However, the qPCR validation confirmed only an age association for *miR-148a-3p* in this thesis, leaving a question mark regarding the role of HRT in the presence of an abundance of circulating microRNA.

Of the adipose tissue microRNAs, miR-16-5p, -451a, -223-3p, -18a-5p, -19a-3p, -363-3p, and -486-5p were higher with more advanced age. These microRNAs showed a similar pattern in the Pre vs. No HRT comparison and Pre vs. HRT comparison, except for miR-19a-3p, which showed no difference between the Pre and HRT groups. MiR-19a-3p expression also differed between the postmenopausal HRT users and non-users when serum E_2 levels were controlled for. These findings indicate that miR-19a-3p is more sensitive to HRT, i.e., to serum E_2 levels, than the other microRNAs measured in the adipose tissue samples. The connection between miR-19a-3p and estrogen signaling has been previously demonstrated as miR-19a-3p has been shown to target ERs (Loven et al. 2010). Only the sensitivity of miR-21-5p and miR-146a-5p to HRT was analyzed in skeletal muscle tissue and blood leukocytes. MiR-146a-5p differed in the skeletal muscle tissue of the postmenopausal co-twins, showing higher levels in the No HRT

women. Previous studies have yielded conflicting results on the role of miR-146a-5p in skeletal muscle. The stretch-induced increment in miR-146a-5p levels in mouse C2C12 cells has been shown to compromise cell differentiation, favoring low levels of miR-146a-5p (Kuang et al. 2009). Furthermore, a recent study by Sun et al. (2017) suggested that miR-146a-5p has an anti-fibrotic function after muscle injury via targeting Smad4. This microRNA-target interaction could be utilized in reducing injury-initiated skeletal muscle fibrosis. These studies, however, have focused on stress responses, revealing little about the normal function of miR-146a-5p in skeletal muscle.

Drawing on the biomarker theory described above (Johnson 2006) and the aging theories described in the introduction of this thesis, circulating tissue-derived microRNAs have, technically, the potential to be indicative of biological age. However, owing to their sensitivity to various intra- and extracellular stimuli, they are easily affected by the environmental stressors. Before the possible utilization of microRNAs as pure aging markers, their behavior in different health and disease conditions, in different tissues and among different populations across the lifespan needs to be addressed. Interestingly, in a very recent study, Huan et al. (2017) measured whole blood-derived microRNA expression in a large study population consisting of 5 221 individuals ranging in age from 24 to 92 years. Based on the expression levels of 80 different microRNAs associated with age-related processes, they proposed the concept of "microRNA age" which was then used to predict the rate of biological age. Huan et al. (2017) showed positive correlations with predicted "microRNA age" and age predicted by DNA methylation state as well as mRNA expression of specific aging-related genes suggesting that "microRNA age" could contribute to the model of epigenetic age. The microRNAs listed above as part of the results of this thesis can be considered microRNAs indicative of age. However, as further discussed below, they are also indicative of many age-associated phenomena, rendering their role in aging much more complex.

6.2 Candidate microRNAs interacting with adiposity and inflammation

The NGS analyses revealed that some microRNA clusters are associated with a healthier phenotype than others (I) (FIGURE 9). This health status association was evaluated by adiposity indicators and classical inflammatory markers. MicroRNAs which differed significantly between the pre- and postmenopausal groups and were linked to serum E_2 levels were exomiRs *miR-106b-5p, -148a-3p, -27b-3p, -28-3p, -126-5p* and *-30a-5p*. Of these microRNAs, the most significant positive associations with adiposity and inflammatory markers were detected for *miR-27b-3p* and *-148a-3p*. The role of miR-27b-3p in inflammation has previously been demonstrated in human macrophages by Jennewein et al. (2010), who showed that miR-27b-3p destabilizes the mRNA expression of peroxisome

proliferator-activated receptor gamma (PPAR-γ), a molecule which has anti-inflammatory potential. A study by Ma et al. (2013) showed that miR-27b-3p belongs to a group of microRNAs that are highly enriched in several types of porcine adipose tissue, and function in adipogenesis and lipid metabolism. Glucocorticoids, molecules known to contribute to metabolic dysfunction, have also been shown to upregulate miR-27b-3p expression in human adipocytes (Kong et al. 2015). Kong et al. (2015) proposed that by targeting PR domain zinc finger protein 16 (Prdm16), miR-27b-3p is likely to have an important role in suppression of white adipose tissue browning and further in the accumulation of fat and development of insulin resistance. These results are in line with the present findings on *miR-27b-3p* and its possible role as one of the regulators of body adiposity and inflammation in women. MiR-148a has been shown to be associated with obesity in both human and mice by upregulating adipogenesis via suppressing target mRNA Wnt1 (Shi et al. 2015). The association of miR-148a with inflammation has not previously been reported except in human aortic valve cells, in which stretch was shown to repress miR-148a levels, which led to increased inflammatory response. This finding does not follow the same pattern as the present findings on serum exomiRs, but indicates tissue specificity in estrogen-sensitive microRNA regulation.

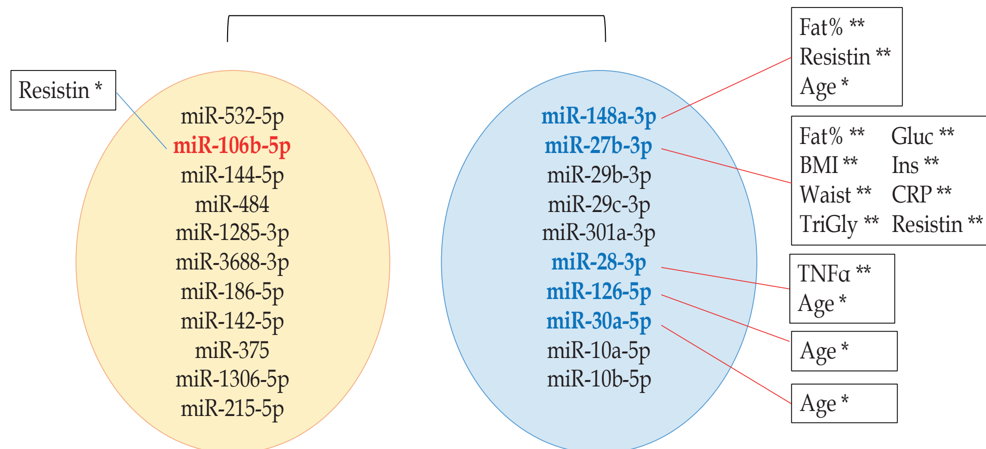


FIGURE 9 Serum exomiR clusters from the SAWEs study detected by NGS. MicroRNAs inside the red circle belong to the "healthier" cluster and those inside the blue circle to the "unhealthier" cluster. MicroRNAs colored in red were positively and those in blue negatively associated with serum E_2 levels. Similarly, red lines represent positive and blue lines negative association. ** FDR<0.05, * p<0.05.

A recent study by Nunez Lopez et al. (2016) showed that a cluster of circulating microRNAs, including many of the microRNAs of interest in the present thesis, i.e., miR-21-5p, *-28-3p*, *-126-5p*, -146a-5p, *-148a-3p*, and *-223-3p*, are, owing to obesity, prediabetes or type 2 diabetes, deregulated together with more classical

inflammatory markers. According to Nunez Lopez et al. (2016), miR-126-5p in particular, could be used as pre-diabetic indicator. Our findings support these previous findings and suggest that exomiRs *miR-126-5p, miR-28-3p* and *miR-148a-3p* are associated not only with metabolic and inflammatory status, but also with higher age and, negatively, with serum E_2 levels. The negative associations with serum E_2 suggest that higher levels of E_2 suppress the number of microRNAs related to unhealthier metabolism and physiological status, indicating possible beneficial interaction between HRT and circulating microRNAs.

When pre- and postmenopausal women were analyzed together (III), miR-21-5p was positively associated with BMI, fat mass and hsCRP, whereas miR-146a-5p was positively associated with LBM. These findings indicate that these two microRNAs play an opposite role in regulating adiposity and overall body composition. The serum levels of miR-21-5p were highest in the postmenopausal No HRT women, suggesting that higher circulating levels of miR-21-5p could be indicative of weaker body composition related to reduced levels of systemic E_2. In contrast, higher levels of miR-146a-5p could be indicative of a healthier phenotype owing to whole-body adiposity. In male athletes, no associations of miR-21-5p with inflammatory or adiposity markers were detected. Instead, miR-146a-5p was negatively associated with hsCRP and positively with LBM. This supports the role of miR-146a-5p as an indicator of a healthier phenotype also in men. Keller et al. (2011) found high miR-21-5p levels in adipose tissue to be associated with adipogenesis and obesity in humans, whereas miR-146a-5p has been shown to inhibit adipogenesis in primary porcine adipocytes after TNFα exposure (Wu et al. 2016). In addition, high levels of miR-146a have been shown to alleviate inflammation in different cell types (Rebane et al. 2014, Li et al. 2014b). Overall, these findings are in line with the present results, further emphasizing the diverse functions of miR-21-5p and miR-146a-5p in regulating adiposity and inflammation. However, whether these two microRNAs were derived from adipose tissue, inflammatory cells or from other tissues, was not directly addressed in this thesis, leaving space for speculation and further experiments.

According to Thomou et al. (2017) adipose tissue is one of the major organs contributing to microRNA secretion. In this thesis, the expression of seven microRNAs i.e. miR-16-5p, -451a, -223-3p, -18a-5p, -19a-3p, -363-3p, and -486-5p, were validated in the adipose tissue of women (II). They all showed a positive age association, whereas only miR-19a-3p showed sensitivity to HRT when E_2 levels were controlled for. Previous studies have shown that miR-223-3p, -18a-5p, -19a-3p, -363-3p, and -486-5p are expressed in the adipose tissue of different organisms, potentially affecting adipogenesis (Wang et al. 2008, Kim et al. 2012, Chen et al. 2014, Chuang et al. 2015, Guan et al. 2015). In original article (II), analyses of the potential microRNA targets suggested that the changes in microRNA levels are linked with estrogen signaling and the homeostasis of adipose tissue, indicating the more proliferative status of adipose tissue in premenopausal women than postmenopausal women. However, the changes in microRNA expressions in adipose tissue were not reflected in the circulation,

thus conclusions about circulating microRNAs derived from adipose tissue could not be drawn.

The circulating pool of microRNAs is most likely to be strongly affected by other tissues with high vascularization or cell types that are highly abundant in the circulating blood. In this thesis, while miR-146a-5p expression in skeletal muscle was relatively low, it was still significantly higher in the postmenopausal women without HRT than in their co-twins on HRT (III). The differential expression pattern between the two groups of postmenopausal co-twins resembled that of serum miR-146a-5p. However, the results are challenging to interpret, as higher miR-146a-5p levels are thought to be associated with overall healthier body composition, while previous studies have demonstrated that the quality of skeletal muscle is better in postmenopausal HRT users than in their non-using co-twins (Ronkainen et al. 2010). These findings are conflicting and do not support a clear association with serum E_2, miR-146a-5p expression and a healthier phenotype in skeletal muscle tissue. It is likely that the effect of miR-146a-5p derives from a different tissue or from cells such as blood leukocytes in which miR-146a-5p expression seemed to be relatively much higher than in skeletal muscle.

6.3 Serum microRNAs as tools for evaluating physical performance

The effects of an acute bout of exercise on serum microRNA expression has been demonstrated by several studies; however, the long-term training effects have been less studied, and we are not aware of any studies in aging athletes. The associations of the microRNAs in focus in this thesis (miR-146a-5p and miR-21-5p, IV) with exercise have been studied to some extent in young athletes and healthy or non-healthy non-athletes but to a lesser extent in masters athletes. MiR-146a-5p has been suggested to be a biomarker of cardiovascular fitness in young male rowers, owing to its correlation with V_{O2max} (Baggish et al. 2011). In the ATHLAS study (IV), V_{O2max} was not measured as it is less crucial in sprint performance. Nevertheless, interesting associations of miR-146a-5p with age and specific physical performance measures were obtained. In the cross-sectional design, serum miR-146a-5p levels were clearly highest among the young athletes, showing a significant negative correlation with age. This finding could indicate that the higher the level of miR-146a-5p, the better not only the cardiovascular fitness but also the capacity for a sprint type of activity. However, the longitudinal results for the masters athletes showed an increase in miR-146a-5p, especially among the oldest sprinters. In theory, this finding could indicate that physical fitness improves with aging. Both training frequency and physical performance, however, showed a modest decline in the oldest age-group, hence this assumption was not supported. Instead, it is likely that changes in miR-146a-5p, which has previously been shown to have role in

the immune system (reviewed by Li, Chen & Li 2010), indicate adaptations to processes related to inflammatory status. Moreover, miR-21-5p has been linked to V_{O2max}. A study by Bye et al. (2013) demonstrated that higher serum miR-21-5p levels were associated with lower V_{O2max} in healthy middle-aged males but not in females. Another study performed on male patients with heart failure found an immediate increase in serum miR-21-5p levels after a maximal symptom-limited cycling test (Xu et al. 2016). Our results showed a concomitant increase in serum miR-21-5p with declining performance in specific physical performance measures. While these declines were in part explained by aging, the GEE model also indicated that the strength measures, including isometric knee flexion and bench press, are partly explained by the serum miR-21-5p levels. MiR-146a and miR-21 have been shown to be associated with aging and many processes linked to it, such as inflammation and cellular homeostasis (reviewed by Olivieri et al. 2013). In addition, their interaction with physical performance and adaptation processes has been recognized (Baggish et al. 2011). The results of this thesis support the previous findings, contributing more detailed information about the timing of the changing levels of these two microRNAs in healthy, physically elite male masters athletes. The results showed that the clearest differences in microRNA levels were between the young athletes and masters athletes, suggesting that the biggest change in microRNA regulation occurs prior to the age of 40, roughly at some point in the age-gap between young and masters athletes. In addition, minor fluctuations also occur within the masters group.

As this thesis shows, the results of the measurement of microRNA levels can be conflicting, depending on whether a cross-sectional or longitudinal design is used. It is, therefore, crucial to follow individual biomarker behavior over time. This was also proposed by Hecksteden et al. (2016). In addition, they suggested that physical training, and especially training type, are confounding factors for microRNA-based disease diagnostics. They found that plasma and blood microRNA profiles differed between strength and endurance athletes and that some of the microRNAs measured were linked with many pathologies, including acute myocardial infarction. It is important to keep this in mind as it also adds to the potential of physical training not only to confound, but more importantly, to counteract disease development via circulating microRNAs. Despite staying active throughout the lifespan, the natural aging process eventually affects the physical functioning, as also shown in this thesis in the case of masters sprinters. MicroRNA profiling from body fluids offers an additional layer of markers with potential for use in estimating the physiological status of an aging individual. When the origins of circulating microRNAs are eventually discovered, microRNA profile will have potential for revealing detailed information about exercise adaptation in specific tissues. Systematic studies on different-aged individuals with diverse training backgrounds, including both sexes, are warranted as it is widely acknowledged that responses differ based on the above-mentioned factors. Their findings will be helpful in planning individual training programs for athletes as well as nonathletes wishing to remain

physically fit in older age. Much research remains to be done, on the functional role of microRNAs in different tissues and the messages they reflect into the circulation.

6.4 Methodological considerations

Studying microRNA contents in body fluids is challenging owing to the relatively low abundance of microRNAs and lack of endogenous controls. In this thesis, as reported in each original article, much effort has been made to optimize the methods used in each study. The lack of endogenous controls has been solved by using spike-in controls, reference samples and stably expressed microRNAs. Another challenge in studying serum samples is that the cells of origin of microRNAs remain unknown, thus leaving much space for speculation on this question. However, the fact that microRNAs are often surrounded by vesicles, such as exosomes, is promising as these structures commonly take a coating or potentially recognizable cell-specific cargo from the cell they are produced in. With the help of these molecules it will be possible to recognize the cell types of origin in the future.

The use of diverse blood/microRNA fractions and detection methodologies can lead to conflicting results (Wang et al. 2012). In this thesis, although only serum samples were obtained, freely circulating microRNAs and exomiRs were both analyzed. These fractions however, could partly overlap, as the freely circulating microRNA fraction most likely includes some exomiR content. Of the possible detection methods, NGS, array and RT-qPCR were utilized here. The results demonstrated that the serum profile of vesicle-free microRNAs and exomiRs differed to some extent. The most prominent difference was in the total number of detected microRNAs, which was clearly greater in the exosomes, when profiled with NGS. This, however, at least in part, is due to the higher sensitivity of NGS and the capacity of the method to detect all the possible microRNAs, an outcome which is not possible with the array-based method, limited in the array used in this thesis to a total of 377 microRNAs. It should also be acknowledged that the contents of exosomes might be more selected compared to the contents of the other freely circulating microRNAs. The number of array-based serum microRNAs seemed to decrease with advanced age and the absence of HRT. In light of these findings, one could speculate whether at a higher age, and to a lesser extent, with lower serum E_2, exosome-specific microRNA messaging becomes emphasized. In addition, the potential role of microRNAs not present in the array used should be borne in mind. Owing to differences in detection and validation techniques, the results are not always completely in line. For example, we obtained opposite results for *miR-28-3p* (I), where for the postmenopausal women NGS yielded the highest values and RT-qPCR validation the lowest. This individual result emphasizes the sensitivity of microRNAs and the importance of standardized workflow throughout the analyses.

This thesis has several strengths. The unique MZ co-twin study design (I, II, III) consisting of the postmenopausal SAWEs study twins, controlled for genes, age, sex, intrauterine and childhood environment, offered the best alternative for studying potential biomarkers strongly affected by the abovementioned factors. In addition, the longitudinal study design (IV), based on the ATHLAS masters athletes study, provided an excellent frame for following individuals over a 10-year period without the effect of genetic variability. The influence of sedentariness is also ruled out among the athletes, whose motivation to achieve to peak performance during the measurements is high compared with that of nonathletes. Therefore, masters athletes offer an ideal model to investigate the fullest physical potential of aging individuals. A particular achievement of the present research was the development of a novel NGS-based method for serum exomiR detection (I).

This thesis was conducted with the best currently available resources and knowledge. However, some limitations remain. Due to the novelty of performing microRNA analyses, a common standardized protocol for the workflow, including the sample collection, equipment, reagents, normalization strategies and data analyses, is absent. This imposes limitations on the comparability of results between different study designs, even where only slightly different methods have been used. As mentioned, microRNAs are sensitive to both internal and external stimuli, and, hence controlling the performance of such measurements in humans is challenging. We were not able to control for all the confounding stimuli, such as small differences in physical activity or training volume, undiagnosed conditions, lack of sleep, personal diet or other environmental aspects/stressors outside the research environment. The relatively small sample size used in all the studies should also be acknowledged, along with the limited amount of biopsy samples, which caused the need for validation of the results from different samples (II). Further, owing to the time of surgery for the miRBody participants (II), it was not always possible to obtain blood samples after overnight fasting. This could be one of the reasons why the validation of the serum profiling results (II) was not successful. The different techniques used in the serum sex hormone measurements should be noted when interpreting the results, especially between the different data sets. This study emphasizes the role of circulating E_2 in microRNA regulation. However, it should be recalled that menopause is also characterized by other hormonal changes, which were not as closely investigated. Functional cell experiments would have added value to the research owing to microRNA-target interactions and exosome secretion/trafficking.

6.5 Future perspectives

The modern lifestyles have imposed various demands on the biomedical sciences and calls for more personalized medical services. People are nowadays increasingly aware of the stressors surrounding them and interested in feeling

and performing better, which creates a need for more efficient tools for prognostics, diagnostics and evaluation of the overall health status. Also, the threat imposed by new as well as the return of old diseases along with rapidly spreading global epidemics, has created a demand for better and faster diagnostics and therapeutic interventions. Moreover, aging populations with deteriorating age-associated conditions, constitute both an individual and societal burden, for which solutions are needed.

Deeper understanding of the interaction between genetics and environmental lifestyle factors opens new opportunities for solving these issues. MicroRNAs can be considered key players in regulating and linking together genetics and environmental effects. They have a notable role in tissue cross-talk, delivering messages and regulating gene function. They maintain cellular homeostasis, as well as participate in various physiological processes and pathologies. The benefits of utilizing circulating microRNAs as tools in detecting changes in gene regulation patterns and as therapeutic agents are recognized. MicroRNAs are stable, especially when attached to carrier molecules or packed inside vesicles, are sensitive, and thus have the potential to reveal early changes in regulatory patterns prior to the actual effects on function, and are non-invasively detectable, relatively easy to manipulate, simple to detect and to some extent tissue-specific. However, some problems have also been raised, such as the reproducibility of microRNA experiments (Leidner, Li & Thompson 2013) and the importance of achieving a standardized workflow. The increasing number of open databases and various tools for microRNA network analyses are key in understanding the complex patterns of microRNA function and their role in health and disease. Collective research performed by the academy, industry and clinicians is also of importance to realize the full potential of microRNAs.

With the right choice of profiling mechanism (sequencing, array, single microRNA validation), microRNA carrier structure (vesicle-free or coated microRNAs), standardized sampling procedures (selection of the body fluid, timing etc.) and data analyses, microRNA profiling offers a potential minimally invasive and reliable screening mechanism with which to evaluate physiological status. In addition, specific screening could be introduced, when seeking to evaluate the effect of various treatments, such as hormonal therapies, on individuals. MicroRNAs are promising additional aids for physicians in diagnosing and evaluating the status of a patient. Their potential has also been recognized in therapeutic treatments, due to the specific binding ability of microRNAs to their mRNA targets. In theory, potentially any gene could be blocked by sequence-specific binding of the regulating microRNA. In addition, the mechanisms by which exosomes enter cells and their ability to cross even the blood-brain barrier creates potential for the utilization of artificial microRNA-packed exosomes as simple intravenous therapeutic shots, including in diseases affecting the brain.

Currently a few microRNA-based therapeutic treatments are already in the clinical phase. A miR-122 biogenesis inhibitor, Miravirsen, which has been

created for the treatment of hepatitis C virus infection, has recently entered clinical phase III (Santaris Pharma-Roche Innovation Center Copenhagen, Copenhagen, Denmark) (Ottosen et al. 2015). A potential microRNA treatment MRX34 (Mirna Therapeutics Inc., Austin, TX, USA) for liver cancer patients has reached open-label phase I in clinical trials (Bouchie 2013). In addition, several tools for cancer diagnostics, based on microRNA profile characterization, have been released (Meiri et al. 2012, Gilad et al. 2012, Gerdes et al. 2014). An ideal biomarker would help to detect the disease/condition, select the right therapy and monitor disease progression and response to the treatment. Altogether, circulating microRNAs have enormous potential for application in monitoring the health and disease stage as well as the effects of an environmental stressor.

The present study is the first to investigate the associations of serum microRNAs with menopausal status and lifetime physical training. The most promising and exciting results were obtained by sequencing serum exomiR content, which revealed health-related microRNA clusters associated with menopausal status and use of HRT. The next logical step would be to deepen understanding of exosome trafficking by determining the origin and the target tissues of these circulating exosomes. A further step would be to perform functional experiments with exosome transfections using both native and modified exosomes in various cell types, and measure the effect on mRNA and protein levels. An alternative approach to the cellular experiments would be to expose different cell types to specific stimuli and to determine the response in exosome secretion and subsequent exomiR content.

7 MAIN FINDINGS AND CONCLUSIONS

The main findings of this thesis are:

1. Several of the studied circulating microRNAs were associated with age (*miR-27b-3p, -148a-3p, -126-5p, -28-3p*, -21-5p, -146a-5p). In addition, three validated serum microRNAs (miR-21-5p, -146a-5p and *-106b-5p*) were sensitive to post-menopausal HRT among women. Adipose tissue showed high microRNA expression and changes with age. Seven microRNAs (miR-16-5p, -451a, -223-3p, -18a-5p, -19a-3p, -363-3p, and -486-5p) showed higher expression at more advanced age when pre- and postmenopausal women were compared. Sensitivity to HRT was detected in miR-19a-3p expression, but only when serum E_2 level was controlled for (owing to high variation in No HRT women, the division into the No HRT and HRT groups was not enough). Consequently, more research on miR-19a-3p is warranted. In skeletal muscle, miR-146a-5p expression was significantly higher in the postmenopausal No HRT women than in their co-twins on HRT.

2. Based on the NGS profiling, clusters of serum exomiRs indicative of healthier or unhealthier phenotypes in women were identified. ExomiR *miR-27b-3p* was positively related to body adiposity and inflammation and *miR-148a-5p* to adiposity, both microRNAs also associating negatively with serum E_2 levels. Of the freely circulating microRNAs, miR-21-5p was associated with higher whole-body adiposity in women, whereas miR-146a-5p was indicative of a healthier phenotype in both women and men.

3. Serum miR-21-5p and miR-146a-5p levels increased among the different-aged masters athletes over a 10-year period. For miR-21-5p, the increment was significant in the age groups 40 to 56 and 69 to 80 years and for miR-146a-5p in the age group 69 to 80 years. When the analysis model was completed with specific physical performance measures, the change in miR-21-5p over time was significantly associated with the change in isometric knee flexion and bench press

strength whereas miR-146a-5p was significantly associated with the change in 60-m running performance, isometric knee flexion and bench press strength.

This thesis provides substantial new findings on serum microRNAs that provide a basis for further research and development in the field. When the present issues of reproducibility, cost and standardization of microRNA analyses are resolved, and the complex network patterns and microRNA trafficking-related behavior more fully revealed, the potential of microRNAs for use as additional tools in evaluating the physiological status of an individual may begin to be realized.

YHTEENVETO (FINNISH SUMMARY)

Ikääntyminen ja mikroRNA-viestintä: yhteydet verenkierron estrogeenitasoihin sekä fyysiseen suorituskykyyn

Ikääntymiseen liittyy monia elimistön muutoksia, joita ohjailevat perimä sekä ympäristö. Epigeneettiset tekijät toimivat edellä mainittujen välillä hienosäätäen geenien toimintaa. Kudoksissa tapahtuvat geenisäätelyn muutokset heijastuvat herkästi verenkiertoon, josta säätelystä kertovat molekyylit voidaan eristää ja määrittää. MikroRNA:t ovat osa epigeneettistä säätelyjärjestelmää. Ne ovat lyhyitä RNA-molekyylejä, jotka osallistuvat geenien säätelyyn sitoutumalla kohdennetusti lähetti-RNA-molekyyleihin estäen näin proteiinien tuottumisen. MikroRNA-säätely perustuu nukleiinihapoille tyypilliseen vastinemästen tunnistukseen/sitoutumiseen ja on näin ollen hyvin tarkoin kontrolloitua. MikroRNA-molekyylejä on osoitettu löytyvän kaikista solutyypeistä ja elimistön nesteistä. Ne toimivat joko soluissa, joissa ne on tuotettu tai ne kulkeutuvat erilaisiin molekyyleihin sitoutuneina tai vesikkeleihin, kuten eksosomeihin pakattuina verenkierron mukana muualle elimistöön. Verenkierron mikroRNA:lla on tärkeä rooli solujen välisessä viestinnässä niin fysiologisissa kuin patologisissakin tilanteissa. Ne reagoivat herkästi ympäristössä tapahtuviin muutoksiin.

Tämän tutkimuksen tarkoituksena oli selvittää, muuttuvatko verenkierrosta mitattavat naisten ja miesten mikroRNA-tasot suhteessa ikään ja ikääntymiseen liittyvissä ilmiöissä. Erityisesti haluttiin tutkia, löydetäänkö estrogeenipohjaisen hormonikorvaushoidon käytön (HRT) ja fyysisen suorituskyvyn sekä verenkierron mikroRNA-tasojen välillä yhteyksiä. MikroRNA-mittauksia tehtiin sekä veri- että kudosnäytteistä.

Tässä väitöskirjatutkimuksessa käytettiin kolmea aineistoa, jotka sisälsivät eri-ikäisiä naisia ja miehiä. SAWEs -aineisto koostui vaihdevuosi-iän ohittaneista identtisistä kaksossisarista (n=11 paria, 54—62 vuotta), joista toinen oli HRT:n käyttäjä ja toinen ei ollut hoidon piirissä. Lisäksi aineistoon kuului nuoria hedelmällisessä iässä olevia naisia (n=30, 29—40 vuotta), jotka eivät käyttäneet hormonaalista ehkäisyä. MiRBody -aineisto koostui yksittäisistä vaihdevuosi-iän ohittaneista naisista, jotka vastasivat iän ja HRT:n käytön suhteen SAWEs-tutkimuksen vaihdevuosi-iän ohittaneita kaksosnaisia. ATHLAS -aineisto koostui pitkän pikajuoksuharjoittelutaustan omaavista veteraaniurheilijamiehistä (n=49, 40—80 -vuotta), jotka osallistuivat tutkimukseen vuosina 2002 ja 2012 ja nuorista pikajuoksijoista (n=18, 20—39 vuotta), jotka mitattiin vuonna 2012.

Tämä väitöskirjatutkimus pohjaa tutkittavilta otettuihin verinäytteisiin ja niistä määritettyihin hormonipitoisuuksiin, tulehdustekijöihin sekä mikroRNA-molekyyleihin. Lisäksi mikroRNA-tasoja määritettiin rasva- ja lihaskudosnäytteistä sekä veren leukosyyteistä. MikroRNA-molekyylien määrityksiin käytettiin erilaisia menetelmiä, jotka sisälsivät seerumin koko mikroRNA-kirjon analysoinnin sekä yksittäisten mikroRNA:ien kohdennettuja määrityksiä. Seerumin eksosomien mikroRNA-kirjon määrityksiin kehitettiin täysin uusi uuden suku-

polven sekvensointi analyysi-menetelmä yhdessä Turun Biolääketieteen laitoksen kanssa. Naisiin kohdistuvissa määrityksissä tutkimuksessa keskityttiin löytämään yhteyksiä ja eroavaisuuksia mikroRNA:ien sekä tulehdustekijöiden, kehon rasvaisuuden ja HRT:n käytön ja iän suhteen. Miespikajuoksijoiden analyyseissä puolestaan määritettiin fyysisen suorituskyvyn, ikääntymisen ja seerumin mikroRNA-tasojen yhteyksiä.

Tämän tutkimuksen tärkeimmät tulokset osoittivat, että seerumin mikroRNA-kirjo vaihtelee niin iän kuin hormonaalisenkin tilan mukaan. Rasvakudoksen ja seerumissa kulkeutuvien mikroRNA:ien yhteyksiä ei pystytty osoittamaan, mutta iän ja HRT:n vaikutukset näkyivät myös rasvakudoksesta mitatuissa mikroRNA-tasoissa. Myös tulehdustekijöissä ja kehon rasvakoostumuksesta kertovissa merkkiaineissa ja mikroRNA-tasoissa havaittiin yhteyksiä, minkä perusteella analysoidut mikroRNA:t kyettiin jakamaan paremmasta tai heikommasta terveydentilasta kertoviin ryhmiin. Lisäksi ATHLAS -tutkimuksessa osoitettiin, että tutkitut mikroRNA:t olivat yhteydessä nopeussuoritus- ja voimantuottokyvyn mittareihin iän karttuessa.

Eliniän odotteen kasvaessa on entistä tärkeämpää ymmärtää, mitkä tekijät edesauttavat yksilön tervettä, hyvinvoivaa ja itsenäistä ikääntymistä. Väitöskirjatutkimukseni antaa uutta tietoa iän, hormonikorvaushoidon sekä elinikäisen fyysisen harjoittelun yhteyksistä geenien säätelyyn mikroRNA-tasolla eri-ikäisillä naisilla ja miehillä. Tutkitut mikroRNA:t tarjoavat uusia mahdollisuuksia elimistön fysiologisen tilan arviointiin.

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ORIGINAL PAPERS

I

AGING AND SERUM EXOMIR CONTENT IN WOMEN -EFFECTS OF ESTROGENIC HORMONE REPLACEMENT THERAPY

by

Kangas R, Törmäkangas T, Fey V, Pursiheimo J, Miinalainen I, Alen M, Kaprio J, Sipilä S, Säämänen A-M, Kovanen V & Laakkonen EK. 2017

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Aging and serum exomiR content in women-effects of estrogenic hormone replacement therapy

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Exosomes participate in intercellular messaging by transporting bioactive lipid-, protein- and RNA-molecules and -complexes. The contents of the exosomes reflect the physiological status of an individual making exosomes promising targets for biomarker analyses. In the present study we extracted exosome microRNAs (exomiRs) from serum samples of premenopausal women (n = 8) and monozygotic postmenopausal twins (n = 10 female pairs), discordant for the use of estrogenic hormone replacement therapy (HRT), in order to see whether the age or/and the use of HRT associates with exomiR content. A total of 241 exomiRs were detected by next generation sequencing, 10 showing age, 14 HRT and 10 age +HRT -related differences. When comparing the groups, differentially expressed miRs were predicted to affect cell proliferation processes showing inactivation with younger age and HRT usage. MiR-106-5p, -148a-3p, -27-3p, -126-5p, -28-3p and -30a-5p were significantly associated with serum 17β-estradiol. MiRs formed two hierarchical clusters being indicative of positive or negative health outcomes involving associations with body composition, serum 17β-estradiol, fat-, glucose- and inflammatory markers. Circulating exomiR clusters, obtained by NGS, could be used as indicators of metabolic and inflammatory status affected by hormonal changes at menopause. Furthermore, the individual effects of HRT-usage could be evaluated based on the serum exomiR signature.

MicroRNAs (miRs) are a class of epigenetic regulators, small non-coding RNAs, which bind to their target mRNAs leading to RNA silencing and further to translational suppression. MiRs are localized in various cell types as well as in all body fluids. To date it has been shown that circulating miRs are either attached to vesicle free protein complexes, such as argonaute proteins (Ago 1–4)[1,2] or high density lipoprotein (HDL) molecules[3], localized inside the apoptotic bodies[4] or exosomes[5]. Especially exosome miRs (exomiRs) are known to be relatively stable in the circulation.

Exosomes are small (<100 nm in diameter) spherical bilayer proteolipid vesicles produced by various cell types under both normal and pathological conditions. They bud from the late endosomes in the cytoplasm forming multivesicular bodies (MVB) and either remain in the cells they are formed or are fused with the plasma membrane and exported to the extracellular matrix or further to the circulation[6]. It has been shown that exosomes consist of lipids, proteins and different sized RNA molecules including miRs[5,7]. The content of the exosomes is affected by their source and also supposedly due to the physiological condition of the exosome secreting cell. Quite recent studies have demonstrated that the messages, delivered by the secreted exosomal RNAs, can generate changes in the gene expressions and protein concentrations of the recipient cells, suggesting the functionality of the exosome content in the target cells[5]. Especially the immuno-stimulating role of the exosomes has been emphasized e.g. by showing that dendritic cells communicate via so called exosome-miR shuttles in mice[8].

Estrogens, female sex steroid hormones, have broad systemic effects on different cell types through their receptors. As known, the levels of systemic estrogens, especially 17β-estradiol (E_2), change dramatically at the

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time of menopause in women. Estrogen sensitivity of miR regulation and their expression in different cell types has been recently demonstrated in several studies[9–13]. Our previous studies have shown that circulating cell free miRs, including miR-21 and miR-146a differ between postmenopausal genetically identical twin sisters who are discordant for the estrogen based hormone replacement therapy (HRT) suggesting that systemic estrogen levels affect miR profile[14].

A very recently published study demonstrated an association between accelerated aging, measured by the epigenetic age i.e. DNA methylation status of blood, and early onset of menopause[15]. In the present study, we investigated whether the age or/and the use of postmenopausal HRT associates with the miR content of the circulating exosomes. We extracted exosomes from the serum of healthy premenopausal women (n = 8) not using external estrogen products and from healthy postmenopausal monozygotic (MZ) twin sisters discordant for the use of HRT (n = 10 pairs). Next generation sequencing (NGS) was used to analyze the exomiR content and CAP-miRSEQ pipeline together with Poisson-normal regression model to identify specific miRs with differential expression pattern. Our results indicated that specific miRs differ in terms of the age of the sample donator and HRT treatment. These miRs have been shown to function as regulators of cellular homeostasis. Hierarchical clustering analysis revealed two miR-clusters, the other indicating negative association with E_2 parallel to positive association with markers of adiposity and inflammation and the other being opposite in the associations with E_2 and metabolic health markers. These results suggest that female E_2 status may be a mediator of the change in exomiR content that in turn is associated with change in health metabolic status known to occur within menopause.

Results

Participant characteristics. Participant anthropometrics and measured blood characteristics as well as group differences are presented in the Table 1. All groups differed significantly from each other in serum E_2 and follicle stimulating hormone (FSH) levels those being highest (E_2) and lowest (FSH) among premenopausal women as expected. Waist circumference was significantly lower among premenopausal women compared to both postmenopausal groups. Fat percentage was significantly lower in postmenopausal HRT users compared to their co-twins. Serum TNF-α and plasma glucose concentrations were also significantly lower among premenopausal women in comparison to postmenopausal women.

ExomiR profile and differentially expressed miRs in NGS. The functionality of the used exosome extraction method was demonstrated by electron microscope imaging (Fig. 1, a representative image obtained from the serum exosomes of a postmenopausal HRT user). Most of the detected lipid vesicles were in normal range for exosomes being under 100 nm in diameter. The exosome quantity, measured by esterase activity, showed no differences between the studied groups (Pre = 17566 ± 1196 RFU/μg, HRT = 17876 ± 5801 RFU/μg, No HRT = 16303 ± 4370 RFU/μg, P = 0.610–0.925). Altogether, 241 different known miRs were detected in the serum exosomes by NGS. The processed sequencing reads are presented in Supplementary data (S1). The most abundant miRs of the exosome cargo include miR-486-5p, -92a-3p, -16-5p, -451a, -22-3p and -423-5p together covering up to 84.6% of the exomiR content (Fig. 2).

The miR read counts from the differentially expressed miRs are presented in Table 2. Altogether, 21 miRs had significant differential expression pattern (p-value with false discovery rate (FDR) correction, $P < 0.05$) in NGS either in one, two or three group comparisons (postmenopausal No HRT group vs. premenopausal group, postmenopausal HRT-user group vs. premenopausal group or postmenopausal HRT-users vs. postmenopausal non-using co-twins). MiR-126-5p, -142-5p, -484 and -10b-5p were differentially expressed between all the studied groups *(P < 0.001)* showing age- and HRT-use associated differences. MiR-27b-3p, -10a-5p, -215-5p and -144-5p were differentially expressed between both postmenopausal groups vs premenopausal women showing age-associated differences with or without HRT ($P < 0.001$). MiR-148-3p and -28-3p were differentially expressed between postmenopausal No HRT group vs premenopausal women (*P = 0.008, P = 0.009, respectively*) and miR-375 and -186-5p between postmenopausal HRT group vs premenopausal women (*P = 0.045, P < 0.001, respectively*). MiR-532-5p, -1285-3p, -30a-5p, -3688-3p, -29b-3p, -106b-5p, -29c-3p, -1306-5p, -148a-3p and -301a were differentially expressed between HRT users and their non-using co-twins suggesting association between HRT-use and miR expression (*P < 0.001, P < 0.001, P = 0.001, P = 0.016, P = 0.029, P = 0.029, P = 0.033, P = 0. 036, P = 0.049, P = 0.049, respectively).*

Functional network analysis. All sequenced miRs were included in the analyses to identify the most prominent networks by explorative comparison analyses by Ingenuity pathway tools (IPA) (Fig. 3). No cut-offs for p-values were used. The age comparison between postmenopausal women without HRT and premenopausal women suggested that estrogen receptors (ESRs) and insulin are predicted to be the biggest upstream regulators of the miRs in the network. Six of the differentially expressed miRs (FDR <0.05) listed in Table 2 were involved in the network and highlighted in the figure. The comparison between postmenopausal HRT users and premenopausal women involved also ESRs and insulin as upstream regulators but only two of the differentially expressed miRs, miR-27b-3p and miR-126-5p. The comparison between the postmenopausal twin sisters involved five of the differentially expressed miRs out of which four are predicted to be regulated by insulin. In addition, miR-106b-5p was predicted to have VEGF and SMAD6/7 as upstream regulators and miR-30a-5p chorionic gonadotropin (Cg).

To focus on the most significant miRs, comparison analysis was performed to differentially expressed miRs (cut-off $P < 0.05$) by IPA, including all the three group comparisons (Fig. 4). Cell proliferation was the most significant downstream target process predicted to be affected in all the comparisons. Comparisons between premenopausal and both postmenopausal groups included the same miRs (miR-27b-3p, -215-5p, -126-5p and -10a-5p), except for miR-148a-3p which was included only in the premenopausal vs postmenopausal No HRT

	PREMENOPAUSAL (N = 8)	POSTMENOPAUSAL NO HRT (N = 10)	POSTMENOPAUSAL HRT (N = 10)	SIGNIFICANT COMPARISON (P < 0.05)
Age (yrs)	32.0 ± 1.6	57.5 ± 2.0	57.5 ± 2.0	a, b
Height (cm)	164.5 ± 4.0	162.2 ± 4.5	162.8 ± 4.7	
Weight (kg)	70.0 ± 11.3	74.7 ± 15.6	67.8 ± 9.1	
BMI	25.9 ± 4.5	28.6 ± 6.8	25.7 ± 4.0	
Waist (cm)	86.4 ± 10.4	104.7 ± 9.6	100.9 ± 6.4	a, b
Fat %	29.7 ± 7.0	35.3 ± 9.3	30.5 ± 7.5	c
hsCRP (mg/l)	0.88 ± 1.1	1.53 ± 0.94	1.09 ± 0.95	
IL-10 (pg/ml)	0.89 ± 1.7	2.50 ± 3.70	2.43 ± 3.17	
IL-6 (pg/ml)	1.05 ± 0.70	1.77 ± 1.29	1.39 ± 0.71	
TNFa (pg/ml)	6.1 ± 1.6	10.5 ± 2.3	10.7 ± 4.8	a, b
WBC (e9/l)	6.1 ± 1.2	5.2 ± 1.4	5.3 ± 1.3	
RBC (e12/l)	4.5 ± 0.2	4.3 ± 0.3	4.4 ± 0.3	
HGB (g/l)	139 ± 5.0	137 ± 9.4	140 ± 9.1	
Plt (e9/l)	242.8 ± 54.2	233.9 ± 39.1	236.8 ± 83.4	
Chol (mmol/l)	4.7 ± 0.8	5.3 ± 0.4	5.3 ± 0.7	
LDL (mmol/l)	2.7 ± 0.8	3.1 ± 0.4	3.1 ± 0.8	
HDL (mmol/l)	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.5	
TriGly (mmol/l)	0.8 ± 0.2	1.2 ± 0.5	1.2 ± 1.0	
Adiponectin (ng/ml)	7432 ± 3606	9382 ± 5273	10267 ± 5350	
Leptin (pg/ml)	23225 ± 10977	18571 ± 13705	12483 ± 8672	
Resistin (ng/ml)	9.4 ± 2.0	10.2 ± 1.2	9.7 ± 1.4	
Ins (mmol/l)	5.3 ± 3.3	8.0 ± 4.5	6.2 ± 3.6	
Gluc (mmol/l, plasma)	4.2 ± 0.4	5.2 ± 0.7	5.0 ± 0.7	a, b
E2 (pmol/l)	496.5 ± 311.9	34.8 ± 28.4	183.0 ± 211.3	a[#], b[#], c[#]
E1 (pmol/l)	369.4 ± 195.3	100.9 ± 26.0	962.0 ± 1517.8	a[#], c[#]
T (nmol/l)	1.02 ± 0.30	0.66 ± 0.27	0.74 ± 0.31	a, c
SHGB (nmol/l)	49.3 ± 17.6	44.1 ± 11.6	72.3 ± 31.1	c
LH (IUl)	12.8 ± 13.2	39.9 ± 30.2	31.6 ± 17.3	a[#], b[#]
FSH (IUl)	6.22 ± 2.85	83.7 ± 32.7	57.0 ± 31.2	a, b, c

Table 1. Participant body anthropometrics and blood characteristics and differences between the studied groups. Significant comparisons between the studied groups are marked with letters a) Pre vs post No HRT b) Pre vs post HRT c) Post HRT vs post No HRT. Independent samples T-test was used for a and b comparisons and paired samples T-test for c comparisons when variables were parametric. [#]Mann Whitney U test (a and b) and Wilcoxon matched pair signed-rank test (c) was used for non-parametric variables. P < 0.05 was considered significant. Results are shown as mean ± S.D. BMI: body mass index, hsCRP: high sensitive c-reactive protein, IL-10: interleukin 10, IL-6: interleukin 6, TNFa: tumor necrosis factor alpha WBC: white blood cell count, RBC: red blood cell count, HGB: hemoglobin, Plt: platelet, Chol: cholesterol, LDL: low density lipoprotein, HDL: high density lipoprotein, TriGly: triglycerides, E2: 17β-estradiol, E1: estrone, T: testosterone, SHGB: sex hormone-binding globulin, LH: lutenizing hormone, FSH: follicle stimulating hormone.

group network. Higher age (or postmenopausal status) was predicted to up-regulate the cell proliferation pathway whether using HRT or not. Other set of miRs (miR-29b-3p, -106b-5p, -142-5p, and -1285-3p) were shown to have an effect when comparing the postmenopausal co-twins with each other. In this comparison, the use of HRT was predicted to down-regulate the cell proliferation pathway. Figure 4 shows the main networks for differentially expressed miRs identified by IPA with its annotation as the top diseases and functions and the interacting molecules i.e., potential miR targets as well as miRs included in the network.

Associations of NGS reads with serum E_2 and other variables. The associations of the differentially expressed miRs with serum E_2 concentration, body composition and fat-, glucose- and inflammation related markers are visualized as a correlation heatmap (Fig. 5). The p-values, FDR-values and correlation coefficients are presented in Supplementary data (S2). Heatmap reveals hierarchically clustered information about the associations of the selected miRs with other measured variables. First of all, six miRs from the differentially expressed NGS results were significantly (FDR < 0.05) associated with serum E_2. MiR-106b-5p was positively associated with serum E_2 concentration, while other miRs, miR-148a-3p, -27-3p, -126-5p, -28-3p and -30a-5p were negatively associated with serum E_2 concentration. In addition, miR-144-5p had indicatively significant positive association and miR-10b-5p indicatively significant negative association with E_2 concentration (P = 0.025, FDR = 0.066 for both associations). Although only the six mentioned miRs possessed significant association with FDR < 0.05, it is clear from the heatmap that the miRs under investigation form two separate clusters the other cluster being indicative of positive and the other of negative health outcomes.

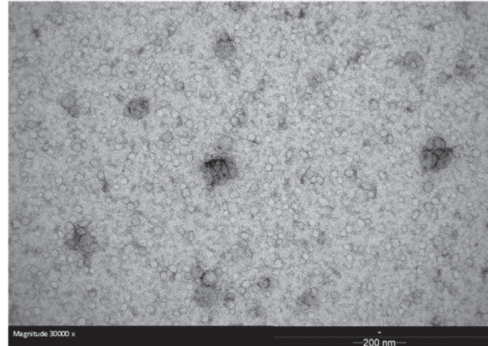


Figure 1. Electron microscope image of the extracted serum vesicles. Typical size of an exosome is less than 100 nm in diameter. A Tecnai G2 Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands).

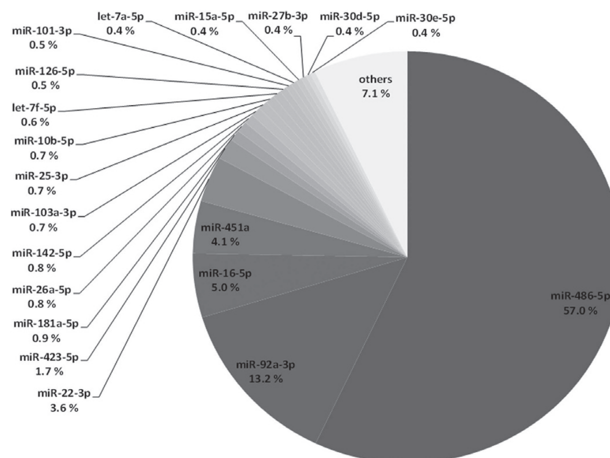


Figure 2. Relative serum exomiR content of all the samples.

The positive health outcome cluster miRs had positive associations with E_2 and the negative health outcome cluster miRs had negative associations with E_2 and concomitant negative and positive associations, respectively, with most of the adiposity and inflammation related variables except that with HDL and adiponectin which were clustered together with E_2 concentration. However, the associations with miRs and HDL count were only indicatively significant for miR-148a-3p and -27b-3p with HDL ($P = 0.009$, $FDR = 0.126$ and $P = 0.012$, $FDR = 0.126$, respectively) and for miR-27b-3p with adiponectin ($P = 0.013$, $FDR = 0.273$) while for the other miRs the associations were non-significant. The leading miRs for the negative health outcome cluster were miR-27b-3p, which had the highest number of significant associations and miR-148a-3p, which had very similar profile although some of the associations were only nominally significant. The higher NGS read count value of miR-27b-3p was significantly associated ($FDR < 0.05$) with greater adiposity (fat percentage, BMI, waist circumference, triglyceride concentration), with higher fasting glucose and insulin and with higher hsCRP, resistin and TNF-α ($P = 0.014$, $FDR = 0.095$) together with lower E_2 concentration. The positive health outcome cluster did not have as prominent leading miR, but the miRs having positive association with E_2, miR-106b-5p and miR-144-5p, had also negative association with TNF-α ($P = 0.027$, $FDR = 0.099$ and $P = 0.008$, $FDR = 0.084$, respectively). In addition, miR-106b-5p, -532-5p and -484 had statistically significant ($FDR < 0.05$) negative associations with resistin.

Validation of differentially expressed sequenced miRs. qPCR validations of the E_2-associated miRs are presented in Fig. 6. For miR-27b-3p, -148a-3p and -126-5p the levels were significantly lower in premenopausal women compared to postmenopausal No HRT group ($P = 0.001$, $P = 0.001$, $P = 0.040$, respectively) and

miR	Pre	Mean count		Overdispersion	Comparison (p-value, FDR)		
		No HRT	HRT		No HRT vs. Pre	HRT vs. Pre	HRT vs. No HRT
miR-126-5p	3942	5822	5601	0.002	<0.001↑	<0.001↑	<0.001↓
miR-142-5p	10591	6254	6669	0.007	<0.001↓	<0.001↓	<0.001↑
miR-484	2106	1201	1471	0.029	<0.001↓	<0.001↓	<0.001↑
miR-10b-5p	8535	6958	5323	0.007	<0.001↓	<0.001↓	<0.001↓
miR-532-5p	395	263	356	0.102	0.184	1	<0.001↑
miR-1285-3p	72	40	97	1.28	1	1	<0.001↑
miR-30a-5p	1121	1880	1485	0.101	0.065	1	0.001↓
miR-3688-3p	6	4	10	6.248	1	1	0.016↑
miR-29b-3p	0	6	17	6.436	0.934	0.494	0.029↑
miR-106b-5p	399	219	333	0.155	0.118	1	0.029↑
miR-29c-3p	174	154	209	0.201	1	1	0.033↑
miR-1306-5p	4	2	5	4.692	1	1	0.036↑
miR-148a-3p	2095	3863	2814	0.094	0.008↑	1	0.049↓
miR-301a-3p	12	38	22	2.061	1	1	0.049↓
miR-375	1056	629	329	0.482	1	0.045↓	0.115
miR-28-3p	319	607	443	0.103	0.009↑	1	0.275
miR-27b-3p	3307	8419	7025	0.034	<0.001↑	<0.001↑	0.325
miR-10a-5p	3958	7682	6252	0.029	<0.001↑	<0.001↑	0.671
miR-215-5p	190	1010	592	0.151	<0.001↑	<0.001↑	0.671
miR-144-5p	585	375	433	0.034	<0.001↓	<0.001↓	1
miR-186-5p	2056	2362	2598	0.026	0.063	<0.001↑	1

Table 2. Read counts of the differentially expressed miRs based on sequencing results. The average read counts of the studied groups are presented on the left (Pre: premenopausal women, HRT: postmenopausal hormone replacement therapy users, No HRT: postmenopausal non-users). FDR corrected group comparisons are presented on the right. Arrow head pointing up indicates upregulation and arrow head pointing down downregulation in the first group of the comparison. Overdispersion is a measure of a greater variability.

HRT group (P = 0.005, P = 0.040, P = 0.005, respectively). MiR-106b-5p was significantly lower in premenopausal women compared to postmenopausal HRT group (P = 0.040) and lower in No HRT women compared to their HRT co-twins (P = 0.043). No differences between any groups were detected for miR-28-3p and -30a-5p (data not shown).

Discussion

This is the first study showing that age and the use of HRT are associated with the miR contents of the circulating exosomes in women. Altogether, 241 distinct known serum exomiRs were detected by NGS among the studied groups from which 21 were differentially expressed. The NGS results showed that exomiR -levels differ between all of the three studied groups significantly in cases of four miRs. In addition, age-associated difference without HRT was detected in six miRs (postmenopausal No HRT vs premenopausal), and with HRT in ten miRs (postmenopausal HRT vs premenopausal) as well as treatment difference in ten miRs (postmenopausal HRT vs No HRT).

Menopause is recognized as a risk factor for the development of metabolic dysfunctions[16,17]. The loss of circulating E_2 is followed by androgen dominance and further by body fat accumulation around the abdominal area[18]. The excess amount of adipose tissue leads to unbalanced cytokine profile which is seen as increased levels of IL-6, TNF-α, IL-1β, resistin and leptin among other inflammatory molecules[19]. These circulating pro-inflammatory cytokines form a base for systemic low-grade inflammation also known to be associated with aging[20]. In addition to adipose tissue initiated release of pro-inflammatory cytokines, also issues in glucose metabolism, such as insulin resistance play a role in the systemic inflammation[21]. Furthermore, endothelic dysfunction and atherosclerosis are adding to the chronic inflammatory state[22]. In our study the premenopausal women had healthier profile in terms of metabolic measures such as waist circumference and plasma glucose levels compared to postmenopausal women. In addition, fat percentage was significantly higher among the postmenopausal women who were not under HRT compared to their co-twins under HRT. We were able to see a slight increase in TNF-α levels due to age, whether under HRT or not, but no significant differences in other classical pro-inflammatory markers between any of the studied groups. However, across the measured inflammatory markers a trend towards the most unbeneficial state was detected among postmenopausal women, who were not under HRT. Clear expected differences were detected in systemic steroid hormone levels as well as gonadotropins, from which high FSH levels are indicators of postmenopausal state. In addition, interesting associations with serum E_2 concentrations and differentially expressed miRs, inflammation, glucose metabolism and adiposity -related markers were found.

The present study revealed six different miRs which were associated (FDR < 0.05) with serum E_2 levels either positively (miR-106b-5p) or negatively (miR-148a-3p, -27-3p, -126-5p, -28-3p and -30a-5p). In addition, two miRs had indicatively positive (miR-144-5p, P < 0.05) and negative (miR-10b-6p, P < 0.05) association with

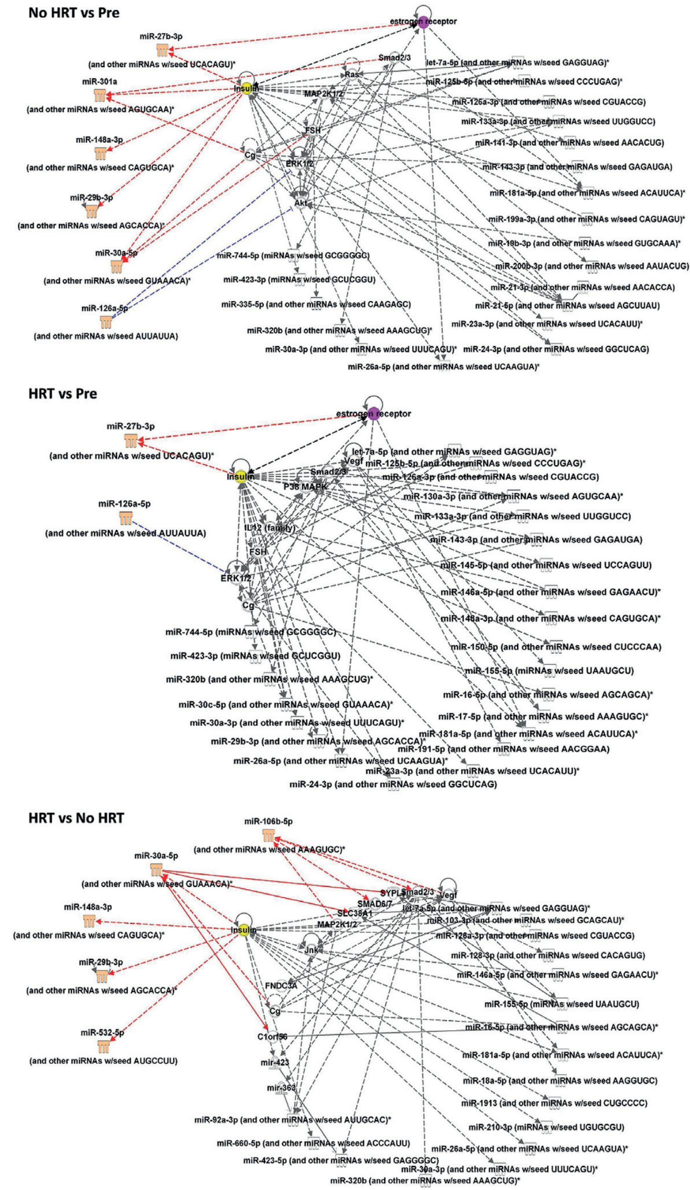
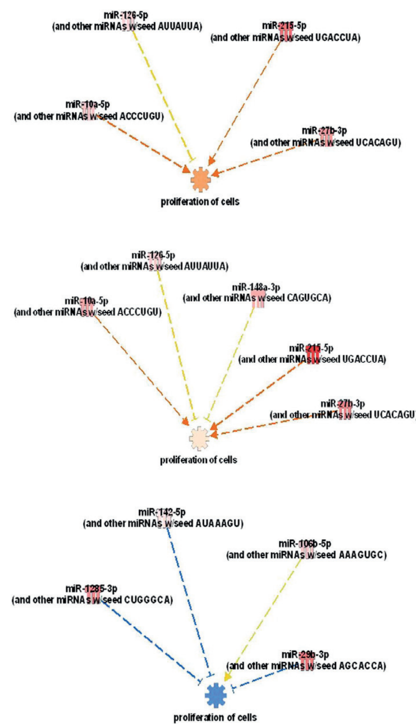


Figure 3. The most prominent network from IPA comparison analyses performed to all sequenced miRs. Differentially expressed miRs (FDR) are highlighted in the figure with coral colour. The arrow head of the red line tells the direction of the predicted affect. Blue line represents the predicted inhibition. Insulin is highlighted in yellow and ESR in pink. Smad2/3: Smad family member 2 or 3, Ras: protein superfamily of small GTPases, MAPK2K1/2: Mitogen activated protein kinase 2K1 or 2, FSH: Follicle stimulating hormone, Cg: Chorionic gonadotrophin, VEGF: Vascular endothelial growth factor, IL12: Interleukin 12, ERK1/2: Extracellular signal regulated kinase 1 or 2, SYPL1: Synaptophysin like 1. SLC38A1: Solute carrier family 38 member 1, Clorf56: Chromosome 1 open reading frame 56 solute, Jnk: Jun kinase, FNDC3A: fibronectin type III domain containing 3A.



Comparison	Diseases and functions	RNA targets in the pathways
HRT vs Pre	Cancer, Organismal injury and abnormalities, Reproductive system diseases	ADAM9, ADORA2B, BIRC5, BRAF, CD44, DDX20, DHFR, DROSCHA, DTL, E2F2, EFNB2, EPHB6, ERK1/2, FOXO1, PHB, PMAIP1, PPP2R2C, ST14, TNFSF12, TP53, TYMS, WEE1, ZEB1, ZEB2
No HRT vs Pre	Cellular development, Cellular growth and proliferation, Cancer	ADAM9, BBC3, BIRC5, BRAF, CYP2A6, EFNB2, EPHB6, ERK1/2, IGF1, IGF1R, MMP13, ODC1, PDPK1, PHB, PPP2R2C, RPS6KA5, ST14, TNFSF12, TYMS, WEE1, ZEB1,
HRT vs No HRT	Cancer, Organismal injury and abnormalities, Reproductive system diseases	ACVR2A, CAV2, CCNE1, CDH1, COL15A1, COL4A1, COL5A2, DNMT3A, DUSP2, FAM3C, FBN1, GRHL3, LOXL2, MYBL2, MYC, NF2, PMP22, PPIC, PPM1D, PURA, Rb, TCL1A, TDG, TP53, Tpm1, ZFP36L1,

Figure 4. IPA comparison analysis of the 3 different comparisons for differentially expressed miRs. The figure shows miRs in each comparison predicted to regulate cell proliferation. Red color indicates activation, blue inhibition and yellow controversial findings of the specific miR. Younger age and the use of HRT were predicted to inhibit the cell proliferation. The table shows the main networks for differentially ex-pressed miRs identified by IPA with its annotation as the top diseases and functions and the interacting molecules i.e., potential miR targets as well as miRs included in the network. The underlined miR targets are also involved in cell proliferation (figure).

serum E_2. To our knowledge two of these miRs have been previously shown to have an association with E_2 in breast cancer cells. Nassa *et al.*[23] have demonstrated that miR-30a-5p is regulated by estrogen receptor β (ESRβ) whereas Tao *et al.*[24] demonstrated that miR-148a is downregulated by E_2 through GPER in breast cancer cells. In addition, 17-β-estradiol–ESRα–miR-27b -connection has been demonstrated on human leukemia cell line[25]. Our NGS data indicated significant differences between postmenopausal twins for miR-30a-5p and between all the studied groups for miR-148a-3p and miR-27b-3p. All miRs had a negative association with serum E_2 which is in line with the mentioned studies.

Based on recent publications, some of the E_2 associated miRs identified in the present study have connections to aging. MiR-10b-5p has been shown to be associated with the motor onset in both Parkinson's and Huntington's diseases[26] indicating its function in the development of age-associated neurodegenerative disorders. Also, the role of circulating miR-10b-5p in osteogenic differentiation after fracture at postmenopausal age has been recognized[27]. In addition, changes in miR-28-3p levels during early senescence were observed in endothelial cells, thus, indicating miR's possible regulatory role in the aging of vascular endothelium. Even though no direct effect of E_2 has been stated in the mentioned studies, it has been well demonstrated that estrogen has a role as a neuro-, osteo- and vascular protector[28–31]. Our earlier studies have also shown beneficial effects of postmenopausal HRT on body composition measures, including fat, muscle and bone properties[32–36]. The connection between lipoproteins, vascular inflammation and exosomes has also been recognized by others[37]. MiR-27b-3p has been shown to regulate fat metabolism and inflammation by targeting RXRα and PPARγ[38,39]. In the present study, mir-27b-3p was one of the most interesting single miRs belonging to the negative outcome cluster. The NGS data indicated higher levels of mir-27b-3p in the circulating exosomes of the postmenopausal twins compared to premenopausal

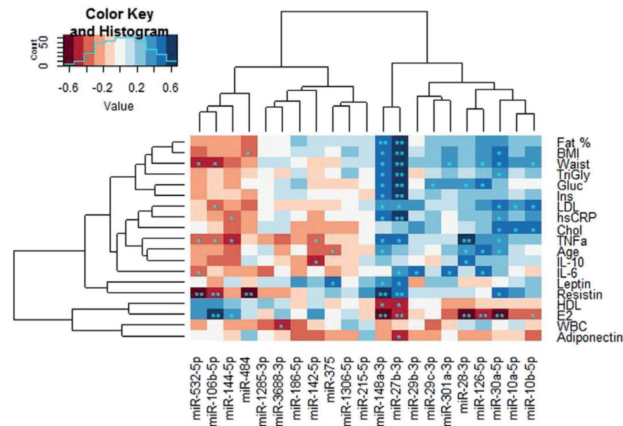


Figure 5. Clustered heatmap of the selected miRs and other measured variables. Blue indicates positive and red negative associations. Spearman correlation coefficient was used for the analyses. *P < 0.05 (nominal), **P < 0.05 (FDR corrected). Positive health outcome cluster is on the left side with positive associations with E2 and negative health outcome cluster on the right side with negative E2 associations. BMI: body mass index, TriGly: triglycerides Gluc: plasma glucose, Ins: serum insulin, hsCRP: high sensitive c-reactive protein, IL-6: interleukin 6, LDL: low densi-ty lipoprotein, TNFa: tumour necrosis factor alpha, IL-10: interleukin 10, Chol: cholesterol, WBC: white blood cell count, HDL: high density lipoprotein, E2: 17β-estradiol. RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

women indicating an age association. This miR was negatively associated with serum E_2, HDL and adiponectin (last 2 nominal) whereas positive associations were detected with other adiposity markers such as fat percentage and triglycerides and nominally with LDL. In addition, the levels of miR-27b-3p were positively associated with serum hsCRP and resistin as well as insulin and plasma glucose concentrations, all of these markers indicative of unbeneficial metabolic and inflammatory status. The associations for miR-148a-3p were similar to miR-27b-3p but only nominally significant in some cases. Altogether, these results support the function of miR-27b-3p (and miR-148a-3p) in the regulation of lipid and glucose metabolism and further reveal its possible negative role in women's aging with hormonal changes.

Different miR carrier systems share a bunch of common miRs but they also seem to have a unique content which differs from the other miR vehicles and, also, from the cell of origin[40]. Exosomes have been shown to deliver gene-based intercellular messages adding to the complexity of cell-cell communication. According to Cheng *et al.*[41], exosomes provide a protected and enriched environment for miRs compared to the intracellular and other cell free miRs. In their study, exosome derived miRs were associated with neuronal signaling whereas the main responsibility of the cell free miRs was the signaling related to the cellular homeostasis, emphasizing the functional differences between cell free miRs and exomiRs. In our previous studies, we have shown that the circulating cell free levels of miR-21 and miR-146a differ between the HRT users and their non-using co-twins[14]. In the present study, we were able to detect the same miRs in the exosomes by NGS, however, significant differences were not obtained. That may be considered as a supporting indicator of the differential roles of the two types of miR transfer. The mechanism how the exomiRs are sorted into the exported exosomes is an unanswered question. It is under active speculation whether the sorting takes place by the miR sequence or whether the exomiR content is a description of the whole miR pool of the exosome forming cell. It is important to recognize that the sequencing data can be interpreted by several ways: one could either focus on the profile as a whole and compare the relative amounts and changes in the relative patterns, or the other option is to focus on specific miRs. Validation of specific miRs is not inevitably the whole truth as very often miRs work as a group fine-tuning each other's function. In the present study, qPCR validation of the six E_2-associated miRs confirmed the NGS results fully for miR-27b-3p and miR-126-5p and partly for miR-148a-3p and miR-106b-3p, however, no differences for miR-28-3p and -30a-5p were detected.

According to the functional network analyses obtained by IPA, the most prominent predicted upstream regulators of the studied miRs include insulin as well as estrogen receptors. These results support the link between the systemic E_2 levels, insulin signaling and the studied miRs. In the comparison analyses performed using only the differentially expressed miRs, the cell proliferation processes were predicted to be inactivated at younger age as well as due to the use of HRT. Interestingly, different set of miRs seem to regulate cell proliferation processes depending on whether comparing only postmenopausal twins or premenopausal and postmenopausal women. Cell proliferation is usually considered to be more active at younger age. However, as we do not know the cell origin or destination of the exomiRs, we cannot say much about the specific processes and cells under the influence. Based on the present findings, the predicted activation of cell proliferation especially among the postmenopausal

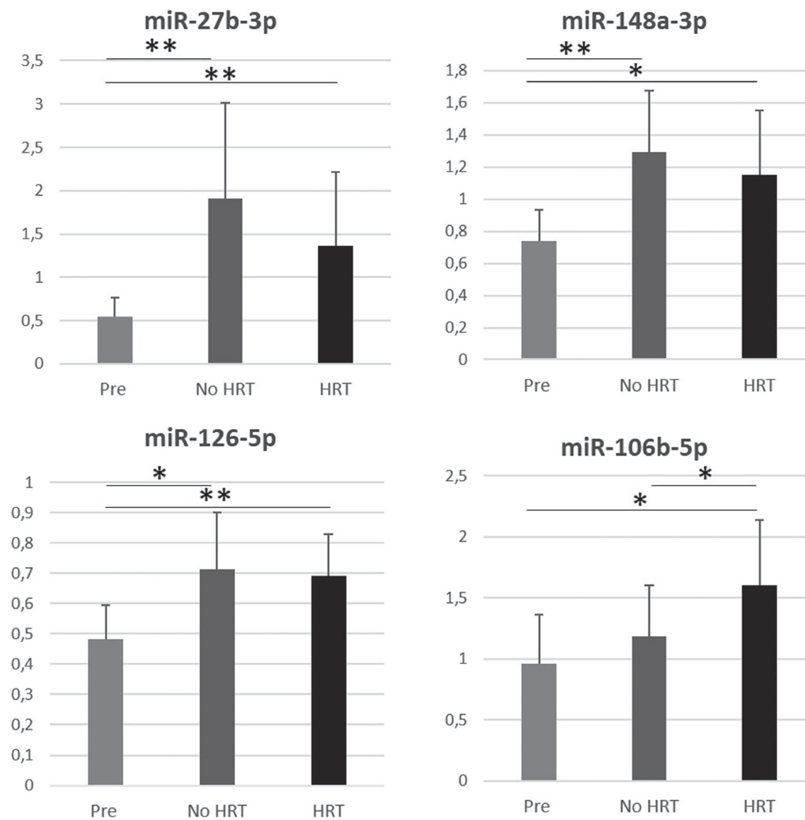


Figure 6. qPCR validation of the miRs associated with systemic E2. Pre: Premenopausal women, No HRT: Postmenopausal women without hormone replacement therapy, HRT: Postmenopausal women using HRT. *P < 0.05, **P < 0.01. Results are presented as mean relative ex-pressions +SD.

women without HRT, could be related to increasing amounts of adiposity, inflammatory cells or possibly tumor cells, all related to natural aging. These results suggest that the postmenopausal HRT users are more similar to premenopausal women in terms of miR regulated cell proliferation than postmenopausal non-users. Based on this genetically controlled study, these effects are achieved to most part by the HRT usage. The study has been performed with a small unique population and thus cannot be generalized directly to female population as a whole. However, as a MZ twin study, it provides a valuable genetically controlled design. Further cell culture experiments are needed in order to say more about the specific functions of HRT sensitive exomiRs on specific cell types.

The recent achieved technologies in molecular biology, including NGS, provide promising tools to discover the broad functions of miRs in both physiological and pathological conditions. To our knowledge, the present study was the first to show a detailed serum exomiR profile of women with different age and hormonal status. We demonstrated that the differential expression patterns were emphasized among exomiRs interplaying with cellular homeostasis, glucose- and lipid metabolism as well as inflammation. Furthermore, we were able to identify miR groups related to more positive or negative health outcomes in which the systemic E_2 concentration played a significant role as a divider. Our findings suggest that the serum exomiRs are sensitive to hormonal changes among women and carry important regulatory messages between cells. The results can be used as a directional starting point for the usage of miR signature, obtained by NGS, as a medical tool for prognostics and diagnostics in aging women. In addition, the miR signature can potentially be applied when evaluating the benefits of HRT usage and its individual suitability in personalized medicine.

Materials and Methods

Study design. The present 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)". A

more detailed design and the recruitment process of the SAWEs- study has been described previously[42,43]. Briefly, the study participants were recruited from the Finnish Twin Cohort (n = 13888 pairs)[44]. The invitation was sent only to women born in 1943-1952 (n = 537 pairs). To be able to take part in the study, both co-sisters needed to participate. This study included 15 MZ female twin pairs who had no contraindications, were discordant for the use of HRT (mean duration of HRT use 6.9 ± 4.1) and were willing to participate to the study. Five of the HRT users were using preparations containing only E_2 (1-2 mg), six used estrogenic (1–2 mg)+ progestogenic compounds and four tibolone (2.5 mg) based treatment. Since the aim of the present study was to investigate the effects of E_2 based HRT, tibolone based HRT users and their co-twins were excluded. Finally, 11 MZ twin pairs were included in the present study, however, the NGS read quality of one sample was not sufficient enough thus, this twin pair was excluded from the analysis. The number of participants is relatively small but comparable with other MZ co-twin studies[45–47] and has enough statistical power to obtain clinically relevant results. No significant differences in physical activity levels (slightly modified Grimby scale[48]), daily energy intake (5-day diary) or smoking habits between the twins were identified. In addition, eight women aged 30 to 40 years with no use of hormonal contraceptives during the last 5 years, were included for the present study representing a premenopausal group. Contraindications for participation were chronic musculoskeletal diseases, type 1 or 2 diabetes, mental disorders, asthma with oral glucocorticosteroid treatment, cancer, drug or alcohol abuse, and Crohn's disease. High blood pressure was the only condition to which daily medication was used (HRT: n = 3, No HRT: n = 4). Other occasional medication included antihistamines, paracetamol and ibuprofein. Overall, the participants were considered as healthy women.

The study was conducted according to the guidelines of the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Central Finland Hospital District (E0606/06). Written informed consent was provided by the study participants prior the laboratory measurements.

Serum analyses. Whole blood samples were collected under standard fasting conditions from antecubital vein in a supine position. The samples of the premenopausal women were collected during the first five days of the menstrual cycle representing the lowest E_2 concentrations. The blood was allowed to clot for 30 mins in room temperature followed by serum separation by centrifugation at 4000 rpm. All the samples were snap frozen and stored in −70 °C in 0.5 ml aliquots.

Exosome isolation, imaging and RNA extraction. Serum exosomes were isolated from 450 μl of sample by using Exoquick Exosome Precipitation Solution according to manufacturer's protocol (#EXOQ5A-1, System Biosciences).

Electron microscopy was used for checking the size of the extracted vesicles. Briefly, the isolated exosomes were deposited on Formvar carbon-coated, glow-discharged grids. After 20 minutes, the grids were washed with PBS and exosomes were fixed in 1% glutaraldehyde for 5 minutes. After washing with distilled water the grids were stained with neutral uranylacetate and embedded in methylcellulose/uranyl acetate and examined in a Tecnai G2 Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands). Images were captured by Quemesa CCD camera using iTEM software (Olympus Soft Imaging Solutions GMBH, Munster, Germany). FLUOROCET Ultrasensitive Exosome Quantitation Assay Kit (System BioSciences), measuring exosome esterase activity, was used to assess the exosome quantity in samples (4 per each studied group) according to manufacturer's protocol. Briefly, the protein concentration of exosome-PBS -solution was measured using BCA protein method by Nanodrop (1000) using wavelength 562 nm. Equal volume of exosome preparation was loaded into each 96-well plate. Promega Glomax Multi + Detection system with fluorescence module was used for the measurement (excitation = 525 nm, emission = 580 to 640 nm). Results are presented as relative fluorescence unit (RFU) per total protein content of the sample (RFU/μg).

Total RNA extraction was performed by using Trisure reagent (Bioline) according to manufacturers' instructions with slight modifications. Additional step was added to the homogenization step where 7 μl of synthetic cel-miR-39 miR mimic ($1,6 \times 10^8$ copies/μl, Qiagen cat. no 219610) was added to each sample to serve as a spike-in control for monitoring the miR purification and amplification. Chloroform was used for the phase separation and 1 μl of nuclease free glycogen (Glycogen RNA Grade, 20 mg/ml, Fermentas) for enhancing the RNA precipitation. RNA concentration was measured by Nanodrop 1000 (Thermo Scientific). Prior the library preparation RNA quality and recovery was checked by qPCR according to manufacturer's protocol (Qiagen miScript Primer assays and II RT kit for cDNA synthesis and MiScript SYBR Green PCR Kit for RT-qPCR) from which the recovery of cel-miR-39 spike-in control ($Ct_{mean} = 24.9 \pm 1.0$) and miR-21-5p ($Ct_{mean} = 27.3 \pm 1.3$) was verified.

cDNA library preparation and small RNA sequencing. The small RNA libraries were prepared using TruSeq Small RNA Sample Preparation Kit (Illumina, USA) with multiplexing adapters. Following the TruSeq Small RNA Sample Preparation Kit user guide (Rev. E), the total RNA, including the small RNA fractions, were ligated to 5′ and 3′ adaptors sequentially before converted to cDNA by reverse transcription. cDNAs were amplified with PCR by using primers containing unique six base index sequences distinguishing different samples from one another. Finally, the samples were subjected to 6% (w/v) non-denaturing polyacrylamide gel electrophoresis (PAGE). cDNA library fragments between 145 and 160 bp corresponding the miR libraries were excised from the gel, purified and eluted. The final miR library pellet was air dried and resuspended in 10 μL nuclease-free water and quantity of the libraries was measured with Qubit fluorometer. Ready miR library pools (8–16 samples on single pool) were loaded to MiSeq V3 flow cell in 12 pM concentrations. To increase signal integrity, 10% of PhiX was spiked in the library pool. MiR libraries were sequenced with MiSeq reagent kit V3 150 cycles using 36 bp reads with single-end chemistry. Three replicates were used in each NGS run.

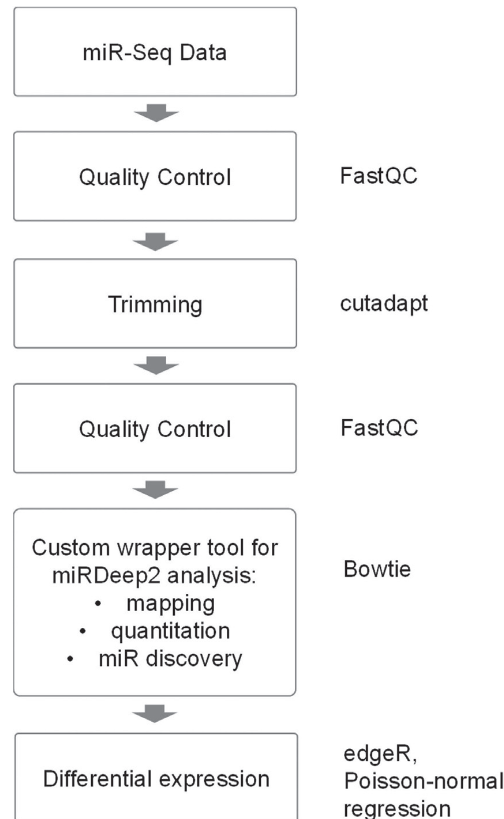


Figure 7. Workflow of the handling of the sequencing data. All the methods and R packages used in the analyses are presented in the figure.

miR validation. The miRs having differential expression in NGS results and significant association with systemic E_2 (FDR < 0.05, Fig. 5) were validated with RT-qPCR. These miRs included miR-27b-3p, -148a-3p, -126-5p, -28-3p, -30a-5p and -106b-5p. Validation was performed using samples from five postmenopausal twin pairs and ten premenopausal women. MiScript II RT Kit was used for cDNA synthesis (Qiagen) according to manufacturer's protocol. cDNA was diluted 1:3 for the RT-qPCR which was performed using miScript SYBR Green PCR Kit and miScript Primer assays (Qiagen: Cat. No. MS00031668, MS00003556, MS00006636, MS00009254, MS00007350, MS00003402, MS00019789) according to manufacturer's protocol. Ct-values less than 36 were included in the analyses. Results were normalized to spike-in cel-miR-39 values of each sample (Ct(av) = 25.1 ± 1.0). A pooled calibrator sample was used across the different plates to obtain 2^{-dCt} (=RQ) results. Subsequently, normalized relative quantities (NRQ) were calculated for each miR of each sample (NRQ = RQ/NF), where normalization factor (NF) presented the geometric mean of RQs of all expressed miRNAs per sample[49].

Bioinformatics and statistical analyses. The workflow for cleaning and analyzing the sequencing results is presented in the Fig. 7. Briefly, quality of the raw reads was assessed with FastQC and reads were trimmed using cutadapt based on the FastQC 'Overrepresented sequences' module output and using a minimum read length filter of nine bases. Trimmed read files were analyzed with miRDeep2, a comprehensive computational tool for miR analysis and discovery which uses a probabilistic model of miR biogenesis to score compatibility of the position and frequency of sequenced RNA with the secondary structure of the miR precursor[50]. Mapping to the reference genome (hg38) was performed using bowtie (version 1.0.1) and miRbase version 18 was used for retrieving miR information. Differential expression analysis was performed on miRDeep2 output data utilizing a custom R script adapted from the differential expression module in the CAP-miRSEQ tool[50,51]. The tools employed in different steps rely on various programming languages. To simplify the workflow a set of R functions was created to carry out QC, trimming, miRDeep analysis and assessment of differential expression in a pipeline-like fashion run from one wrapper script. In addition, Poisson-normal regression model was created to analyze the related

participants (unpublished manuscript). Briefly, read counts were modeled as Poisson distributed variables with over distribution modeled through a normally distributed random variable. The advantage of this model over e.g. the negative-binomial model is that it allows flexible modeling of dependency among related subjects through the random effect correlation matrix. The model was applied on each miR on MPlus version 7 and FDR approach was used to adjust for multiple testing. All other statistical computations were done in R software (versions 3.1.3–3.3.1). R packages used in the bioinformatics workflow are mentioned in Fig. 7.

The group analyses are based on three groups: (1) premenopausal women ($n = 8$), (2) postmenopausal No HRT ($n = 10$) and (3) postmenopausal HRT users ($n = 10$), the latter two forming MZ twin pairs. Based on the normal distribution of the studied variables, tested by Shapiro-Wilk test, the group comparisons were performed either with Independent samples T test and paired samples T test (twins), in case of parametric variables, or with Mann Whitney U test and Wilcoxon signed-rank test (twins) in case of non-parametric variables. Two-sided tests were used. Ingenuity pathway analyses (IPA) tool was used for pathway and comparison analyses of all sequenced miRs and differentially expressed miRs. $P < 0.05$ was considered significant. Spearman's rank correlation coefficient was used for correlation analyses and R packages "gplots" and "RColorBrewer" were used for creating the clustered heatmap (Fig. 5). Data analyses and visualizations were carried out using Eclipse IDE Luna (4.4.2) and the StatET plugin for R (3.4.2) with RJ 2.0, IBM SPSS Statistics (version 23.0, Chicago, IL) and R Studio (R Studio Team 2015, Boston, MA).

Data availability. The processed read counts are provided in the Supplemetary data (S1). The raw sequence read files are open at ArrayExpress database (www.ebi.ac.uk/arrayexpress) upon publication under access number E-MTAB-5245. Computer code availability is provided on request.

Limitations. The methods of sample collection, RNA extraction and further analyses are not yet standardized in NGS. The enzymatic steps in small RNA cDNA library preparation might favor certain miRs over others due to sequence-specific biases. However, same protocol was used across the study, therefore such bias would occur similarly in each sample and would not affect the results of the group comparisons. Also exosome extraction methods lack standardization.

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Author Contributions

R.K. is the corresponding author and has mainly performed all the laboratory work. E.K.L. and V.K. have jointly supervised the work and contributed to the writing. T.T. and V.F. have contributed equally to the statistical analyses and writing the manuscript. A.-M.S., J.P., S.S., M.A., J.K. and I.M. have contributed to the writing. All authors reviewed the manuscript.

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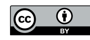

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MENOPAUSAL STATUS ASSOCIATES WITH SPECIFIC MICRORNA AND TARGET EXPRESSIONS IN SUBCUTANEOUS ADIPOSE TISSUE

by

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Menopausal status associates with specific microRNAs and target expressions in subcutaneous adipose tissue

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Summary

Aging causes metabolic changes, thus affecting overall body composition. Menopause, characterized by a decline in 17β-estradiol (E_2) levels in the circulation, is known to alter the whole-body fat distribution in women, but the underlying molecular mechanisms are not completely known. Tissue-specific effects of E_2, whether induced by ovarian E_2 or E_2-based hormone replacement therapy (HRT), are delivered via estrogen receptors known to be also regulated by microRNAs (miRs). By using explorative microarray for 377 miRs, we found 203 miRs to be expressed in subcutaneous adipose tissue and 92 miRs in serum from premenopausal women and/or postmenopausal monozygotic twin sister pairs discordant for the use of HRT. Seven miRs were validated by qPCR in an independent set adipose tissue samples from postmenopausal HRT users and non-users and the differential expression of miR targets was determined by qPCR and Western blotting. Here we report differential expression of a set of subcutaneous adipose tissue miRs, i.e., miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-19a-3p, miR-486-5p and miR-363-3p in pre- and postmenopausal women. These miRs were found to have common targets on the estrogen signaling pathways. Of these targets AKT1, BCL-2 and BRAF were expressed differently at the protein level and AKT1, ESR1 and CCND1 at the mRNA level associating with age and E_2 concentration. The E_2-sensitive miR-mediated regulation identified likely affects the cellular homeostasis of adipose tissue, thereby contributing to the postmenopausal change in body composition.

Abbreviations: ESR1: estrogen receptor 1, AKT1: AKT serine/threonine kinase 1, BRAF: B-Raf proto-oncogene, serine/threonine kinase, BCL-2: B-cell lymphoma 2, CCND1: cyclin D1

Introduction

Human aging is a lifelong process characterized by a dynamic phenotype that changes over time with different trajectories, likely associated with sex hormone differences in men and women (Ostan et al., 2016). Women's aging is tied to the change in hormonal milieu during menopausal transition: the concentration of circulating 17β-estradiol (E_2) decreases concomitantly with an increase in follicle-stimulating hormone (FSH). Declining amounts of ovarian E_2 lead to changes in the distribution of body fat (Tremollieres et al., 1996) emphasizing the importance of ovarian E_2 in regulating the lipid metabolism. The effects of E_2 in adipose tissue are likely to be mediated through estrogen receptors of which ESR1 is known to affect whole-body metabolism as well as adipocyte cell growth and differentiation (Pedram et al., 2016, Hevener et al., 2015). Postmenopausal women using E_2-based hormone replacement therapy (HRT) have healthier adipokine/cytokine profile and less centrally located body fat than women not using HRT (Ahtiainen et al., 2012, Ronkainen et al., 2009, Chen et al., 2005). This evidence is relevant given that adipose tissue is metabolically active and, owing to its secretory activity, thus, it is one of the main contributors to the crosstalk between different tissues (reviewed by Kershaw and Flier, 2004). Furthermore, the role of adipose tissue in human aging is central owing to its influence on sustaining the pro-inflammatory microenvironment, the main premise underlying the "inflammaging" and its propagation mechanisms (Franceschi et al., 2007, Franceschi 2017). However, adipose tissue is not only responsive to fluctuations in the systemic levels of hormones, but it actively produces and secrets multitude of molecules including E_2 and other hormones, inflammatory factors and microRNAs (miRs).

MiRs are small RNAs that contribute to gene regulation by binding to their mRNA targets, a process that induces mRNA cleavage or seizure and, eventually, a possible reduction in the abundance of the functional target protein. Many of the miRs expressed by adipose tissue take part in adipogenesis and lipid homeostasis (Chen et al., 2014, Kang et al., 2013, Tang et al., 2009, Mysore et al., 2015). Aging has been shown to change the expression of adipose miRs in humans (Mori et al., 2012), which may lead to changes in the abundancy of miR targets either locally or in

systemic manner far from the site of exocytosis (Thomou et al., 2017). We have previously shown that specific circulating miRs (Kangas et al., 2014, Kangas et al., 2017) and skeletal muscle miRs (Olivieri et al., 2014) of pre- and postmenopausal women are associated with serum E_2 levels. These findings indicate that sex steroid hormones are part of miR-mediated signal transduction in aging women.

The aim of this study was to investigate if the expression of adipose tissue- and serum-derived miRs and their targets differ owing to age-associated hormonal status or the use of HRT. First, using explorative microarray for 377 miRs we found 203 miRs expressed in subcutaneous adipose tissue and 92 miRs in serum from premenopausal women and/or postmenopausal monozygotic (MZ) twin sister pairs discordant for the use of HRT. Second, we selected seven miRs that were expressed in adipose tissue for validation by quantitative PCR (qPCR). In addition, six of them were also expressed in serum samples. Third, the differential expression of miR targets in adipose tissue was determined by qPCR and Western blotting. These methods enabled us to determine age- or HRT-related differences in the expression of miRs in female white adipose tissue. Together, our findings indicate that miR-16-5p, miR-451a, miR-18a-5p, miR-19a-3p, miR-486-5p and miR-363-3p strongly correlate with circulating E_2 levels and that miR-mediated regulation, based on the studied miRs, associates with cellular homeostasis in adipose tissue.

Results

Participant characteristics

Two different sample and data sets originating from 1) the SAWEs and 2) miRBody studies were analyzed (Figure 1A). The SAWEs study consists of a cross-sectional design with premenopausal women (Pre) without any E_2-containing hormonal treatments, and a co-twin design of postmenopausal MZ twin sister pairs with approximately 7 years of E_2-based HRT discordance (HRT and No HRT) i.e., one sister was a current HRT user and the other had never used HRT. MiRBody is a cross-sectional study with a postmenopausal cohort of unrelated women age-matched with the SAWEs MZ twins and who formed two groups, one using and the other not using

HRT. The SAWEs samples were used in the miR profiling. The miRBody samples (postmenopausal HRT and No HRT women) and SAWEs samples (premenopausal women) were available for the validation of the profiling data. FIGURE 1 B presents the scheme of comparisons among the identified three groups.Anthropometrics, serum inflammatory markers and hormone levels among the groups in the different data sets are presented in **Table** 1 and clinical data in Table S1. No differences in body composition measures or inflammatory markers were observed between the groups. Serum E_2 and FSH levels differed, as expected, most significantly between the premenopausal women and postmenopausal non-users, with E_2 showing the highest values in the premenopausal women and the lowest values in the postmenopausal non-users and vice versa for FSH. Comparisons were also performed between the SAWEs and miRBody participants. The E_2 levels of the postmenopausal No HRT group in the miRBody study were higher than those of the SAWEs No HRT women ($p < 0.001$) and the E_2 levels of the SAWEs No HRT women differed significantly from the miRBody HRT women ($p < 0.001$). The differences between the No HRT groups might in part be due to the different methods used to analyze the E_2 levels in these two studies (see methods). Otherwise no group differences between postmenopausal SAWEs and miRBody women were detected.

miR profiling

TaqMan human microRNA Array containing 377 miRs was used for miR profiling in the subcutaneous adipose tissue and serum samples from the premenopausal women and postmenopausal MZ twin sister pairs of the SAWEs study. In total, 203 miRs were detected in the adipose tissue and 92 miRs in the serum (Figure 2A). Three types of comparisons were performed: 1) Postmenopausal No HRT group vs. premenopausal women, 2) Postmenopausal HRT group vs. premenopausal women and 3) postmenopausal HRT group vs. postmenopausal No HRT group. MiRs with a fold change (FC) value 1.9 or more are presented. A list of all the FCs obtained is given as supplemental data (Table S2). The experimental design (Figure 1B) and miR filtering procedure yielded the following results (Figure 2A):

1. Age associations without HRT (No HRT vs. Pre) were defined for 16 adipose tissue and 23 serum miRs. Of these, 10 adipose tissue miRs and 1 serum miR had a higher expression level and 6 adipose tissue and 22 serum miRs had a lower expression level in the postmenopausal No HRT women compared with the premenopausal women.
2. Age associations with HRT (HRT vs. Pre) were defined for 24 adipose tissue and 8 serum miRs. Of these, 2 adipose tissue and 5 serum miRs had a higher expression level and 22 adipose tissue and 3 serum miRs had a lower expression level in the postmenopausal HRT women compared with the premenopausal women.
3. The associations found for HRT treatment (HRT vs. No HRT) were defined for 31 adipose tissue and 27 serum miRs. Of these, 1 adipose tissue miR and 27 serum miRs had a higher expression level and 30 adipose tissue miRs had a lower expression level in postmenopausal HRT users compared with non-users.

Comparison analyses using Ingenuity Pathway Analysis (IPA) were performed to identify the potential biological processes with possible interactions with the profiled adipose tissue and serum-derived miRs (Figure 2B). The analyses predicted differences in up- or downregulated biological functions between adipose tissue and blood circulation. In the No HRT vs. Pre -comparison biological processes necrosis, apoptosis and DNA damage were predicted to be affected based on the observed differences in miR expression. In the HRT vs. No HRT comparison the predicted pathways were cell proliferation of hepatoma cell lines and DNA damage. The HRT vs. Pre -comparison showed no statistically significant predictions in any biological processes. The identified biological processes showed the opposite pattern in the adipose tissue and serum samples.

Validation of the miR profiling

The miR profiling was validated by qPCR, using samples from the premenopausal women (SAWEs) and the independent clinical cohort of postmenopausal HRT users and non-users (miRBody). For validation, we selected miRs which were detected in all measured samples. Further, we filtered six relevant miRs (FC ≥1.9) expressed in both adipose and blood tissue.

Interestingly, these were all expressed in the opposite direction in adipose tissue and serum (miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-19a-3p, miR-486-5p). One additional miR showing the highest FC in adipose tissue (miR-363-3p), was also selected, as reported in Figure 2A. The main results for the adipose tissue (Figure 3) were:

1. Age associations without HRT (No HRT vs. Pre) were found for miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-19a-3p, miR-363-3p, and miR-486-5p, the values being significantly higher in the adipose tissue of the postmenopausal non-users than in that of the premenopausal women ($p < 0.001$; $p < 0.001$; $p = 0.003$, $p = 0.003$; $p = 0.006$; $p < 0.001$; $p = 0.002$, respectively). These results, except for miR-223-3p and miR-363-3p, were in accordance with the miR profiling.
2. Age associations with HRT (HRT vs. Pre) were found for miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-363, and miR-486-5p, the values being significantly higher in the adipose tissue of the postmenopausal HRT users than in that of the premenopausal women ($p = 0.002$; $p = 0.014$; $p = 0.009$; $p = 0.024$; $p = 0.036$; $p = 0.039$, respectively). No significant differences were detected for miR-19a-3p. The profiling indicated opposite direction for the FCs other than miR-16-5p, thus results for adipose tissue were, in general, not confirmed.
3. The associations found for HRT treatment (HRT vs. No HRT) were not significant, although the miRs showed trends towards higher levels of expression in No HRT women, as also found in the profiling. Owing to the relatively high E_2 levels in some of the No HRT participants of the miRBody, we performed additional analyses by excluding the No HRT women with E_2 levels higher than 110 pmol/l which was the lowest E_2 level found in the HRT group. These analyses revealed significantly higher miR-19a-3p levels in the postmenopausal low-E_2 No HRT group compared to HRT group ($p = 0.043$, data not shown). A similar trend in miR-18a-5p levels was seen between the low-E_2 No HRT group and HRT group ($p = 0.094$, data not shown).

Single miR analyses by qPCR, using serum samples, did not confirm the data obtained by the miR profiling. No significant differences were observed even after excluding the No HRT women with the higher E_2 levels (data not shown). Therefore, further analyses of the miR targets were performed only for adipose tissue.

Adipose tissue miRs associate with age and circulating hormones

A clustered correlation heatmap was created to determine the associations of the validated adipose tissue miRs with age, body mass index (BMI) and circulating high sensitivity C-reactive protein (CRP), E_2 and FSH levels (Figure 4). All the adipose tissue miRs correlated positively with age (false discovery rate corrected p-value (FDR) < 0.05) and all, except miR-223-3p, negatively with serum E_2 levels (FDR < 0.05). In addition, miR-18a-5p, -16-5p and -363-3p showed positive correlations with serum FSH levels. None of these miRs were significantly associated with BMI or CRP. Numerical details of the correlations are given as supporting information (Table S3).

mRNA and protein expression of miR targets in adipose tissue

A bioinformatic approach, looking at experimentally validated miR-target pairs described in the literature, was applied for the validated miRs. The common targets (at least two miRs targeting the same mRNA) identified were ESR1, AKT1, BRAF, BCL-2 and CCND1 (Figure 5). For these targets, both mRNA and protein levels were measured in adipose tissue samples (Figure 6).

1. Age associations without HRT (No HRT vs. Pre) were found for ESR1, AKT1 and CCND1 mRNA, the values being significantly lower in the postmenopausal No HRT group when compared to the premenopausal women ($p < 0.001$, $p = 0.005$, $p = 0.003$, respectively). At the protein level, AKT1, BRAF and BCL-2 were significantly less abundant with higher age ($p = 0.005$, $p = 0.003$, $p = 0.028$, respectively).
2. Age associations with HRT (HRT vs. Pre) were found for AKT1 and CCND1 mRNA, the values being lower in the postmenopausal HRT group when compared to the premenopausal women ($p = 0.023$, $p = 0.014$, respectively). At the protein level, AKT1,

BRAF and BCL-2 were significantly less abundant among the older HRT users (p = 0.013, p = 0.020, p = 0.024, respectively).

3. Associations with HRT treatment (HRT vs. No HRT) were found for ESR1 mRNA with higher values in the postmenopausal HRT group compared to No HRT group (p = 0.026). At the protein level, ESR1 values were similar in both groups.

Discussion

In the current study, we focused on miR expressions in adipose tissue and blood and on the differences in their expression between healthy pre- and postmenopausal women, the latter group subdivided into users and non-users of E_2-based HRT. We adopted an exploratory miR profiling approach to determine the differences in miR expression, assuming causality due to menopausal status or postmenopausal HRT treatment. We detected 203 miRs in the adipose tissue and 92 in the serum samples. Functional analysis with IPA predicted necrosis, apoptosis and DNA damage-related biological processes to be the main downstream mechanisms affected by these miRs. The IPA results support the general hypothesis that inflammaging is fueled by the age-related production of cell debris or misplaced cell-molecules released during repair or damage processes (Franceschi et al., 2017). Based on that and taking also into account that cells, organs and tissues communicate by releasing different type of secretomes, including miRs, we performed qPCR validation for six miRs found to be present in both the adipose and serum samples (miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-19a-3p, miR-486-5p) and for the most highly expressed miR in adipose tissue (miR-363-3p). In line with the results obtained by the profiling, qPCR validation confirmed the age-associations for five miRs in adipose tissue i.e., miR-16-5p, miR-451a, miR-18a-5p, miR-19a-3p, and miR-486-5p. The validated miRs were shown to target ESR1, AKT1, BCL-2, BRAF and CCND1 in common, thereby indicating their role in the regulation of adipocyte cell fate, death or proliferation.

Two of the validated miRs, miR-18a-5p and 19a-3p, have previously been shown to target ESR1 (Ferraro et al., 2012, Zhu et al., 2015, Yoshimoto et al., 2011, Castellano et al., 2009). These miRs

are part of a miR-17-92 cluster, which has been shown to participate in the regulation of adipocyte development by accelerating preadipocyte differentiation (Wang et al., 2008). Our results showing the opposite expression pattern between targeting miRs, miR-18a-5p and -19a-3p, and ESR1 mRNA levels is in line with the expected regulatory function of miR-18a-5p and -19a-3p in ESR1 expression. Interestingly, miR-19a-3p levels differed significantly between the postmenopausal groups (HRT vs. No HRT) when the highest E_2 blood levels in the No HRT group were excluded, suggesting an association with HRT treatment. Furthermore, our results indicate that miR-223-3p, -16-5p, -363-3p and 486-5p, which are predicted to target ESR1 based on their complementary sequence structure, also participate in the regulation of ESR1 expression, owing to their similar expression pattern with miR-18a-5p and -19a-3p and negative correlation with E_2 (except for miR-223-3p) in the hierarchical clustering analysis. Altogether, the lower amount of the miRs targeting ESR1 and the concomitantly higher amount of ESR1 in the adipose tissue of the premenopausal women indicate higher E_2 responsiveness when compared with the postmenopausal non-users. Nevertheless, the protein levels of ESR1 appear well preserved in all groups, thus suggesting other regulatory layers (Greenbaum et al., 2003). In addition, the low number of samples in the protein analyses and the potential effect of local production of E_2 in adipose tissue, which we had no opportunity to investigate here, should be acknowledged as possible limitations of this work.

miR-363-3p and miR-486-5p have previously been involved in adipose tissue remodeling (Chen et al., 2014, Kim et al., 2012). A study performed by Chen et al. (2014) demonstrated that miR-363 is downregulated during adipogenic differentiation in the adipose tissue-derived stromal cells. Based on those findings, our data suggests that the aged adipose tissue having the highest miR-363-3p expression is likely associated with reduced differentiation capacity. This finding is supported by the common notion that a younger tissue is more prone to proliferate and differentiate than older tissue. Further, the expression of miR-486-5p in human adipose tissue-derived mesenchymal stem cells has been shown to induce replicative senescence (Kim et al., 2012). Our study demonstrates the age-related association of this miR in adipose tissue, thereby emphasizing the possible role of

the menopausal loss of ovarian function in changing the cellular homeostasis or early phase of replicative senescence in adipose tissue.

To our knowledge, no previous studies exist on miR-451a expression in adipose tissue. Instead, the association of miR-451a with aging has been identified in the skeletal muscle (Mercken et al., 2013). In their study, miR-451a levels were shown to be higher in the skeletal muscle of old monkeys compared to younger ones. In addition, decreased expression of miR-451a in non-alcoholic steatohepatitis human liver was associated with an increase in pro-inflammatory cytokines (Hur et al., 2015), suggesting the role for this miR in inflammation. In the current study, miR-451a was highly expressed at more advanced ages in adipose tissue independently of HRT use. Interestingly, a higher miR levels with age in adipose tissue appears to be a common trend shared by other metabolic organs such as the human liver, as recently reported (Capri et al., 2016).

MiR-16-5p and miR-223-3p are widely studied miRs in different cellular contexts. Age-related changes in miR-16-5p have been shown to be associated with vascular and neurodegenerative diseases and B-cell function (Muller et al., 2014, Yentrapalli et al., 2015) whereas, with advanced age, an increase in miR-223-3p in inflammatory cells has been reported (Teteloshvili et al., 2015). MiR-223-3p has also been shown to regulate macrophage activation resulting in the suppression of pro-inflammatory responses in the adipose tissue of mice (Zhuang et al., 2012). In the current study, the highest expression of both miRs was found in the adipose tissue of postmenopausal women without HRT, strengthening their association with aging: however, the possible contribution to the level of miR-223-3p of adipose tissue macrophages cannot be excluded.

The inability to verify HRT-treatment associations for miR-223-3p, 363-3p, -486-5p, 16-5p and -451a in the adipose tissue, suggests that HRT potentially has a specific effect only on miR-19a-3p and a weak effect on miR-18a-5p levels (data not shown). Interestingly, we have previously demonstrated an association between HRT treatment and miR-223-3p in the skeletal muscle of

these same postmenopausal HRT-discordant MZ twins, which suggests tissue-specific regulation of miR-233-3p expression (Olivieri et al., 2014).

Despite the profiling results, the selected miRs were not differentially expressed in the serum, suggesting not only a specific effect of HRT treatment on adipose tissue but also no associations of age with the selected miRs in the blood. Recently, however, we demonstrated that age and HRT differentially modulate the expression of specific miRs, that are packed in the circulating exosomes (Kangas et al., 2017). The inability to validate the profiling results from the serum samples in the current study is likely due to a different carrier mechanism (not only exosomes were included) or high variation of E_2 levels in the postmenopausal No HRT group of miRBody participants. This variation has to be acknowledged as one of the limitations of the current study together with the differences of the two cohorts (SAWEs, miRBody) owing to their health status.

The ESR1 signaling pathway in adipose tissue is likely to be followed by the activation of AKT1, leading in turn to more proliferative or anti-apoptotic processes. AKT1 has also been previously linked to estrogen signaling (Zhao et al., 2016). Our results showed that postmenopausal women have lower amounts of AKT1 in their subcutaneous adipose tissue than premenopausal women. This age-association was consistent for both the mRNA and protein levels independently of HRT use. AKT1 affects cellular homeostasis and it is considered as a survival factor suppressing apoptosis. The role of AKT1 in human adipocytes has previously been demonstrated by Fischer-Posovszky et al. (2012), who showed that AKT1 takes part in insulin-induced metabolic signaling. In the current study, the lower amount of AKT1 in the postmenopausal women could indicate a weakened sensitivity to insulin and also reduced proliferative status. BCL-2 and CCND1, which have been shown to be induced by E_2 in breast cancer cells (Yu et al., 2012) are downstream from AKT1. We showed that BCL-2, an anti-apoptotic protein, is less abundant at higher ages, suggesting that the rate of apoptosis could be activated in older women. CCND1 mRNA levels were negatively associated with age, the premenopausal women showing significantly higher values. However, the protein concentrations did not differ between the groups, suggesting more complex post-transcriptional regulation. The mRNA expression of BRAF, a molecule regulating cell

growth independently of AKT1, did not differ between the studied groups. However, the protein concentrations were lower with higher age, suggesting, also in this case, further levels of regulation. These findings indicate lower proliferation capacity of adipose tissue in postmenopausal than premenopausal women being in line with previous studies showing sex-dependent differences in healthy adipose tissue remodeling (Wu et al., 2017). Further, our results also support lower expression of anti-apoptotic mediators with higher age.

Altogether, our data indicate that the menopausal transition acts as a crossway between apoptotic and proliferative signaling. Lower levels of specific proteins, such as AKT1, BCL2 and BRAF after menopause, suggest that the proliferative activity is likely to be slowed down in the subcutaneous adipose tissue obtained from the abdominal region. Based on the current findings, we can hypothesize that adipocytes are addressed towards deficiency in cellular remodeling and/or early phase of cellular senescence following menopause. The subcutaneous adipose tissue, even if it is not representative of all the different types of fat in humans, shows an age-related increase of miR expressions involved in the estrogen signaling pathway. The interplay of age, systemic E_2 levels and tissue-specific miRs highlights the critical role of HRT that partially could counteract the effects of menopausal estrogen decline in a tissue-specific manner. However, longitudinal studies with greater numbers of participants are needed to confirm the proposed causality of the present findings and evaluate the miRs not included in the validation phase.

Experimental procedures

Experimental design

The current study is based on two different sample and data sets (Figure 1): 1. Sarcopenia and Skeletal Muscle Adaptation to Postmenopausal Hypogonadism: Effects of Physical Activity and Hormone Replacement Therapy in Older Women – a Genetic and Molecular Biology Study on Physical Activity and Estrogen- related Pathways (SAWEs-study); 2. Circulating microRNAs and body composition (miRBody-study). The recruitment process and exclusion criteria for participation for both studies are presented in Figure 1A and the analysis strategy of the current

miR-study in Figure 1B. Briefly, the SAWEs-study investigated a group of healthy premenopausal women (n=17, 32.9±3.3 years), with a natural menstrual cycle and no estrogen/progesterone-based treatments for at least the previous 5 years (Pöllänen et al., 2011) and a group of postmenopausal MZ twin sister pairs (n=5 pairs, 57.4±1.5 years), who were discordant for E_2-based HRT (mean duration of HRT use 6.9±4.1 years; Ronkainen et al., 2009). The miRBody-study investigated 33 independent postmenopausal women either using estrogen-based HRT (mean duration of HRT use 6.6±6.3 years, n=16, age 57.7±2.9 years) or not (n=17, age 58.8±3.0 years). The study protocols were approved by the Ethics Committee of the Central Finland Health Care District (SAWEs: 7.6.2006 and 22.11.2006 E0606/06; miRBody: 3.2.2015 1U/2015). All the study participants gave their written informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki.

Participant characteristics

BMI was calculated from body weight and height (kg/cm^2). Body fat percentage and lean body mass (LBM) were measured with bioelectrical impedance (inBody 720, Biospace Co. Ltd., Seoul, Korea). Serum E_2 levels were measured using an extraction radioimmunoassay (SAWEs; Ronkainen et al, 2009) and with solid-phase, chemiluminescent immunometric assay (miRBody) which was also used for the serum CRP and FSH measurements (Immulite 1000, Diagnostic Products, Los Angeles, CA, USA).

Sampling

In the SAWEs study, adipose tissue biopsies were obtained from below the navel by a physician with the needle-aspiration method. Blood traces were cleaned with 0.9 % NaCl and samples were snap frozen in liquid nitrogen and stored at -80°C until further analysis. The samples used in the mirBody-study were obtained from abdominal subcutaneous adipose tissue in the proximity of the navel during gynecological surgery. Samples were stored in All Protect Reagent (Qiagen) at -20°C until further analysis. Blood samples, collected in standard conditions, were allowed to clot for 30 mins at room temperature before serum separation by centrifugation at 4000 rpm. Serum samples were stored at -80°C in 0.5 ml aliquots until further analysis. The samples from the premenopausal

women were collected during the first five days of the menstrual cycle to obtain the lowest E_2 concentrations.

RNA extraction

Total RNA (~20 mg of tissue) was extracted from adipose biopsies with a mirVana miRNA isolation kit (Ambion, by Life Technologies, NY, USA) according to manufacturer's protocol. Total RNA was extracted from the serum samples (100 µl) with Total RNA Purification kit (Norgen Biotek Corp, Thorold, Canada) according to the manufacturer's protocol. In addition, 20 fmol of spike-in cel-miR-39 (Qiagen, Hilden, Germany) was added to the serum samples at the lysis step to control for the RNA extraction efficiency.

MicroRNA profiling

To assess global miR expression, 10 SAWEs adipose tissue samples (4 premenopausal, 3 HRT users, 3 non-users) and 13 serum samples (4 premenopausal, 5 HRT users, 4 non-users) were screened using a TaqMan human MicroRNA Array A (Applied Biosystems, by Life Technologies, NY, USA) containing 377 of the most common human miR assays. MiR profiling protocol is described in details in Supplemental Experimental Procedures.

Confirming the profiling results

For qPCR validation, six miRs (miR-16-5p, miR-451a, miR-223-3p, miR-18a-5p, miR-19a-3p and miR-486-5p) expressed in both adipose tissue and serum samples across all the participants were selected from the profiling analysis assuming a possible tissue cross-talk. In addition, miR-363 was validated owing to its extreme pattern of differential expression in adipose tissue between the studied groups. Samples from the premenopausal SAWEs women (n=9) and postmenopausal miRBody women (HRT: n=9, No HRT: n=12) were used for validation (see in details in Supplemental Experimental Procedures).

microRNA target analyses from adipose tissue

Only previously validated miR targets, based on the literature, were analyzed. SAWEs samples from the premenopausal women (n=12) and mirBody samples from the postmenopausal women (HRT; n=9, No HRT; n=12) were used to assess the mRNA expression of the targets by qPCR. miR target protein abundancy was analyzed by Western blotting (n=5/each group). RNA and protein analyses are described in details in Supplemental Experimental Procedures.

Statistical analysis

The normal distribution of the studied variables, was tested by the Shapiro-Wilk test and the group comparisons for anthropometric, serum inflammatory markers and hormones were performed either with Independent samples T test and paired samples T test (within twin pairs) for parametric variables, or with Mann Whitney U test and Wilcoxon signed-rank test (within twin pairs) for non-parametric variables. The Kruskall-Wallis test for non-parametric variables was used for comparisons of miR expressions across groups. MiR profiling data with FC values greater than 1.9 were used in the IPA comparison analyses (Qiagen) and an activation Z-score greater than 2 was considered statistically significant. Diana tools (http://diana.imis.athena-innovation.gr/DianaTools/index.php) and miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) were used for identifying the common validated target genes of the analyzed miRs. Spearman's rank correlation coefficient was used for the correlation analyses and R packages "gplots" and "RColor-Brewer" were used to create the clustered heatmap. P-values less than 0.05 were considered statistically significant.

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Author contributions

R.K and C.M equally contributed to the experiments, data analyses and drafting the manuscript. G.P and C.L. contributed to the experiments and data analyses. M.C., E.K.L and V.K. planned and supervised the study. P.A. contributed to the study as a physician. M.C., E.K.L, V.K., S.S and J.K contributed to writing the manuscript. Critical advice was provided by C.F. All authors critically reviewed the manuscript and approved the final version prior to submission.

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Supporting information

Table S1: Medical history, current medication and health characteristics of the pre- and postmenopausal women in both studies. Related to the Figure 1 and Table 1.

Table S2: Fold changes (FC) of the profiled miRs in adipose tissue (A) and serum (B). Related to the Figure 2.

Table S3: Numerical values for validated miR correlations illustrated in clustered heatmap. Related to the Figure 4.

Supporting Experimental Procedures

Tables

Table **1**: **The participant characteristics. See also table S1.**

	Premenopausal women (SAWEs; n=17)	**Postmenopausal HRT women (SAWEs; n=5)**	**Postmenopausal No HRT women (SAWEs; n=5)**	**Postmenopausal HRT women (miRBody; n=16)**	**Postmenopausal No HRT women (miRBody; n=17)**
Age (yrs)$^{\#}$	32.9±3.3	57.4±1.5*	57.4±1.5*	57.7±2.9*	58.8±3.0*
Years on HRT	-	8.4±4.7	-	6.6±6.3	-
BMI (kg/m2)	27.1±5.7	26.6±3.3	27.3±5.6	27.7±4.6	27.9±3.8
Fat %	32.3±9.2	31.6±7.3	33.3±9.3		
LBM (kg)	45.5±4.6	45.1±2.0	44.2±4.0		
CRP (mg/l)	1.4±1.7	1.3±1.1	1.3±0.7	2.4±2.2 (n=15)	3.5±3.8 (n=15)
HGB (g/l)	136.5±7.1	146.0±8.1$^{*\$}$	138.8±12.4	138.7±10.1 (n=12)	135.0±10.1 (n=11)
WBC (e9/l)	5.7±1.3	5.9±1.1	5.7±1.8	6.4±1.5 (n=12)	6.1±1.8 (n=11)
E2 (pmol/l)$^{\#}$	355.4±288.5	250.4±296.4*†	23.8±10.6*	284.4±177.4$^{\$\dagger}$	118.4±46.9*†
FSH (IU/l)$^{\#}$	6.0±2.3	39.8±32.0*	75.1±46.5*	32.9±24.6*	58.2±28.4*

*Results are shown as mean ± S.D. For parametric variables, independent samples T-test was used to compare the postmenopausal groups with the premenopausal group and to compare mirBody postmenopausal HRT group with the NO HRT group, while paired samples T-test was used to compare the SAWEs HRT women with the NO HRT co-twins. For non-parametric variables, $^{\#}$Mann Whitney U test was used to compare the postmenopausal groups with the premenopausal group and to compare mirBody postmenopausal HRT group with the NO HRT group, while Wilcoxon matched pair signed-rank test was used to compare the SAWEs HRT women with the NO HRT co-twins. Significant comparisons between the studied groups: *P<0.05 compared to the Premenopausal group; †P<0.05 compared to the SAWEs Postmenopausal NO HRT group; $^{\$}$P<0.05 compared to the miRBody No HRT group. BMI: body mass index, LBM: lean body mass (fat free), CRP: high sensitivity C-reactive protein, HGB: hemoglobin, WBC: white blood cell count, E2: 17β-estradiol, FSH: follicle-stimulating hormone*

Figure legends:

Figure 1: Recruitment of the participants and group comparisons under interest. Panel A) shows the flowchart of the recruitment process of the two different data sets used in the study. Panel B) shows the studied associations. Pre=premenopausal women, No HRT=postmenopausal women without estrogen-based hormone replacement therapy, HRT=postmenopausal women with estrogen-based hormone replacement therapy. See also Table S1.

Figure 2: MiR profiling of the adipose tissue and serum miRs. A) Discovery phase of the miRs performed on miR arrays for both tissues (FC ≥ ±1.9). Numerical FC values for the comparisons are given as supplementary data (Table S2). Venn diagram presents number of all the detected miRs and the miRs chosen for RT-qPCR validation. **http://bioinfogp.cnb.csic.es/tools/venny/index.html.** B) IPA comparison analyses were performed between adipose tissue and serum miRs in each comparison (FC ≥ ±1.9, Z-activation score ≥ ± 2.0). The results show the differences among diseases and biological functions between the two tissues. Orange indicates up- and blue downregulation.

Figure 3: qPCR validation of selected miRs from adipose tissue. * $P < 0.05$, ** $P < 0.001$. Pre = premenopausal women (n=9), HRT = postmenopausal HRT users (n=9), No HRT = postmenopausal non-users (n=12). Results are shown as mean±SD.

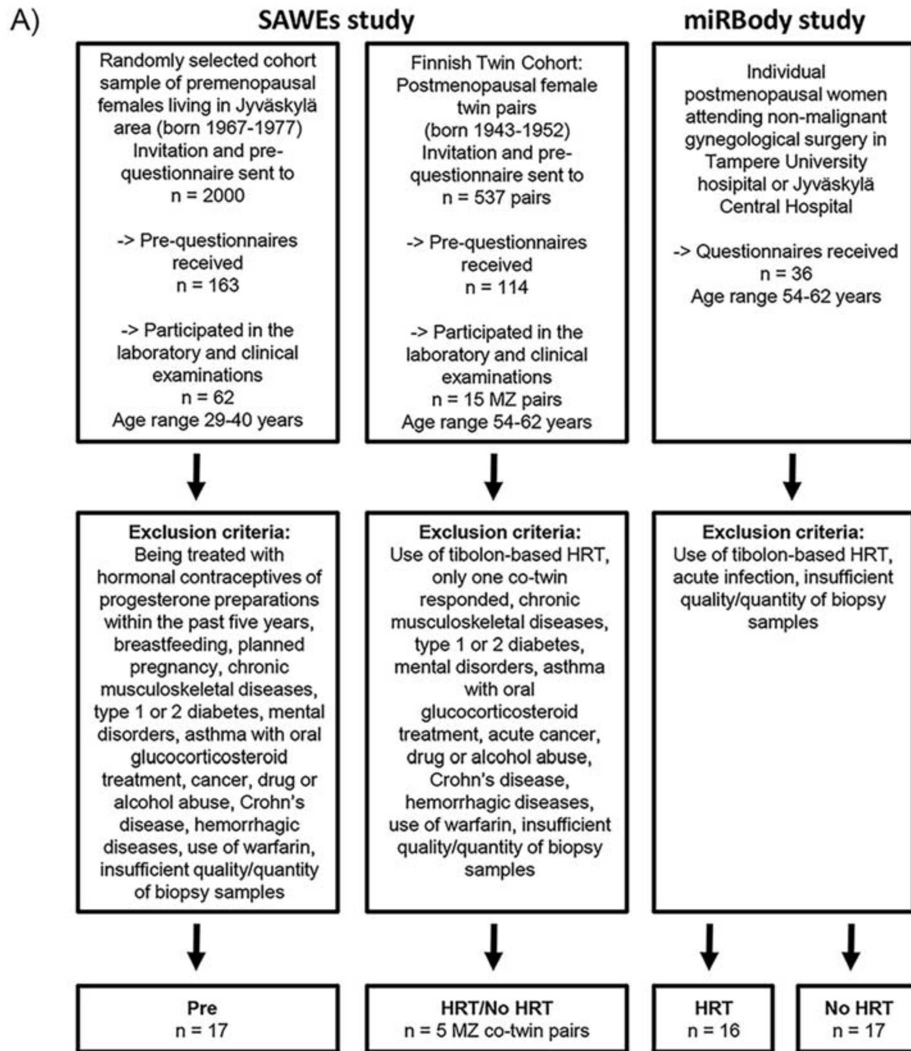
Figure 4: Clustered heatmap including the validated adipose tissue miRs and their associations with serum hormones, CRP and BMI. * $P < 0.05$, ** $P < 0.05$ with FDR correction. The numerical values of the Spearman correlation, P -values and FDR corrected P -values are given as supplementary data (Table S2). FSH: follicle-stimulating hormone, CRP: high sensitivity C-reactive protein, BMI: body mass index, E2: 17β-estradiol. See also Table S3.

Figure 5: Illustration of potential estrogen signaling pathway affecting adipose cell apoptosis and proliferation. MiRs of the experimentally validated miR-target pairs are bolded and underlined. The other miRs indicate putative miR-target pairs. Studied target molecules are highlighted in yellow. Data is obtained from **http://diana.imis.athena-**

innovation.gr/DianaTools/index.php and http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/. References for experimentally validated miR-target pairs are: Ferraro et al., 2012, Yoshimoto et al., 2011, Zhu et al., 2015, Nan et al., 2010, Castellano et al., 2009, Loven et al., 2010, Leivonen et al., 2009, Meyer-Rochow et al., 2010, Li et al., 2016, Liu et al., 2008, Yu et al., 2008, Cimmino et al., 2005.

Figure 6: Relative mRNA expression and protein concentration of the miR targets in the studied groups. * $P < 0.05$, ** $P < 0.001$. Pre = premenopausal women (n_{mRNA}=12, n_{prot}=5), HRT = postmenopausal HRT users (n_{mRNA}=9, n_{prot} =5), No HRT = postmenopausal non-users (n_{mRNA}=12, n_{prot}=5). Results are shown as mean±SD.

FIGURE 1



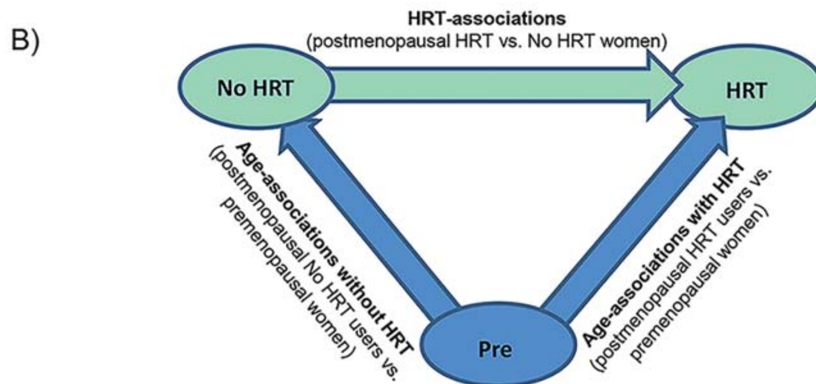


FIGURE 2

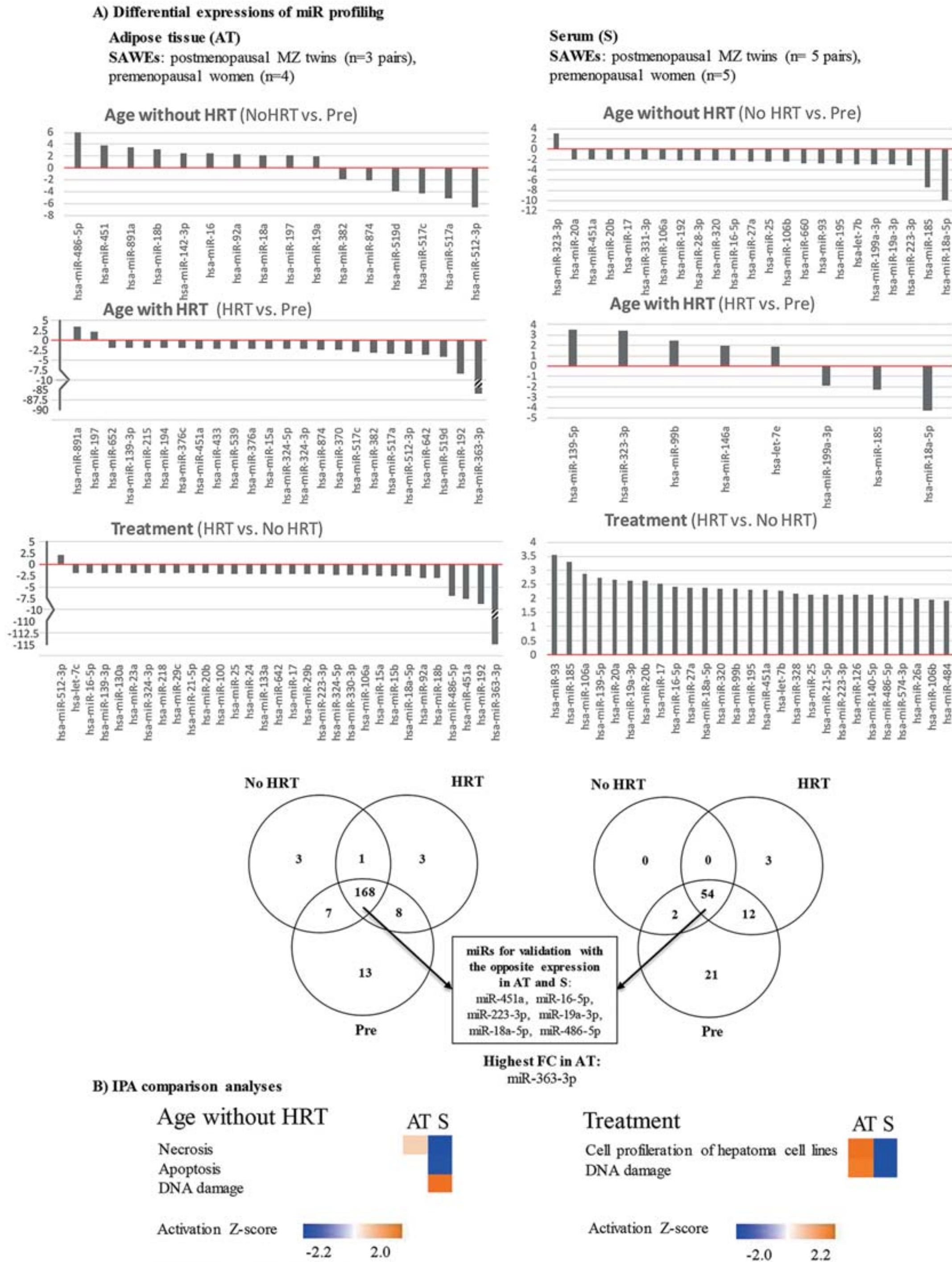


FIGURE 3

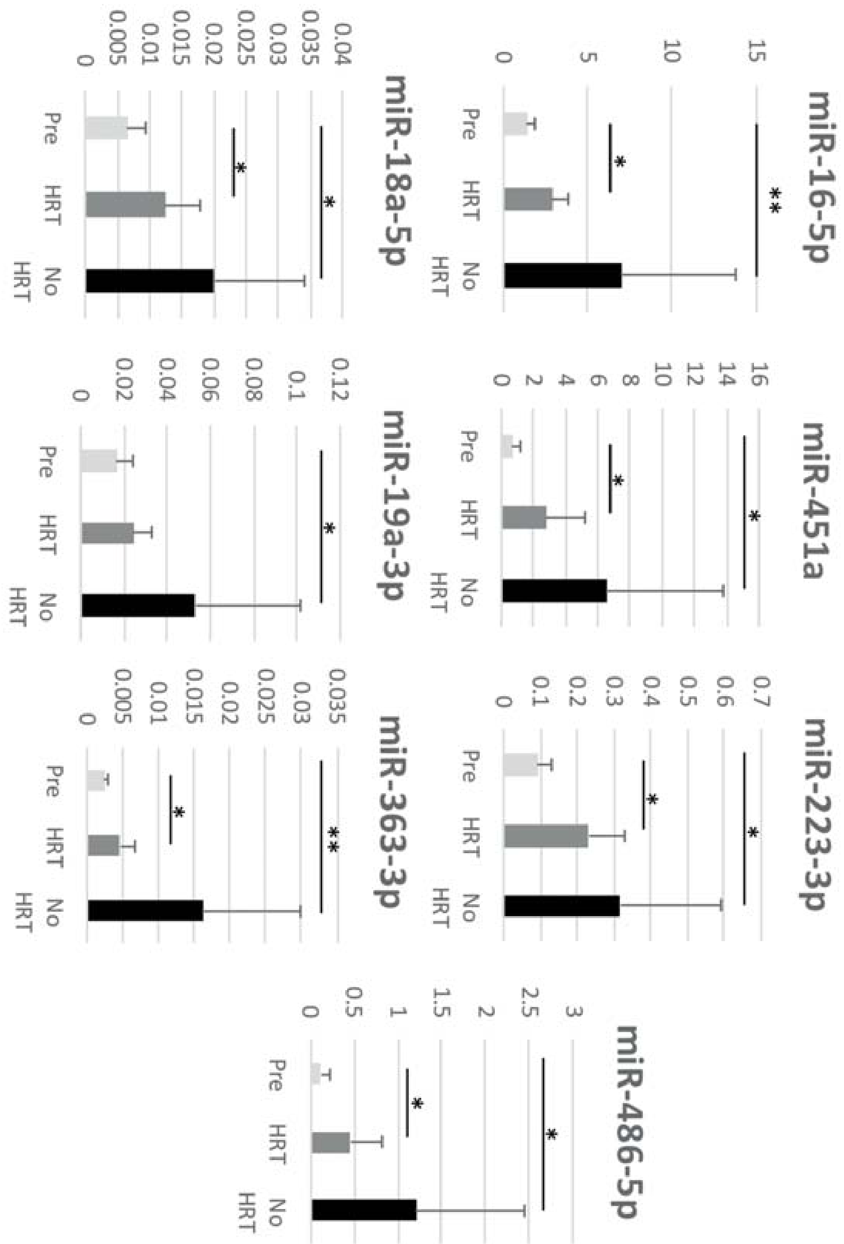


FIGURE 4

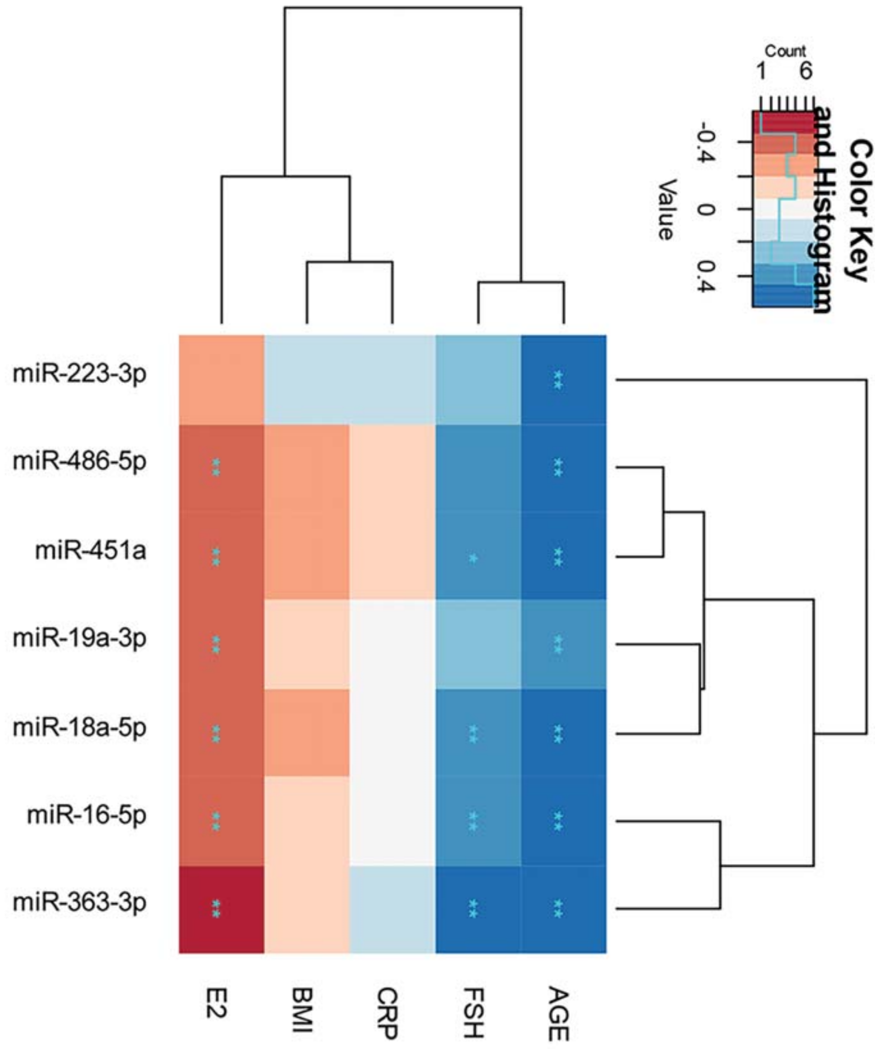


FIGURE 5

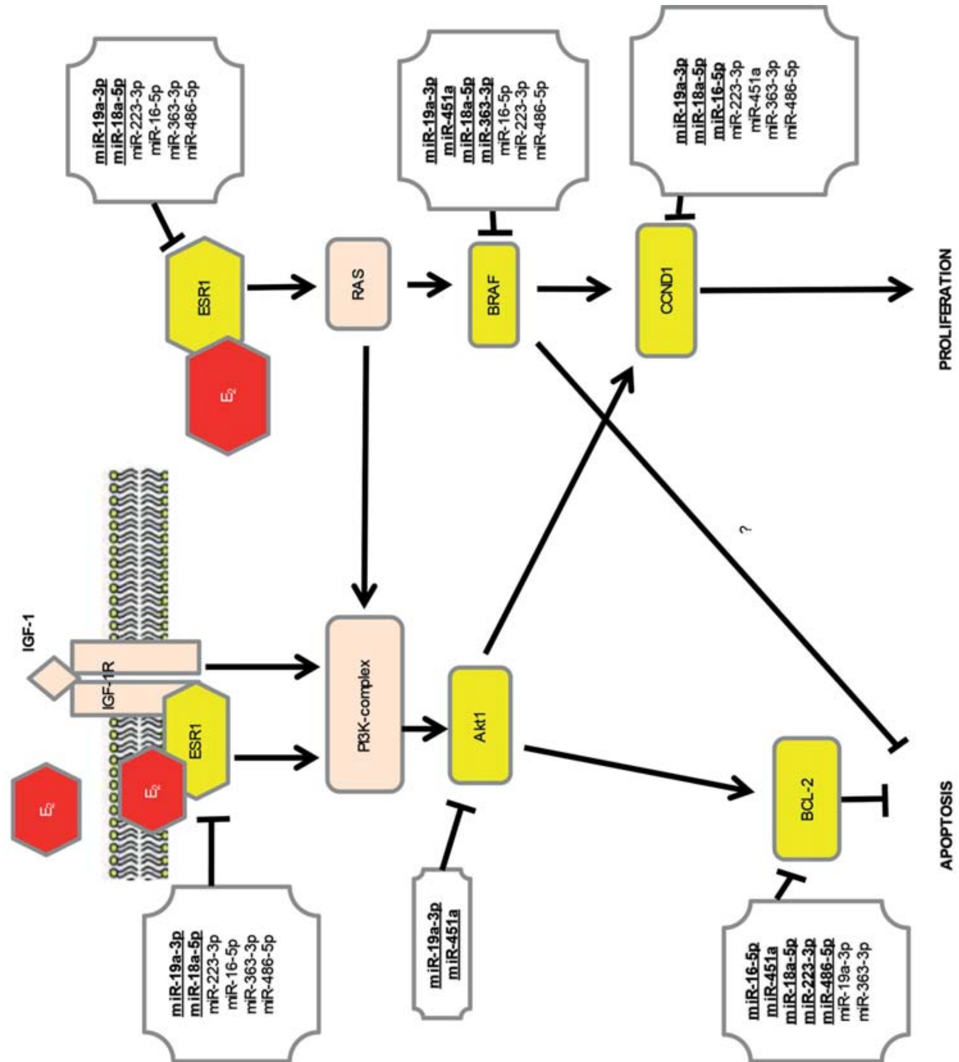
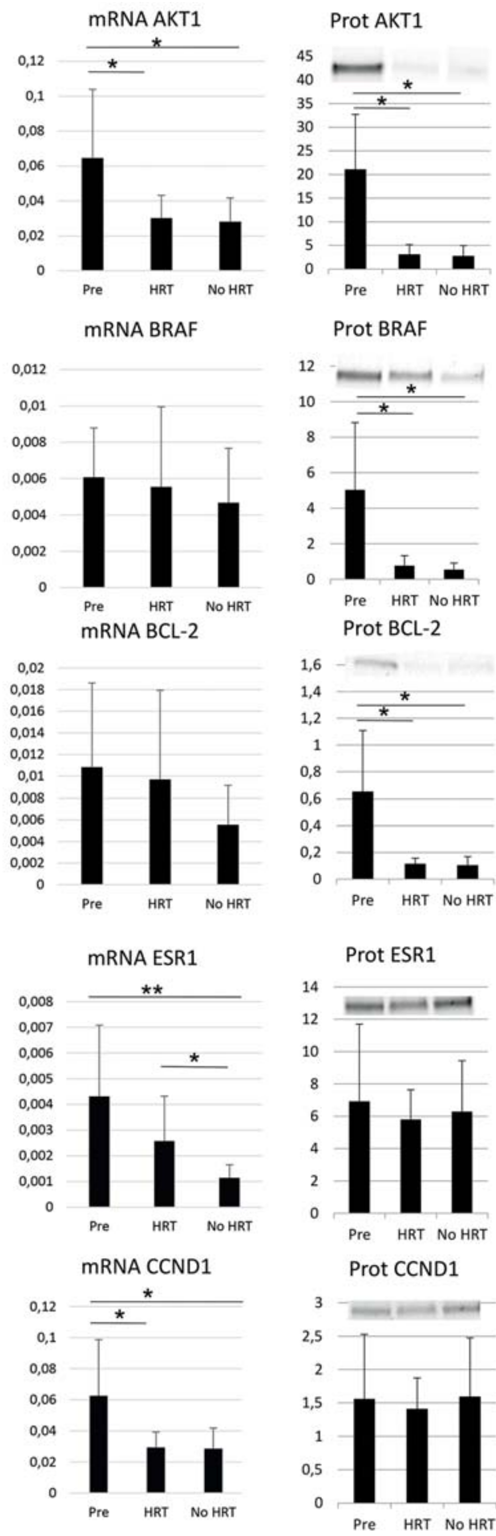


FIGURE 6



III

CIRCULATING MIR-21, MIR-146A AND FAS LIGAND RESPOND TO POSTMENOPAUSAL ESTROGEN-BASED HORMONE REPLACEMENT THERAPY – A STUDY WITH MONOZYGOTIC TWIN PAIRS

by

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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, which are partly due to changes in the hormonal profile. MicroRNAs regulate various processes associated with cell senescence; differentiation, replication and apoptosis. Serum microRNAs have 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 biopsy samples from 54 to 62 year-old postmenopausal monozygotic twins ($n = 11$ pairs) discordant for HRT usage. Premenopausal 30–35 year-old women ($n = 8$) were used as young controls. We focused on the hormonal aging and on the interaction between HRT use and the modulation of miR-21, miR-146a and classical inflammation markers. Fas-ligand was analysed since it functions in both apoptosis and inflammation.

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

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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

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(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 (Pacifici et al., 1989, 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,b), 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-kβ-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-κB-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-1β) 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 signalling 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 E_2 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 = 13,888 pairs) (Kaprio and Koskenvuo, 2002). Invitations were sent to all postmenopausal MZ twin sister pairs born from 1943 to 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 and 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 $\leq$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 E_2 (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 E_2 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.

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

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 μl 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 μl, containing 3.35 μl of sample RNA, 2 μl of primers, 1.26 μl of RNase inhibitor (diluted 1:10), 1 μl of 10× buffer, 1 μl of 10 mM dNTP's, 0.66 μl of reverse transcriptase and 0.73 μl nuclease free H_2O. 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 μl, containing 0.5 μl 20× Taqman MicroRNA Assay (5′-FAM PCR primers and probes), 5 μl of Taqman® Universal Master Mix 2× (Applied Biosystems) and 1.33 μl 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 μl, which contained 1.67 μl of sample RNA, 1 μl of each stem-loop primer, 0.6 μl of RNA inhibitor (diluted 1:10), 0.5 μl of 10× buffer, 0.4 μl of 10 mM dNTP's, 0.3 μl reverse transcriptase and 0.5 μl H_2O. 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 μl and contained 0.25 μl of the 20x Taqman MicroRNA Assay (5′-FAM PCR primers and probes), 2.75 μl of the TaqMan Fast Universal PCR Master Mix (2×) (Applied Biosystems) and 2.25 μl 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 $\Delta Ct = \text{mean } Ct_{miR\ X} - \text{mean } Ct_{miR\ 17}$ and $\Delta Ct = \text{mean } Ct_{miR\ X} - \text{mean } Ct_{RNU44}$ 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® 1000 (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 Jylhava et al. (2012). Serum 17β-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

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.
Statistically significant results are marked as bold.

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 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, clear 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 ($p = 0.030$). 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

Fig. 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) (Fig. 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 ($r = 0.709$; $p = 0.022$).

4. Discussion

The purpose of this study was to investigate if estrogen or estrogen and progesterone containing postmenopausal HRT interplays with

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.
Statistically significant results are marked as bold.

Table 4
Serum (s-) and tissue miR relative expressions in arbitrary units (AU) and serum FasL concentrations and tissue transcript levels.

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**$^{\mathbf{M}}$	**0.001**$^{\mathbf{M}}$	**0.018**$^{\mathbf{W}}$	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**$^{\mathbf{W}}$	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**$^{\mathbf{M}}$	**0.021**$^{\mathbf{W}}$	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.
Statistically significant results are marked as bold.

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 E_2 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 E_2 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". 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

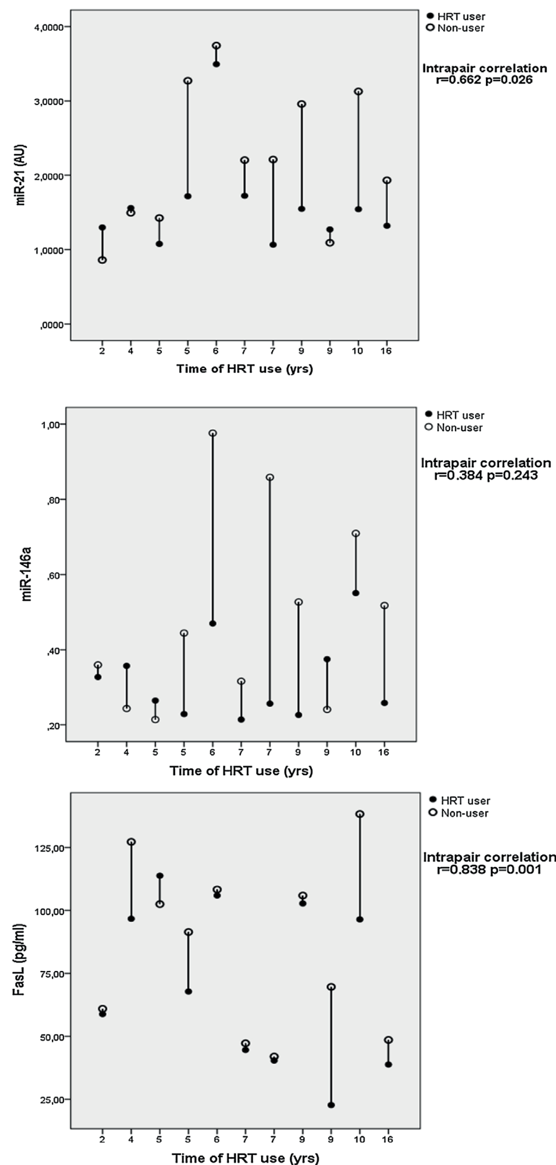

Fig. 1. Pairwise values in relation to the time of HRT use in years, and overall intrapair correlations of circulating miR-21, miR-146a transcripts and FasL serum concentrations.

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 sgb130, were lower in HRT users than the non-user co-twins, suggesting the E_2 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-1B, IL-6, TNFα 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 E_2 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

Table 5
Spearman correlations of circulating miR-21 and miR-146a transcript levels and FasL concentrations with measured inflammation parameters.

	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[a]	0.519
sgbp130 (ng/ml)	0.393	0.257	0.055	0.881	0.295[a]	0.243
cfDNA (μg/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						
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[a]	0.109
sgbp130 (ng/ml)	0.287	0.422	−0.018	0.960	0.154[a]	0.496
cfDNA (μg/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						
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[a]	0.907
sgbp130 (ng/ml)	0.098	0.789	**0.709**	**0.022**	**0.525**[a]	**0.040**
cfDNA (μg/ml)	−0.009	0.979	−0.064	0.853	0.033	0.894

Statistically significant results are marked as bold.
[a] $n = 10$ pairs.

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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2014.11.001.

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IV

DECLINING PHYSICAL PERFORMANCE ASSOCIATES WITH SERUM FASL, MIR-21, AND MIR-146A IN AGING SPRINTERS

by

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Research Article

Declining Physical Performance Associates with Serum FasL, miR-21, and miR-146a in Aging Sprinters

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Aging is associated with systemic inflammation and cellular apoptosis accelerating physiological dysfunctions. Whether physically active way of life affects these associations is unclear. This study measured the levels of serum inflammatory and apoptotic molecules, their change over 10 years, and their associations with physical performance in sprint-trained male athletes. HsCRP, cell counts, HGB, FasL, miR-21, and miR-146a were measured cross-sectionally ($n = 67$, 18–90 yrs) and serum FasL, miR-21, and miR-146a and their aging-related associations with physical performance were assessed over a 10-year follow-up ($n = 49$, 50–90 yrs). The cross-sectional study showed positive age correlations for neutrophils and negative for lymphocytes, red blood cells, HGB, FasL, and miR-146a. During the 10-year follow-up, FasL decreased ($P = 0.017$) and miR-21 ($P < 0.001$) and miR-146a ($P = 0.005$) levels increased. When combining the molecule levels, aging, and physical performance, FasL associated with countermovement jump and bench press ($P < 0.001$), miR-21 and miR-146a with knee flexion ($P = 0.023$; $P < 0.001$), and bench press ($P = 0.004$; $P < 0.001$) and miR-146a with sprint performance ($P < 0.001$). The studied serum molecules changed in an age-dependent manner and were associated with declining physical performance. They have potential as biomarkers of aging-related processes influencing the development of physiological dysfunctions. Further research is needed focusing on the origins and targets of circulating microRNAs to clarify their function in various tissues with aging.

1. Introduction

Physical exercise affects inflammatory state. The common understanding is that an acute bout of exercise results in a temporal inflammatory response, while regular training has a protective anti-inflammatory effect [1–4]. In addition to immune cells, skeletal muscle tissue contributes to the inflammatory response by releasing inflammatory molecules, such as TNF-α and IL-6, following of acute exercise [5]. During inflammation, whether induced by age, disease, or acute exercise, controlling the cellular balance of the inflammatory cells is important. Apoptosis, or programmed cell destruction, is a crucial mechanism for maintaining cellular balance in all tissues. Disruptions in the cellular homeostasis lead to either an accumulation of poorly functioning cells or a deficit of important cells, both recognized in aging. One way to regulate immune cell homeostasis, and subsequent immune response, is through Fas ligand - Fas receptor (FasL-Fas) interaction on the cell surfaces, which leads to target cell destruction [6]. There are indications that habitual training influences the apoptotic processes of the immune system. According to Mooren et al. [7], the basal levels of leukocyte apoptosis as well as the levels of exercise-induced apoptosis are distinct between highly trained and poorly trained men, suggesting training-induced adaptation to leukocyte homeostasis.

Traces from inflammation and cellular apoptosis can be detected from the circulation by measuring specific molecules from the blood. In addition to the classical inflammation (e.g., CRP, IL6, and TNF-α) and apoptotic markers

Cross-sectional 2012	*Group A*	*Group B*	*Group C*	*Group D*
Age (yrs)	18 to 39	50 to 66	66 to 79	79 to 90
N	18	16	18	15

Follow-up baseline in 2002, end-point in 2012	Baseline	End-point	Baseline	End-point	Baseline	End-point
Age (yrs)	40 to 56	50 to 66	56 to 69	66 to 79	69 to 80	79 to 90
N	16	16	18	18	15	15

Figure 1: Descriptions of the study designs needed in the current study.

(cytochrome c, Fas, and FasL), the small RNA molecules called microRNAs (miRs) are novel and potentially even more sensitive tools for screening these physiological processes. miRs are noncoding RNAs regulating gene expression by blocking the translation of specific target mRNA into proteins. miRs are released, either actively or passively, from different cell types into the blood stream, where they reflect the gene regulation changes in their cells of origin. Their role in intercellular communication has also been recognized [8]. In healthy conditions, circulating miRs most likely originate from blood or epithelial cells or from organs with high vascularization, including skeletal muscle cells [9]. Owing to trauma, cancer, cardiovascular disease, or other conditions influencing metabolism, the miR signature in the circulation changes.

mir-21 and miR-146a are among the miRs that seem to be responsive to various physiological stimuli. These miRs are associated with aging-related processes such as senescence and inflammation, miR-21 having proinflammatory and miR-146a having anti-inflammatory effects [10, 11]. Both miRs induce apoptosis by targeting FasL-Fas signaling, which strengthens the interplay of these molecules with each other and their role as regulators of immune cell homeostasis [12, 13]. Circulating miR-21 has also been shown to promote cachexia-related apoptosis in skeletal muscle cells [14]. Physical exercise creates a whole body adaptive responses that also affect miR regulation and expression in different cell types and subsequently in the circulation [15–17]. miR-21 and miR-146a have also been shown to be highly responsive to physical exercise. The pioneering work by Baggish et al. [15] demonstrated that these miRs were associated with cardiovascular/musculoskeletal adaptations and low-grade inflammation and changed in response to acute exercise as well as sustained training. Circulating miR-146a levels were upregulated by acute cycling exercise before and after 90 days of sustained row training, whereas miR-21 was only upregulated by acute exercise prior to the sustained training period. In addition, the highest levels of miR-146a during exercise were found to correlate with peak VO_{2max} [15], whereas miR-21 levels have been shown to be upregulated in males with low VO_2 max, indicating the contrasting roles of these two miRs [18]. Nielsen et al. [17] demonstrated that miR-146a decreased immediately after a bout of acute exercise, whereas the basal levels of miR-21 were downregulated after 12 weeks of endurance training. Both miR-21 and miR-146a have also been shown to exist at different levels in the plasma of young male endurance and strength trained athletes [19]. The above-mentioned studies demonstrate that these specific miRs detected in the circulation are affected differently by exercise type and duration and have potential as indicators of physiological changes [15, 16, 19, 20].

In order to evaluate if circulating FasL, miR-21, and miR-146a have the potential as biomarkers of training adaptations in aging athletes, longitudinal studies are needed. The aim of this study was to determine whether circulating FasL, miR-21, and miR-146a levels change over 10-year period among competitive male masters sprinters with a long-term training background and to assess their associations with physical performance measures and aging.

2. Methods

2.1. Study Design and Ethics. This study is part of an ongoing Athlete Aging Study (ATHLAS) [21–25] on young adult athletes and masters athletes from different sport disciplines. The participants were recruited from the memberships of Finnish athletic organizations. The present study comprised male sprinters. The sprinters had a long-term background in sprint training and had been successful in national or international 100–400 m sprinting events. Both cross-sectional and longitudinal study designs were used (Figure 1). In the cross-sectional analysis (using data from follow-up measurements conducted in 2012), the sprinters were divided into four age groups: (A) 18 to 39 years ($n = 18$), (B) 50 to 66 years ($n = 16$),

(C) 66 to 79 years ($n = 18$), and (D) 79 to 90 years ($n = 15$). In addition, baseline measurements (conducted in 2002) were available for 49 older masters sprinters (aged 50 to 90 years at follow-up) belonging to age groups B, C, and D, thereby allowing longitudinal analysis. Both the baseline and the follow-up measurements consisted of similar standardized two-day measurements.

Prior to the measurements, training and health history were elicited and evaluated using questionnaires. The average number of training years among the masters athletes at baseline was 34.1 ± 15.1 years. More detailed self-reported training histories (training frequency, sprint-specific training hours, and other training hours) and their changes over 10 years are presented in Tables 1 and 3. The sprint-specific training hours per week included sprints, jumps, and strength training, while the other training hours included all other notably strenuous physical exercises. Subjects over age 55 underwent a medical examination. Current ability to participate in physically demanding measurement was assessed ad hoc, individually by the study physician. The health of all the participants was in general good with no acute conditions (infections, traumas) or functionally limiting chronic neurological, cardiovascular, endocrinological, or musculoskeletal conditions. In a few cases, some physical tests were not performed due to local musculoskeletal pain.

The study was conducted according to the guidelines of the Declaration of Helsinki. All participants were informed a priori about the possible risks and discomfort of the physical and clinical measurements. Written informed consent, including permission for the use of the gathered data for research purposes only, was provided by the study subjects. The study protocol was approved by the ethics committees of the University of Jyväskylä (in 2002 for baseline) and the Central Finland Healthcare District (in 2012 for follow-up).

2.2. Physical Performance and Body Composition Measurements. Participants were instructed to rest (no heavy training or competition) two days before the measurements. On the first measurement day, the participants performed a maximal 60 m sprint twice on an indoor running track with spiked running shoes. Explosive force production of the lower limbs was measured by a vertical countermovement jump (CMJ). Isometric knee flexion force and isometric upper limb force were measured by bench press performance using a David 200 dynamometer (David Fitness and Medical Ltd., Outokumpu, Finland). The best of 2 or 3 trials was recorded in the subsequent analyses. In addition, the assessment of total body fat and lean mass (LBM) was performed with bioelectrical impedance (Spectrum II, RJL System, Detroit, MI). More detailed descriptions of the measurements are given elsewhere [22–25]. Participants' meals during the measurement days were arranged by the research organizers.

2.3. Serum Analyses. To obtain serum, venous blood was collected on the second measurement day under standard fasting conditions at least 12 h after exercise. The serum samples were stored at −80°C until analyzed. Serum FasL concentration was measured using a Human Fas Ligand/TNFSF6 Quantikine® ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Reactions were performed as duplicates and a common sample was added to each plate in order to observe whether controlling for interassay variation was necessary. HsCRP measurements at baseline and follow-up were performed with an Immulite 1000 Immunoassay System. Leukocyte counts were part of the standard medical complete blood count analysis.

2.4. RNA Extraction and miR Analyses. The RNA extraction methods and miR-21 and miR-146a analyses have been described previously [26]. Briefly, total RNA was isolated from 100 μl of serum with a total RNA purification kit (Norgen Biotek Corporation, Thorold, ON, Canada) according to the manufacturer's protocol. Synthetic *C. elegans* Cel-miR-39 (5′-UCA CCG GGU GUA AAU CAG CUU G-3′, Invitrogen) (25 fM, concentration determined by dilution series) was added at the lysis step to all of the samples as a spike-in control in order to monitor the efficiency and uniformity of the RNA extraction and qPCR procedure. RNA was reverse-transcribed to cDNA ($V_{tot} = 10\,\mu l$) by a Taqman reverse transcription kit and the qPCR ($V_{tot} = 10\,\mu l$) was performed with a Taqman Universal Mastermix II NO Ung using Taqman miR assays (hsa-miR-21: 5′-UAGCUUAUCAGACUGAUGUUGA-3′, hsa-miR-146a: 5′-UGAGAACUGAAUUCCAUGGGUU-3′) (Device: Applied Biosystems, ABI 7300).

Ct values less than 35 were accepted for the analysis. All the samples were normalized to a reference sample of an average sprint athlete across the plates. ΔCt values were calculated as $\Delta Ct = \text{mean } Ct_{miR\text{-}X} - \text{mean } Ct_{miR\,(reference)}$. Each reaction was performed in duplicate and the relative expressions were calculated by using the $2^{-\Delta Ct}$ method. To observe the possible contaminations and primer-dimers, no template controls (NTCs) were used in either the RT or qPCR reactions.

2.5. Statistical Analyses. In the age-correlation analyses of the cross-sectional design, Pearson's correlation coefficient was used for continuous variables and Spearman's correlation coefficient for ordinal variables. The paired sample t-test for parametric variables and Wilcoxon signed rank test for nonparametric variables were used in the longitudinal comparison in the 10-year follow-up design. In addition, Generalized Estimating Equations (GEE) models were constructed to study the association of FasL, miR-21, and miR-146a with the performance measures over age. The model was based on the two measurements points (2002, 2012) used in the longitudinal study with unstructured working correlation matrix specification and athletes' age as the descriptive metric of time. As the effect of age is generally nonlinear we used polynomial terms (quadratic and cubic) of age to include curvature in modeling the age-related effect of the FasL and the miRs on the outcomes. The results were adjusted for LBM. More detailed prediction equations based on GEE-models are presented in the supplementary data (S1 in Supplementary Material available online at https://doi.org/10.1155/2017/8468469).

Table 1: Self-reported training history and physical performance measures in different age groups and correlations of the variables with age.

	A 18–39 yrs (n = 18)	B 50–66 yrs (n = 16)	C 66–79 yrs (n = 18)	D 79–90 yrs (n = 15)	Correlation with age (all groups)/P value	95% CI/coefficient of determination	Correlation with age (only B, C, D)/P value	95% CI/coefficient of determination
Self-reported training								
Frequency (times/wk)	6.8 ± 2.3 (n = 15)	3.6 ± 1.6 (n = 14)	3.5 ± 1.3	3.1 ± 1.1 (n = 14)	−0.659 (n = 61) **P < 0.001**	−0.8 to −0.5 0.434	−0.176 (n = 46) P = 0.241	−0.4 to 0.1 0.031
Sprint training (h/wk)	7.0 ± 5.5	2.6 ± 1.7 (n = 14)	3.2 ± 3.2	2.7 ± 2.3 (n = 14)	-0.380^{S} (n = 64) **P = 0.002**	−0.6 to −0.1 0.144	-0.085^{S} (n = 46) P = 0.573	−0.3 to 0.2 0.007
Other training (h/wk)	2.5 ± 4.2	0.4 ± 0.6 (n = 14)	1.3 ± 2.0	2.0 ± 2.5 (n = 14)	0.179^{S} (n = 64) P = 0.157	−0.1 to 0.4 0.032	0.310^{S} (n = 46) **P = 0.036**	0.0 to 0.6 0.096
Physical performance								
Sprint 60 m (s)	7.52 ± 0.36 (n = 12)	8.43 ± 0.63 (n = 13)	9.29 ± 0.61 (n = 13)	10.81±1.30 (n = 10)	0.898^{S} (n = 48) **P < 0.001**	0.8 to 0.9 0.806	0.841^{S} (n = 36) **P < 0.001**	0.7 to 0.9 0.707
CMJ (cm)	45.7 ± 11.8 (n = 15)	32.0 ± 4.2 (n = 12)	25.8 ± 5.0 (n = 12)	18.9 ± 4.7 (n = 11)	−0.810 (n = 50) **P < 0.001**	−0.9 to −0.7 0.656	−0.782 (n = 35) **P < 0.001**	−0.9 to −0.6 0.612
Isometric knee flexion (N)	326 ± 86 (n = 17)	253 ± 57 (n = 15)	227 ± 34 (n = 14)	190 ± 49 (n = 12)	−0.647 (n = 58) **P < 0.001**	−0.8 to −0.5 0.419	−0.469 (n = 41) **P = 0.002**	−0.7 to −0.2 0.220
Isometric bench press (N)	1307 ± 347 (n = 17)	945 ± 159 (n = 14)	717 ± 175 (n = 13)	565 ± 126 (n = 11)	−0.777 (n = 55) **P < 0.001**	−0.9 to −0.6 0.604	−0.776 (n = 38) **P < 0.001**	−0.9 to −0.6 0.602

Table is formed based on the cross-sectional study design (2012) including all the athletes from ages 18 to 90 yrs. Results are presented as means ± SD. Age correlations are presented: (1) all athletes and (2) masters athletes only. CMJ: countermovement jump. ^{S}Spearman's correlation coefficient.

3. Results

3.1. Participant Characteristics and Serum FasL and miR Levels in Different Age Groups. Participants' characteristics in the different age groups and age correlations of the variables are presented in Tables 1 and 2. The age correlations are presented in two different ways: (1) inclusive of all the participants (ages 18 to 90) and (2) inclusive only of the masters athletes (ages 50 to 90). A modest negative correlation was observed between age and training frequency when all the athletes were included in the analyses ($r = -0.659$, 95% CI = −0.8 to −0.5, $P < 0.001$), with age accounting for 43.3% of the variation in training frequency. The same correlation was very low when only the masters athletes were included ($r = -0.176$, 95% CI = −0.4 to 0.1, $P = 0.241$), with age accounting only for 3.1% of the variation. Among the physical performance measures, a high positive correlation was found between age and 60 m sprint time among all the athletes ($r = 0.898$, 95% CI = 0.8 to 0.9, $P < 0.001$), with age accounting for 80.6% of the variation in sprint time. The pattern was similar when only the masters athletes were included in the analyses ($r = 0.841$, 95% CI = 0.7 to 0.9, $P < 0.001$), with age accounting for 70.7% of the variation in sprint time. The other physical performance measures showed a modest to high negative correlation with age when all the athletes were included (CMJ: $r = -0.81$, 95% CI = −0.9 to −0.7, $P < 0.001$, 65.6%; knee flexion: $r = -0.647$, 95% CI = −0.8 to −0.5, $P < 0.001$, 41.9%; and bench press: $r = -0.777$, 95% CI = −0.9 to −0.6, $P < 0.001$, 60.4%). When only the masters athletes were included, high negative correlations were observed between age and CMJ and age and bench press (CMJ: $r = -0.782$, 95% CI = −0.9 to −0.6, $P < 0.001$; bench press: $r = -0.776$, 95% CI = −0.9 to −0.6, $P < 0.001$), with age accounting for 61.2% and 60.2% of the variation, respectively. Between age and knee flexion, only a modest negative correlation was detected ($r = -0.469$, 95% CI = −0.7 to −0.2, $P = 0.002$), with age accounting for 22% of the variation in knee flexion strength.

Among the body anthropometric variables, modest negative correlations were observed between age and height, age and weight, and age and LBM when all the athletes were included (height: $r = -0.512$, 95% CI = 0.7 to −0.3, $P < 0.001$; weight: $r = -0.419$, 95% CI = −0.6 to −0.2, $P < 0.001$; LBM: $r = -0.525$, 95% CI = −0.7 to −0.3, $P < 0.001$), with age accounting for 26.2%, 17.6%, and 27.6% of the variation, respectively. Of the blood cell counts, low to modest negative correlations were detected between age and lymphocyte percentage and age and RBC when all the athletes were included (LYM%: $r = -0.384$, 95% CI = −0.6 to −0.2, $P = 0.002$; RBC: $r = -0.433$, 95% CI = −0.6 to −0.2, $P < 0.001$), with age accounting for 14.7% and 18.7% of the variation, respectively. In addition, a modest positive correlation was detected between age and neutrophil percentage ($r = 0.411$, 95% CI = 0.2 to 0.6, $P = 0.001$), with age accounting for 16.9% of the variation in neutrophils. When only the masters athletes were included, the pattern was similar in slightly weaker age correlations (LYM%: $r = -0.315$, 95% CI = −0.6 to 0.0, $P = 0.031$; RBC: $r = -0.370$, 95% CI = −0.6 to −0.1, $P = 0.010$; and NEUT%: $r = 0.326$, 95% CI = 0.0 to 0.6, $P = 0.025$), with age accounting for 9.9%, 13.7%, and 10.6% of the variation, respectively. A low negative correlation was observed between age and HGB both when all the athletes were included ($r = -0.383$, 95% CI = −0.6 to −0.2, $P = 0.002$) and when only the masters athletes were included ($r = -0.370$, 95% CI = −0.6 to −0.1, $P = 0.010$), with age accounting for 14.7% and 13.7% of the variation in HGB, respectively. Of the studied serum molecules, a modest negative correlation between age and FasL was detected when all the athletes were included in the analyses ($r = -0.596$, 95% CI = −0.7 to −0.4, $P < 0.001$), with age accounting for 35.5% of the variation in FasL concentration. A modest positive correlation was detected between age and miR-146a when all the athletes were included ($r = -0.611$, 95% CI = −0.7 to −0.4, $P < 0.001$), with age accounting for 37.3% of the miR-146a variation. No significant correlations between age and the serum molecules were detected when only masters athletes were included in the analyses.

3.2. Changes in the Physical Performance of Masters Athletes over 10 Years. Changes in the training frequencies and the change percentage of the specific physical performance measures in 10 years are presented in Table 3. The results are shown for masters sprinters as a whole and divided into 3 age groups (B: 50–66 yrs, C: 66–79 yrs, and D: 79–90 yrs presenting the end-point ages). The self-reported weekly training frequency, sprint, and other training hours decreased when all the masters athletes were involved in the analyses ($P < 0.001$, $P < 0.001$, and $P = 0.003$, resp.). When grouping the athletes, the declines in all the self-reported training activities remained significant for the group B ($P = 0.011$, $P = 0.009$, and $P = 0.008$, resp.). For group C, no significant changes in the training activities over 10 years were reported. For the group D, weekly training frequency and sprint training hours decreased significantly ($P = 0.004$, $P = 0.023$, resp.). From the physical performance measures 60 m sprint, CMJ and isometric bench press changed during the 10 years' follow-up period when all the masters sprinters were included ($P < 0.001$ for all measures). When the masters sprinters were divided into 3 age groups the changes remained for all groups B ($P = 0.001$, $P = 0.001$, and $P = 0.036$, resp.), C ($P = 0.002$, $P = 0.001$, and $P = 0.008$, resp.), and D ($P = 0.005$, $P < 0.001$, and $P < 0.001$, resp.), with the highest deterioration seen among the oldest athletes (group D). There were no significant changes in the knee flexion strength in 10 years.

3.3. Changes in the Serum hsCRP, FasL, miR-21, and miR-146a Levels of Masters Athletes over 10 Years. The change percentages of the blood parameters, hsCRP, FasL, miR-21, and miR-146a over 10 years are presented in Table 3. The results are shown for the masters sprinters both as a whole and divided into 3 age groups (B: 50–66 yrs, C: 66–79 yrs, and D: 79–90 yrs presenting the end-point ages). Serum hsCRP did not change over the 10-year period among the athletes. The serum FasL concentrations decreased ($P = 0.017$) and serum miR-21 and miR-146a levels increased ($P < 0.001$, $P < 0.005$, resp.) among the masters sprinters as a single group during the 10 years. At the age-group level, the changes were

Table 2: Participant characteristics and blood parameters in different age groups and correlations of the variables with age.

	A 18–39 yrs (n = 18)	B 50–66 yrs (n = 16)	C 66–79 yrs (n = 18)	D 79–90 yrs (n = 15)	Correlation with age (all groups)/P value	95% CI/coefficient of determination	Correlation with age (only B, C, D)/P value	95% CI/coefficient of determination
Anthropometrics and body composition								
Height (cm)	180.1 ± 5.0	178.2 ± 8.1	172.8 ± 4.7	172.4 ± 4.9	−0.512 (n = 67) **P < 0.001**	−0.7 to −0.3 0.262	−0.464 (n = 49) **P = 0.001**	−0.7 to −0.2 0.215
Weight (kg)	78.3 ± 6.8	80.2 ± 9.7	71.0 ± 6.2	69.4 ± 6.4	−0.419 (n = 67) **P < 0.001**	−0.6 to −0.2 0.176	−0.568 (n = 49) **P < 0.001**	−0.7 to −0.3 0.323
LBM (kg)	67.2 ± 5.7	66.3 ± 7.0	60.2 ± 4.1	58.3 ± 4.8	−0.525 (n = 67) **P < 0.001**	−0.7 to −0.3 0.276	−0.564 (n = 49) **P < 0.001**	−0.7 to −0.3 0.318
Body fat mass (kg)	11.2 ± 4.1	13.9 ± 6.1	11.1 ± 3.7	11.2 ± 3.6	−0.028 (n = 67) P = 0.857	−0.3 to 0.2 0.001	−0.301 (n = 49) **P = 0.035**	−0.5 to 0.0 0.091
Blood cell count								
WBC	5.6 ± 1.4	5.6 ± 1.0	5.7 ± 2.2 (n = 17)	5.4 ± 0.8 (n = 14)	−0.057 (n = 65) P = 0.652	−0.3 to 0.2 0.003	0.099 (n = 47) P = 0.509	−0.2 to 0.4 0.010
LYM%	39.2 ± 7.2	35.9 ± 6.2	34.2 ± 5.6 (n = 17)	31.0 ± 9.4 (n = 14)	−0.384 (n = 65) **P = 0.002**	−0.6 to −0.2 0.147	−0.315 (n = 47) **P = 0.031**	−0.6 to 0.0 0.099
MXD%	12.3 ± 4.8	11.5 ± 3.0	11.5 ± 1.9 (n = 17)	10.9 ± 2.4 (n = 14)	−0.174 (n = 65) P = 0.165	−0.4 to 0.1 0.030	−0.112 (n = 47) P = 0.455	−0.4 to 0.2 0.013
NEUT%	48.6 ± 8.5	52.6 ± 6.9	54.3 ± 6.6 (n = 17)	58.1 ± 9.5 (n = 14)	0.411 (n = 65) **P = 0.001**	0.2 to 0.6 0.169	0.326 (n = 47) **P = 0.025**	0.0 to 0.6 0.106
RBC	5.1 ± 0.4	4.9 ± 0.4	4.9 ± 0.4 (n = 17)	4.4 ± 0.3 (n = 14)	−0.443 (n = 65) **P < 0.000**	−0.6 to −0.2 0.187	−0.370 (n = 47) **P = 0.010**	−0.6 to −0.1 0.137
PLT	211.9 ± 44.0	242.3 ± 44.3	210.0 ± 83.6 (n = 17)	199.7 ± 42.9 (n = 14)	−0.100 (n = 65) P = 0.429	−0.3 to 0.1 0.010	−0.375 (n = 47) **P = 0.009**	−0.6 to −0.1 0.141
HGB	154.3 ± 9.3	152.9 ± 12.4	149.4 ± 9.3 (n = 17)	140.3 ± 8.0 (n = 14)	−0.383 (n = 65) **P = 0.002**	−0.6 to −0.2 0.147	−0.443 (n = 47) **P = 0.002**	−0.6 to −0.2 0.187
Serum molecules								
hsCRP (mg/L)	1.6 ± 5.1	1.7 ± 3.3	2.3 ± 6.0 (n = 17)	1.3 ± 1.2 (n = 14)	−0.035 (n = 65) 0.784	−0.3 to 0.2 0.001	−0.102 (n = 47) P = 0.999	−0.4 to 0.2 0.010
FasL (pg/ml)	92.3 ± 24.7	56.8 ± 21.9 (n = 14)	56.0 ± 18.2 (n = 14)	52.3 ± 23.4 (n = 13)	−0.596 (n = 59) **P < 0.001**	−0.7 to −0.4 0.355	−0.181 (n = 41) P = 0.258	−0.5 to 0.1 0.033
miR-21 (RE)	1.81 ± 0.75	2.22 ± 1.41	1.74 ± 1.05	1.65 ± 0.62	-0.095^{S} (n = 67) P = 0.444	−0.3 to 0.1 0.009	-0.118^{S} (n = 49) P = 0.419	−0.4 to 0.2 0.014
miR-146a (RE)	5.82 ± 2.66	1.83 ± 1.16	1.50 ± 0.71	1.51 ± 0.96	-0.611^{S} (n = 67) **P < 0.001**	−0.7 to −0.4 0.373	-0.105^{S} (n = 49) P = 0.473	−0.4 to 0.2 0.011

Table is formed based on the cross-sectional study design (2012) including all the athletes from ages 18 to 90 yrs. Results are presented as means ± SD. Age correlations are presented in two ways: (1) one including all the athletes and (2) one including only masters athletes. LBM: lean body mass, WBC: white blood cells, LYM: lymphocytes, MXD: mixed leukocytes, NEUT: neutrophils, RBC: red blood cells, PLT: platelet, HGB: hemoglobin, hsCRP: high sensitivity c-reactive protein, FasL: Fas-ligand, and RE: relative expression. ^{S}Spearman's correlation coefficient.

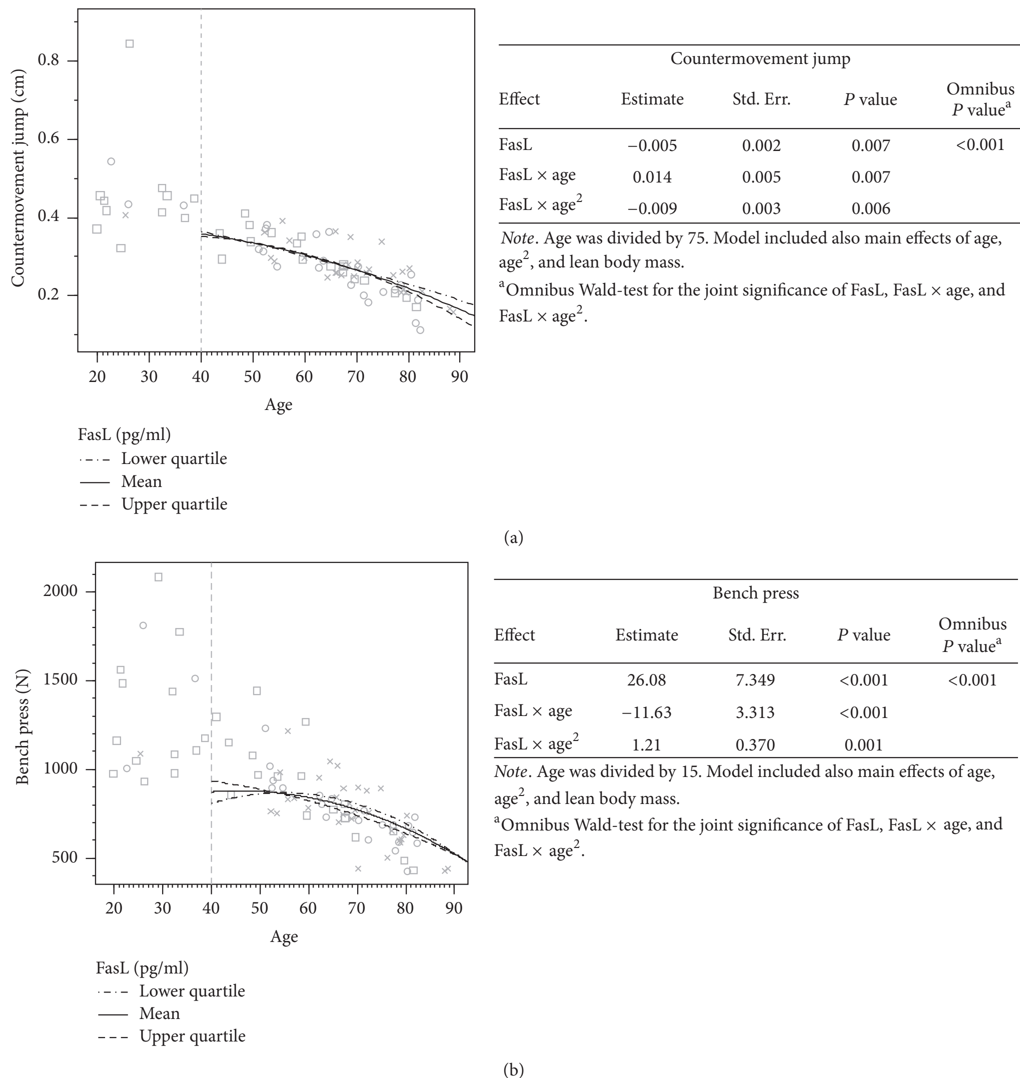
Table 3: The change in physical performance measures, self-reported training amounts, and serum molecule levels among all and age-grouped masters sprinters in the 10-year follow-up.

	All 50–90 yrs	B 50–66 yrs	C 66–79 yrs	D 79–90 yrs
Training frequency				
Change (times/wk)	−0.89 ± 1.22 (n = 42)	−0.91 ± 1.15 (n = 14)	−0.73 ± 1.43 (n = 15)	−1.06 ± 1.09 (n = 13)
P value	**P < 0.001**	**P = 0.011**	P = 0.067	**P = 0.004**
Sprint training				
Change (h/wk)	−1.84 ± 3.00 (n = 42)	−1.80 ± 2.25 (n = 14)	−1.94 ± 4.08 (n = 15)	−1.77 ± 2.43 (n = 13)
P values	**P < 0.001**	**P = 0.009**	P = 0.078	**P = 0.023**
Other training				
Change (h/wk)	−1.02 ± 2.62 (n = 32)	−1.53 ± 1.61 (n = 11)	−0.93 ± 3.44 (n = 11)	−0.56 ± 2.49 (n = 10)
P value	**P = 0.006**	**P = 0.008**	P = 0.221	P = *0.424*
Sprint 60 m (s)				
Change%	11.9 (6.7–16.5) (n = 35)	8.2 (5.7–10.0) (n = 13)	12.2 (5.7–16.4) (n = 12)	15.8 (12.1–22.8) (n = 10)
P value	**P < 0.001**^{W}	**P = 0.001**^{W}	**P = 0.002**^{W}	**P = 0.005**^{W}
CMJ (cm)				
Change%	−15.4 ± 10.2 (n = 32)	−9.2 ± 6.3 (n = 11)	−15.7 ± 10.0 (n = 11)	−22.0 ± 10.4 (n = 10)
P value	**P < 0.001**	**P = 0.001**	**P = 0.001**	**P < 0.001**
Knee flexion (N)				
Change%	−1.8 ± 23.6 (n = 40)	5.5 ± 29.0 (n = 15)	−3.6 ± 20.1 (n = 13)	−9.07 ± 18.0 (n = 12)
P value	P = 0.062	P = 0.896	P = 0.287	P = 0.111
Isometric bench press (N)				
Change%	−10.9 ± 11.9 (n = 36)	−5.3 ± 9.8 (n = 14)	−14.0 ± 14.7 (n = 12)	−14.9 ± 8.51 (n = 10)
P value	**P < 0.001**	**P = 0.036**	**P = 0.008**	**P < 0.001**
hsCRP				
Change%	112 ± 349 (n = 47)	203 ± 512 (n = 16)	71.5 ± 230 (n = 17)	56.8 ± 220 (n = 14)
P value	P = 0.841	P = 0.404	P = 0.868	P = 0.220
FasL				
Change%	−5.3 ± 26.3 (n = 41)	−18.3 ± 16.7 (n = 14)	1.22 ± 29.1 (n = 14)	1.78 ± 27.9 (n = 13)
P value	**P = 0.017**	**P = 0.001**	P = 0.746	P = 0.587
miR-21				
Change%	77.0 (1.1–689) (n = 49)	107 (21.0–662) (n = 16)	37.9 (−34.7–834) (n = 18)	136 (21.3–995) (n = 15)
P value	**P < 0.001**^{W}	**P = 0.007**^{W}	P = 0.267^{W}	**P = 0.017**^{W}
miR-146a				
Change%	95.4 (−7.4–631) (n = 49)	93.0 (−3.0–1101) (n = 16)	18.6 (−26.0–444) (n = 18)	143 (53.6–861) (n = 15)
P value	**P = 0.005**^{W}	P = 0.079^{W}	P = 0.500^{W}	**P = 0.011**^{W}

The age ranges of the groups (B, C, and D) represent the ages at follow-up. Data are presented as means ± SD for parametric variables and as median (IQR) for nonparametric variables. ^{W}P value was calculated with paired t-test for normally distributed variables and with Wilcoxon signed rank test for nonparametric variables. ^{W}Wilcoxon signed rank test for nonparametric variables. CMJ = countermovement jump, hsCRP = high sensitivity C-reactive protein, and FasL: Fas-ligand.

significant for FasL and for miR-21 among the youngest masters sprinters (group B; $P = 0.001$; $P = 0.007$, resp.) and for miR-146a among the oldest group (D; $P = 0.011$). The change was also significant for miR-21 in the oldest group (group D, $P = 0.017$). The original values for the blood parameters are presented in supplementary data (S2).

3.4. FasL, miR-21, and miR-146a Associations with Physical Performance Measures in the 10-Year Follow-Up. A GEE model was constructed to combine the effects of a specific serum molecule (FasL, miR-21, or miR-146a) and the physical performance measures (60 m sprint, CMJ, knee flexion, or bench press) over time. The association curves for the physical performance measures according to the different measured circulating molecule levels are presented in Figures 2, 3, and 4. Only significant or trending curves are presented. The results for the models were adjusted with the LBM. Figure 2 shows that the combination of the effects of FasL



Countermovement jump				
Effect	Estimate	Std. Err.	P value	Omnibus P value[a]
FasL	−0.005	0.002	0.007	<0.001
FasL × age	0.014	0.005	0.007	
FasL × age^2	−0.009	0.003	0.006	

Note. Age was divided by 75. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of FasL, FasL × age, and FasL × age^2.

Bench press				
Effect	Estimate	Std. Err.	P value	Omnibus P value[a]
FasL	26.08	7.349	<0.001	<0.001
FasL × age	−11.63	3.313	<0.001	
FasL × age^2	1.21	0.370	0.001	

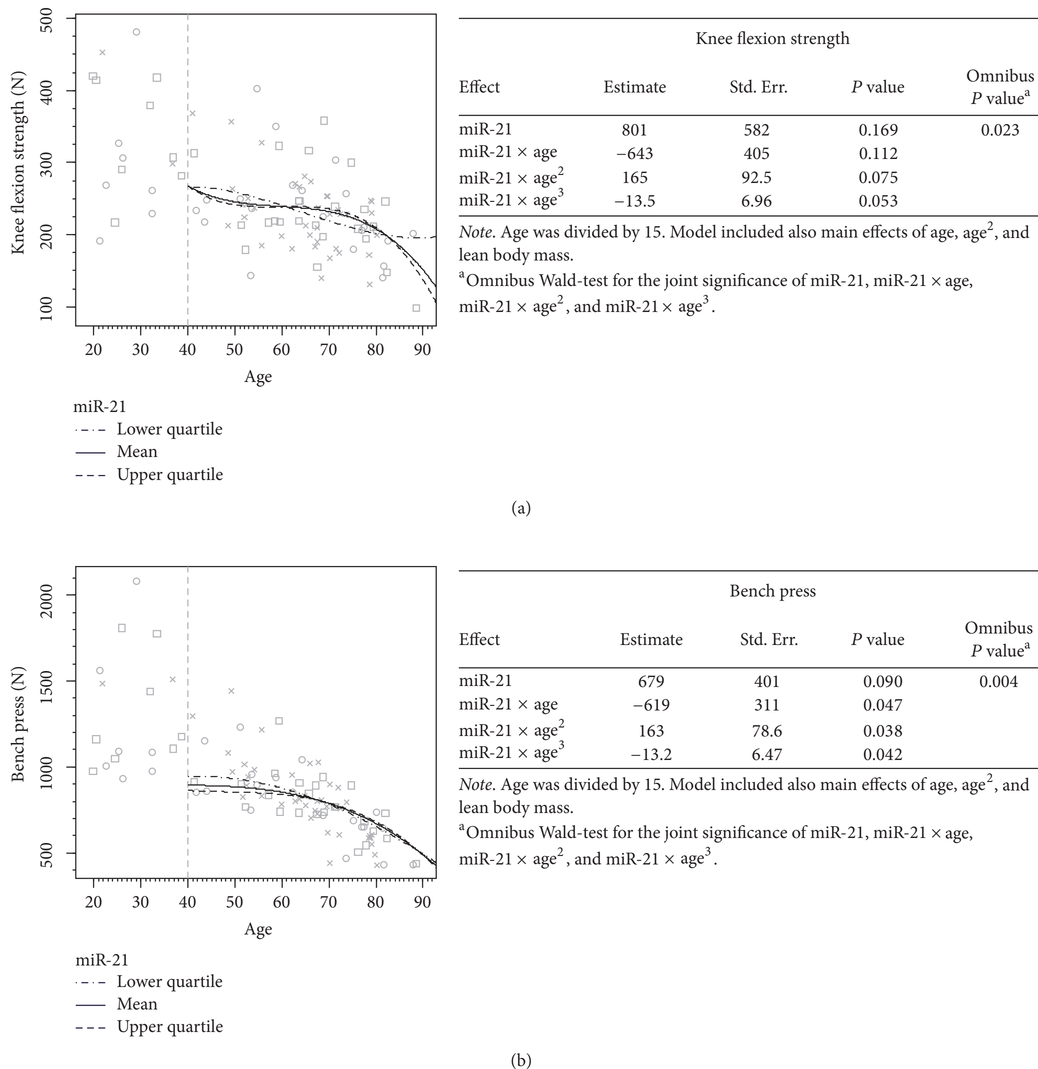
Note. Age was divided by 15. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of FasL, FasL × age, and FasL × age^2.

Figure 2: The association of serum FasL concentration with physical performance over time. Values for the younger participants (<40 years) without follow-up measures are presented on the left side of the images. The associations are based on the follow-up design (n = 49, >40 yrs). Cross (×) indicates that case is located within 0–37.5%, circle (○) within 37.5–67.5%, and square (□) within 67.5–100% of the cumulative share of the FasL distribution. The 3 different lines present the associations of the different serum marker levels with the physical performance measures over time. The tables next to the curves present the model used in forming the prediction curves, in greater detail, including statistics on the main effects of the studied serum marker and the possible quadratic and cubic effects.

and its interactions with age was statistically significant for the CMJ ($P < 0.001$) and bench press ($P < 0.001$). Also, all coefficient estimates for these outcomes were statistically significant indicating both a significant linear and quadratic curvature term. The model for the CMJ, when the serum FasL concentration was taken into account (Figure 2(a)), predicted a steeper decline in performance after age of 70 with slightly higher FasL serum concentrations than with lower FasL levels. For bench press, higher FasL levels at the younger ages predicted a steadier decline in performance while lower FasL values predicted better sustained performance at the older ages (Figure 2(b)).

Significant effects of miR-21 and age combined (Figure 3) were detected for knee flexion ($P = 0.023$) and bench press strength ($P = 0.004$). Statistically significant coefficient estimates relate mainly to miR-21 interaction with quadratic of



Knee flexion strength				
Effect	Estimate	Std. Err.	*P* value	Omnibus *P* value[a]
miR-21	801	582	0.169	0.023
miR-21 × age	−643	405	0.112	
miR-21 × age^2	165	92.5	0.075	
miR-21 × age^3	−13.5	6.96	0.053	

Note. Age was divided by 15. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of miR-21, miR-21 × age, miR-21 × age^2, and miR-21 × age^3.

Bench press				
Effect	Estimate	Std. Err.	*P* value	Omnibus *P* value[a]
miR-21	679	401	0.090	0.004
miR-21 × age	−619	311	0.047	
miR-21 × age^2	163	78.6	0.038	
miR-21 × age^3	−13.2	6.47	0.042	

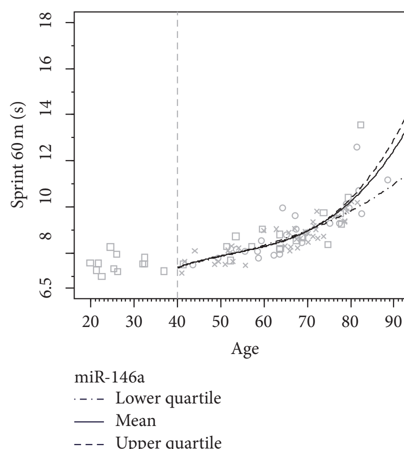
Note. Age was divided by 15. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of miR-21, miR-21 × age, miR-21 × age^2, and miR-21 × age^3.

Figure 3: The association of serum miR-21 level with physical performance over time. Values for the younger participants (<40 yrs) without follow-up measures are presented on the left side of the images. The predictions are based on the follow-up design ($n = 49$, >40 yrs). Cross (×) indicates that case is located within 0–37.5%, circle (○) within 37.5–67.5%, and square (□) within 67.5–100% of the cumulative share of the miR-21 -distribution. The 3 different lines present the associations of the different serum marker levels with the physical performance measures over time. The tables next to the curves present the model used in forming the prediction curves, in greater detail, including statistics on the main effects of the studied serum marker and the possible quadratic and cubic effects.

cubic terms of age indicating that curvature has a stronger role in the prediction. For knee flexion strength, when serum miR-21 levels were taken into account, low miR-21 levels predicted best performance prior to age 60 and high levels best performance between ages 60 and 80 (Figure 3(a)). Low miR-21 levels predicted highest performance in the bench press until age 65, after which the prediction curves were very similar to each other (Figure 3(b)).

Significant combination effects of miR-146a and age (Figure 4) were detected for sprint ($P < 0.001$), knee flexion ($P < 0.001$), and bench press strength ($P < 0.001$). The miR-146a levels predicted the largest differences in 60 m sprint performance after the age of 70, after which the lowest values predicted the best sprint performance (Figure 4(a)). For knee flexion strength, the lowest miR-146a levels predicted the best performance until age 60, after which, until age

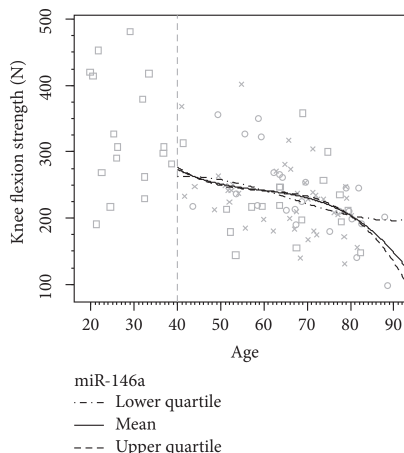


Sprint				
Effect	Estimate	Std. Err.	P value	Omnibus P value[a]
miR-146a	−7.43	2.24	0.001	<0.001
miR-146a × age	8.35	2.49	0.001	
miR-146a × age ^{2}	−3.06	0.908	0.001	
miR-146a × age ^{3}	0.367	0.108	0.001	

Note. Age was divided by 20. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of miR-146a, miR-146a × age, miR-146a × age ^{2}, and miR-146a × age ^{3}.

(a)

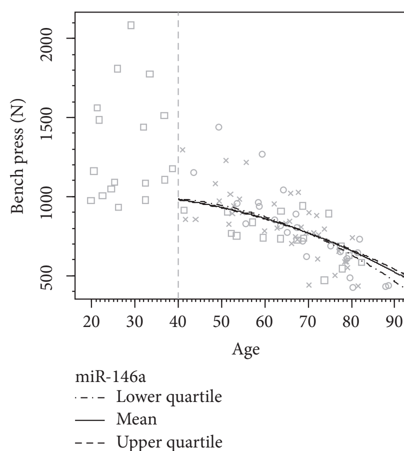


Knee flexion strength				
Effect	Estimate	Std. Err.	P value	Omnibus P value[a]
miR-146a	759	202	<0.001	<0.001
miR-146a × age	−788	197	<0.001	
miR-146a × age ^{2}	264	63.3	<0.001	
miR-146a × age ^{3}	−28.7	6.72	<0.001	

Note. Age was divided by 20. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of miR-146a, miR-146a × age, miR-146a × age ^{2}, and miR-146a × age ^{3}.

(b)



Bench press				
Effect	Estimate	Std. Err.	P value	Omnibus P value[a]
miR-146a	137	44.5	0.002	<0.001
miR-146a × age	−115	31.5	<0.001	
miR-146a × age ^{2}	21.7	5.39	<0.001	

Note. Age was divided by 20. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of miR-146a, miR-146a × age, miR-146a × age ^{2}, and miR-146a × age ^{3}.

(c)

Figure 4: The association of serum miR-146a level with physical performance over time. Values for the younger participants (<40 years) without follow-up measures are presented on the left side of the images. The predictions are based on the follow-up design (n = 49, >40 yrs). Cross (×) indicates that case is located within 0–37.5%, circle (○) within 37.5–67.5%, and square (□) within 67.5–100% of the cumulative share of the miR-146a distribution. The 3 different lines present the associations of the different serum marker levels with the physical performance measures over time. The tables next to the curves present the model used in forming the prediction curves, in greater detail, including statistics on the main effects of the studied serum marker and the possible quadratic and cubic effects.

80, the highest serum levels predicted the best performance in the knee flexion strength (Figure 4(b)). For bench press strength, the lowest miR-146a values predicted the highest performance until age 65, after which the lowest miR-146a levels predicted the steepest decline (Figure 4(c)).

4. Discussion

This study investigated the associations of circulating levels of traditional (hsCRP, leukocyte count, and FasL) and novel (miR-21 and miR-146a) inflammation- and apoptosis-related molecules with physical performance in competitive male sprinters of different ages. In addition, the associations of serum FasL, miR-21, and miR-146a levels with specific physical performance measures and aging were determined. We used both cross-sectional and follow-up study designs, with an emphasis on the latter, which focused on older masters sprinters. In the cross-sectional analysis, which included sprinters from ages 18 to 90 years, anthropometrics, LBM, physical performance measures, RBC, and HGB were, as expected, negatively associated with aging. For the traditional inflammation markers, no age-association was observed with hsCRP; instead, the percentage of serum lymphocytes decreased and that of neutrophils increased, with age. Serum FasL concentration and miR-146a levels correlated negatively with age when all the sprinters were included. However, when only the masters sprinters were studied, the age correlation was not significant, indicating that the most radical changes in these molecules generally occur during the interval between being a young sprinter and becoming a masters sprinter. The 10-year follow-up study design, which concerned masters sprinters only, showed, as expected, a worsening of physical performance in parallel with the decrement in the serum FasL and increment in the serum miR-21 and miR-146a levels. Interestingly, when grouped into 3 different age groups we obtained novel information about the time frames of the changes. For FasL and miR-21 the changes were significant for the 50- to 66-year-old (end-point age) sprinters and for miR-21 and miR-146a for the 79- to 90-year-old sprinters. No significant changes were observed in the 66–79-year-old athletes. In addition, associations with serum molecules, physical performance and aging were determined. We found nonlinear associations of circulating FasL concentration with CMJ height and bench press strength. MiR-21 levels were associated with knee flexion and bench press strength and miR-146a levels with sprint time, knee flexion, and bench press strength. The associations were based on the 10-year-follow-up data and age was used as a continuous determinant. Based on the constructed model, it is possible to predict whether and how the different levels of the studied serum molecules explain the declining physical performance measures over time.

Aging is accompanied with declining skeletal muscle properties and increasing numbers of systemic classical inflammatory markers, which, in general, affect physical functioning [27]. Inflammation and apoptosis are two crucial processes known to be altered during the aging process having broad physiological or even pathological influences in the body [28, 29]. Prolonged physical training has been shown to improve the systemic inflammatory state, especially by lowering hsCRP and IL-6 levels, as well as preventing the loss of muscle mass [3]. In the present study, hsCRP neither differed between the studied age groups nor changed during the 10-year follow-up among the masters athletes. Therefore, we focused on the more specific circulating molecules, FasL, miR-21, and miR-146a, interplaying with aging, inflammation, apoptosis, and skeletal muscle tissue [6, 10–14].

4.1. FasL as a Potential Biomarker. Serum FasL contributes to cellular homeostasis by inducing apoptosis of the target cells, especially T lymphocytes [6]. Serum FasL concentrations have been shown to decrease with aging [29, 30], with higher serum FasL levels being associated with diseases related to imbalanced homeostasis of the immune cells [31–33]. As the follow-up results show, the most radical change in serum FasL levels in the present study had occurred by age 66, with the levels having decreased significantly by that age. This could be interpreted as a decrement in the apoptotic rate. Lower serum FasL level predicted better overall performances (CMJ, bench press). In light of both these studies and our findings, the natural decrement in FasL during aging could be beneficial for balancing the changing metabolism and inflammatory status. However, conflicting studies and theories exist. The shift towards reduced apoptosis, measured by decreased serum FasL levels, could be followed by an accumulation of immune cells, resulting in "inflammaging" or an accumulation of other cell types, thereby increasing the risk for cancer development (reviewed by Tower [34]). However, the traditional inflammation marker hsCRP levels of the oldest athletes in the present study would appear to be in the normal healthy range and show no indication of an increased inflammatory state. Instead, the measured higher count of circulating neutrophils among older sprinters could be an indication of slightly higher inflammatory status compared to younger athletes. However, the role of neutrophils as driving forces of tissue repair and regeneration has also recently been discussed (reviewed by Jones et al. [35]). Immune cell homeostasis has been shown to differ between men with opposite training background [7]. The authors showed that the basal level of lymphocyte apoptosis, controlled by Fas-FasL interaction, is distinctly different between high and low trained men, being higher among the former. However, right after a bout of acute exercise, the high-trained men seemed to be more resistant to exercise-induced apoptosis. It is possible that, through physiological adaptations induced by long-term training, the basal levels of serum FasL could be kept at low levels without adding to inflammatory status; instead the postexercise condition would function in its own, adapted, way. However, this notion needs to be addressed by a study that also includes nontrained sedentary people.

4.2. miR-21 as a Potential Biomarker. miRs are regulating several biological processes in cells including those associated with adaptation to exercise, inflammation, and apoptosis (see S3). miR-21 is widely known as an antiapoptotic-miR owing to its presence at high levels in several malignancies. Its

systemic levels have also been shown to be upregulated in elderly people and its possible role as an inflammatory marker has been discussed [10]. In the present study, miR-21 levels increased significantly in the earlier years (40+), leveled out through the middle years (56+) and again increased significantly in the later years (69+). The self-reported training histories showed that the most significant decline in training occurred among the 40+ group and 69+ group, showing the opposite pattern to that of the miR-21 levels in those age groups. However, the associations between the change in sprint-specific training and the change in miR-21 levels (data not shown) were analyzed and no significant correlations were found ($R^2 = 0.074$; $P = 0.081$), indicating that the decline in training did not explain the increments in serum miR-21. Our association analyses indicate that lower miR-21 levels are more beneficial for knee flexion and bench press performance. These findings support the idea that the higher the level of miR-21, the more unbeneficial it is for physiological status. Our results are also in line with the study by Wardle et al. [19], who reported that young male strength athletes have lower levels of plasma miR-21 than endurance athletes, which could be a beneficial result favoring strength training.

4.3. miR-146a as a Potential Biomarker. miR-146a has been proposed as an anti-inflammatory miR, negatively regulating the inflammatory response by targeting TNF receptor-associated factor 6 (TRAF-6) and IL-1R-associated kinase (IRAK-1) [11]. In the present study, miR-146a serum levels increased significantly among the oldest participants after age 69. This finding raises the question of whether circulating miR-146a is one of the regulators and a component of the training-induced adaptation system, needed to balance the inflammatory status in the elderly. In the sprint association, the miR-146a levels at the earlier ages did not seem to predict performance in the later years; however, with lower levels after age 70, better 60 m sprint time was obtained. In the knee flexion association, similar results were obtained for miR-21: with lower levels, better performance was obtained in the later years. In the bench press association, with higher miR-146a levels, slightly better results in bench press performance were obtained in the later years. These results for miR-146a and physical performance associations in aging showed a distinct pattern for the sprint versus bench press, with lower levels being more beneficial for sprint and higher levels for bench press in the later years.

Masters athletes demonstrate that, with an active, motivated, and healthy lifestyle, aging does not inevitably lead to physical frailty and disability [36]. In our study, even explosive strength and sprinting performances were preserved at relatively high levels into old age, even if some athletes reported a decline in their training activity over the 10-year follow-up. However, despite habitual training, after 80 years of age the hitherto modest decline in the performance assumes a more radical form. It has been suggested that in old age the curvilinear decline in physical performance may be explained by a concomitant deterioration in several physiological systems [37]. The role of circulating molecules delivering intercellular messages in these deteriorative events is evident, however, very complex. In the present study, the decline in the physical performance measures over time was partially explained by changes in the serum FasL, miR-21, and miR-146a levels, molecules associated with inflammation and cellular homeostasis. More detailed functional and tissue specific studies are thus needed to better understand the role and regulation of these potential biomarkers in aging and training adaptations.

5. Conclusions

The main focus of the study was to determine whether specific circulating inflammation- and apoptosis-related molecules, that is, FasL, miR-21, and miR-146a, are associated with physical performance and aging among masters sprinters. Previous studies have demonstrated distinct associations of the studied molecules with physical performance and with age, but longitudinal combined associations have not been reported. We showed that the systemic levels of these molecules change over 10-year period and that associations exist between the molecules, specific physical performance measures, and aging. In addition, the associations with physical performances were slightly different depending on the age of the masters sprinters. Lower levels of FasL and miR-21 seemed to have more beneficial association with the performance measures generally, whereas the associations between miR-146a and performance are more dependent on the specific type of physical performance measure used. Further research with well-controlled study designs and populations are needed to determine whether these molecules are useful as biomarkers in the prediction of successful aging or identification of individuals at high risk for deterioration in performance with older age. In addition, the origin of the circulating biomarkers remains to be clarified.

Additional Points

Limitations and Benefits. This study only concerned relatively healthy males with a regular training background. However, it has been emphasized that athletes themselves are the best controls in the study of inherent aging [37]. Small percentage of the masters sprinters, who had taken part in the baseline measurements in 2002, was not available for the study follow-up in 2012. It is likely, therefore, that the follow-up setting is based on the fittest sprinters; this should be considered when interpreting the results. It should also be noted that the greatest emphasis in the physical performance association curves is on the middle age range, where the number of the participants was the greatest. The predictions are the most reliable until the age of 85 years from which onwards the thinning of the subjects might have unwanted effects on the predictions. It is important to bear in mind that miRs are sensitive and easily affected by external and internal stimuli. It was not possible to control for all the potentially confounding stimuli that could have affected the circulating miR levels (such as personal diet, lack of sleep, and nondiagnosed conditions). Previous studies have reported that miR-146a

reacts acutely to exercise [17, 38]. However, the present study population represents relatively healthy, regularly training males with a long training history. With their bodies being well adapted to even rather heavy exercise, no surprising acute effects on blood parameters during the brief physical measurements on the previous day of blood sampling were expected. The participants had a similar background in their training and other living habits (no regular smokers, no heavy drinking). Also, the fact that the study design was similar in both years strengthens longitudinal comparability. When studying athletes, the motivation to reach one's personal peak in measurements of performance is likely to be higher than in nonathletes. One of the major strengths of the study is the 10-year follow-up design: it is unique and leaves no space for genetic variation, which is an issue in cross-sectional designs, especially when studying partly genetically regulated biomarkers, as shown in our previous study with monozygotic twins [26].

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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ORIGINAL PAPERS

I

AGING AND SERUM EXOMIR CONTENT IN WOMEN -EFFECTS OF ESTROGENIC HORMONE REPLACEMENT THERAPY

by

Kangas R, Törmäkangas T, Fey V, Pursiheimo J, Miinalainen I, Alen M, Kaprio J, Sipilä S, Säämänen A-M, Kovanen V & Laakkonen EK. 2017

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Aging and serum exomiR content in women-effects of estrogenic hormone replacement therapy

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Exosomes participate in intercellular messaging by transporting bioactive lipid-, protein- and RNA-molecules and -complexes. The contents of the exosomes reflect the physiological status of an individual making exosomes promising targets for biomarker analyses. In the present study we extracted exosome microRNAs (exomiRs) from serum samples of premenopausal women (n = 8) and monozygotic postmenopausal twins (n = 10 female pairs), discordant for the use of estrogenic hormone replacement therapy (HRT), in order to see whether the age or/and the use of HRT associates with exomiR content. A total of 241 exomiRs were detected by next generation sequencing, 10 showing age, 14 HRT and 10 age + HRT -related differences. When comparing the groups, differentially expressed miRs were predicted to affect cell proliferation processes showing inactivation with younger age and HRT usage. MiR-106-5p, -148a-3p, -27-3p, -126-5p, -28-3p and -30a-5p were significantly associated with serum 17β-estradiol. MiRs formed two hierarchical clusters being indicative of positive or negative health outcomes involving associations with body composition, serum 17β-estradiol, fat-, glucose- and inflammatory markers. Circulating exomiR clusters, obtained by NGS, could be used as indicators of metabolic and inflammatory status affected by hormonal changes at menopause. Furthermore, the individual effects of HRT-usage could be evaluated based on the serum exomiR signature.

MicroRNAs (miRs) are a class of epigenetic regulators, small non-coding RNAs, which bind to their target mRNAs leading to RNA silencing and further to translational suppression. MiRs are localized in various cell types as well as in all body fluids. To date it has been shown that circulating miRs are either attached to vesicle free protein complexes, such as argonaute proteins (Ago 1–4)[1,2] or high density lipoprotein (HDL) molecules[3], localized inside the apoptotic bodies[4] or exosomes[5]. Especially exosome miRs (exomiRs) are known to be relatively stable in the circulation.

Exosomes are small (<100 nm in diameter) spherical bilayer proteolipid vesicles produced by various cell types under both normal and pathological conditions. They bud from the late endosomes in the cytoplasm forming multivesicular bodies (MVB) and either remain in the cells they are formed or are fused with the plasma membrane and exported to the extracellular matrix or further to the circulation[6]. It has been shown that exosomes consist of lipids, proteins and different sized RNA molecules including miRs[5,7]. The content of the exosomes is affected by their source and also supposedly due to the physiological condition of the exosome secreting cell. Quite recent studies have demonstrated that the messages, delivered by the secreted exosomal RNAs, can generate changes in the gene expressions and protein concentrations of the recipient cells, suggesting the functionality of the exosome content in the target cells[5]. Especially the immuno-stimulating role of the exosomes has been emphasized e.g. by showing that dendritic cells communicate via so called exosome-miR shuttles in mice[8].

Estrogens, female sex steroid hormones, have broad systemic effects on different cell types through their receptors. As known, the levels of systemic estrogens, especially 17β-estradiol (E_2), change dramatically at the

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time of menopause in women. Estrogen sensitivity of miR regulation and their expression in different cell types has been recently demonstrated in several studies[9–13]. Our previous studies have shown that circulating cell free miRs, including miR-21 and miR-146a differ between postmenopausal genetically identical twin sisters who are discordant for the estrogen based hormone replacement therapy (HRT) suggesting that systemic estrogen levels affect miR profile[14].

A very recently published study demonstrated an association between accelerated aging, measured by the epigenetic age i.e. DNA methylation status of blood, and early onset of menopause[15]. In the present study, we investigated whether the age or/and the use of postmenopausal HRT associates with the miR content of the circulating exosomes. We extracted exosomes from the serum of healthy premenopausal women (n = 8) not using external estrogen products and from healthy postmenopausal monozygotic (MZ) twin sisters discordant for the use of HRT (n = 10 pairs). Next generation sequencing (NGS) was used to analyze the exomiR content and CAP-miRSEQ pipeline together with Poisson-normal regression model to identify specific miRs with differential expression pattern. Our results indicated that specific miRs differ in terms of the age of the sample donator and HRT treatment. These miRs have been shown to function as regulators of cellular homeostasis. Hierarchical clustering analysis revealed two miR-clusters, the other indicating negative association with E_2 parallel to positive association with markers of adiposity and inflammation and the other being opposite in the associations with E_2 and metabolic health markers. These results suggest that female E_2 status may be a mediator of the change in exomiR content that in turn is associated with change in health metabolic status known to occur within menopause.

Results

Participant characteristics. Participant anthropometrics and measured blood characteristics as well as group differences are presented in the Table 1. All groups differed significantly from each other in serum E_2 and follicle stimulating hormone (FSH) levels those being highest (E_2) and lowest (FSH) among premenopausal women as expected. Waist circumference was significantly lower among premenopausal women compared to both postmenopausal groups. Fat percentage was significantly lower in postmenopausal HRT users compared to their co-twins. Serum TNF-α and plasma glucose concentrations were also significantly lower among premenopausal women in comparison to postmenopausal women.

ExomiR profile and differentially expressed miRs in NGS. The functionality of the used exosome extraction method was demonstrated by electron microscope imaging (Fig. 1, a representative image obtained from the serum exosomes of a postmenopausal HRT user). Most of the detected lipid vesicles were in normal range for exosomes being under 100 nm in diameter. The exosome quantity, measured by esterase activity, showed no differences between the studied groups (Pre = 17566 ± 1196 RFU/μg, HRT = 17876 ± 5801 RFU/μg, No HRT = 16303 ± 4370 RFU/μg, P = 0.610–0.925). Altogether, 241 different known miRs were detected in the serum exosomes by NGS. The processed sequencing reads are presented in Supplementary data (S1). The most abundant miRs of the exosome cargo include miR-486-5p, -92a-3p, -16-5p, -451a, -22-3p and -423-5p together covering up to 84.6% of the exomiR content (Fig. 2).

The miR read counts from the differentially expressed miRs are presented in Table 2. Altogether, 21 miRs had significant differential expression pattern (p-value with false discovery rate (FDR) correction, $P < 0.05$) in NGS either in one, two or three group comparisons (postmenopausal No HRT group vs. premenopausal group, postmenopausal HRT-user group vs. premenopausal group or postmenopausal HRT-users vs. postmenopausal non-using co-twins). MiR-126-5p, -142-5p, -484 and -10b-5p were differentially expressed between all the studied groups *(P < 0.001)* showing age- and HRT-use associated differences. MiR-27b-3p, -10a-5p, -215-5p and -144-5p were differentially expressed between both postmenopausal groups vs premenopausal women showing age-associated differences with or without HRT ($P < 0.001$). MiR-148-3p and -28-3p were differentially expressed between postmenopausal No HRT group vs premenopausal women (*P = 0.008, P = 0.009, respectively*) and miR-375 and -186-5p between postmenopausal HRT group vs premenopausal women (*P = 0.045, P < 0.001, respectively*). MiR-532-5p, -1285-3p, -30a-5p, -3688-3p, -29b-3p, -106b-5p, -29c-3p, -1306-5p, -148a-3p and -301a were differentially expressed between HRT users and their non-using co-twins suggesting association between HRT-use and miR expression (*P < 0.001, P < 0.001, P = 0.001, P = 0.016, P = 0.029, P = 0.029, P = 0.033, P = 0. 036, P = 0.049, P = 0.049, respectively*).

Functional network analysis. All sequenced miRs were included in the analyses to identify the most prominent networks by explorative comparison analyses by Ingenuity pathway tools (IPA) (Fig. 3). No cut-offs for p-values were used. The age comparison between postmenopausal women without HRT and premenopausal women suggested that estrogen receptors (ESRs) and insulin are predicted to be the biggest upstream regulators of the miRs in the network. Six of the differentially expressed miRs (FDR <0.05) listed in Table 2 were involved in the network and highlighted in the figure. The comparison between postmenopausal HRT users and premenopausal women involved also ESRs and insulin as upstream regulators but only two of the differentially expressed miRs, miR-27b-3p and miR-126-5p. The comparison between the postmenopausal twin sisters involved five of the differentially expressed miRs out of which four are predicted to be regulated by insulin. In addition, miR-106b-5p was predicted to have VEGF and SMAD6/7 as upstream regulators and miR-30a-5p chorionic gonadotropin (Cg).

To focus on the most significant miRs, comparison analysis was performed to differentially expressed miRs (cut-off $P < 0.05$) by IPA, including all the three group comparisons (Fig. 4). Cell proliferation was the most significant downstream target process predicted to be affected in all the comparisons. Comparisons between premenopausal and both postmenopausal groups included the same miRs (miR-27b-3p, -215-5p, -126-5p and -10a-5p), except for miR-148a-3p which was included only in the premenopausal vs postmenopausal No HRT

	PREMENOPAUSAL (N = 8)	POSTMENOPAUSAL NO HRT (N = 10)	POSTMENOPAUSAL HRT (N = 10)	SIGNIFICANT COMPARISON (P < 0.05)
Age (yrs)	32.0 ± 1.6	57.5 ± 2.0	57.5 ± 2.0	a, b
Height (cm)	164.5 ± 4.0	162.2 ± 4.5	162.8 ± 4.7	
Weight (kg)	70.0 ± 11.3	74.7 ± 15.6	67.8 ± 9.1	
BMI	25.9 ± 4.5	28.6 ± 6.8	25.7 ± 4.0	
Waist (cm)	86.4 ± 10.4	104.7 ± 9.6	100.9 ± 6.4	a, b
Fat %	29.7 ± 7.0	35.3 ± 9.3	30.5 ± 7.5	c
hsCRP (mg/l)	0.88 ± 1.1	1.53 ± 0.94	1.09 ± 0.95	
IL-10 (pg/ml)	0.89 ± 1.7	2.50 ± 3.70	2.43 ± 3.17	
IL-6 (pg/ml)	1.05 ± 0.70	1.77 ± 1.29	1.39 ± 0.71	
TNFa (pg/ml)	6.1 ± 1.6	10.5 ± 2.3	10.7 ± 4.8	a, b
WBC (e9/l)	6.1 ± 1.2	5.2 ± 1.4	5.3 ± 1.3	
RBC (e12/l)	4.5 ± 0.2	4.3 ± 0.3	4.4 ± 0.3	
HGB (g/l)	139 ± 5.0	137 ± 9.4	140 ± 9.1	
Plt (e9/l)	242.8 ± 54.2	233.9 ± 39.1	236.8 ± 83.4	
Chol (mmol/l)	4.7 ± 0.8	5.3 ± 0.4	5.3 ± 0.7	
LDL (mmol/l)	2.7 ± 0.8	3.1 ± 0.4	3.1 ± 0.8	
HDL (mmol/l)	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.5	
TriGly (mmol/l)	0.8 ± 0.2	1.2 ± 0.5	1.2 ± 1.0	
Adiponectin (ng/ml)	7432 ± 3606	9382 ± 5273	10267 ± 5350	
Leptin (pg/ml)	23225 ± 10977	18571 ± 13705	12483 ± 8672	
Resistin (ng/ml)	9.4 ± 2.0	10.2 ± 1.2	9.7 ± 1.4	
Ins (mmol/l)	5.3 ± 3.3	8.0 ± 4.5	6.2 ± 3.6	
Gluc (mmol/l, plasma)	4.2 ± 0.4	5.2 ± 0.7	5.0 ± 0.7	a, b
E2 (pmol/l)	496.5 ± 311.9	34.8 ± 28.4	183.0 ± 211.3	a#, b#, c#
E1 (pmol/l)	369.4 ± 195.3	100.9 ± 26.0	962.0 ± 1517.8	a#, c#
T (nmol/l)	1.02 ± 0.30	0.66 ± 0.27	0.74 ± 0.31	a, c
SHGB (nmol/l)	49.3 ± 17.6	44.1 ± 11.6	72.3 ± 31.1	c
LH (IUl)	12.8 ± 13.2	39.9 ± 30.2	31.6 ± 17.3	a#, b#
FSH (IUl)	6.22 ± 2.85	83.7 ± 32.7	57.0 ± 31.2	a, b, c

Table 1. Participant body anthropometrics and blood characteristics and differences between the studied groups. Significant comparisons between the studied groups are marked with letters a) Pre vs post No HRT b) Pre vs post HRT c) Post HRT vs post No HRT. Independent samples T-test was used for a and b comparisons and paired samples T-test for c comparisons when variables were parametric. #Mann Whitney U test (a and b) and Wilcoxon matched pair signed-rank test (c) was used for non-parametric variables. P < 0.05 was considered significant. Results are shown as mean ± S.D. BMI: body mass index, hsCRP: high sensitive c-reactive protein, IL-10: interleukin 10, IL-6: interleukin 6, TNFa: tumor necrosis factor alpha WBC: white blood cell count, RBC: red blood cell count, HGB: hemoglobin, Plt: platelet, Chol: cholesterol, LDL: low density lipoprotein, HDL: high density lipoprotein, TriGly: triglycerides, E2: 17β-estradiol, E1: estrone, T: testosterone, SHGB: sex hormone-binding globulin, LH: lutenizing hormone, FSH: follicle stimulating hormone.

group network. Higher age (or postmenopausal status) was predicted to up-regulate the cell proliferation pathway whether using HRT or not. Other set of miRs (miR-29b-3p, -106b-5p, -142-5p, and -1285-3p) were shown to have an effect when comparing the postmenopausal co-twins with each other. In this comparison, the use of HRT was predicted to down-regulate the cell proliferation pathway. Figure 4 shows the main networks for differentially expressed miRs identified by IPA with its annotation as the top diseases and functions and the interacting molecules i.e., potential miR targets as well as miRs included in the network.

Associations of NGS reads with serum E_2 and other variables. The associations of the differentially expressed miRs with serum E_2 concentration, body composition and fat-, glucose- and inflammation related markers are visualized as a correlation heatmap (Fig. 5). The p-values, FDR-values and correlation coefficients are presented in Supplementary data (S2). Heatmap reveals hierarchically clustered information about the associations of the selected miRs with other measured variables. First of all, six miRs from the differentially expressed NGS results were significantly (FDR < 0.05) associated with serum E_2. MiR-106b-5p was positively associated with serum E_2 concentration, while other miRs, miR-148a-3p, -27-3p, -126-5p, -28-3p and -30a-5p were negatively associated with serum E_2 concentration. In addition, miR-144-5p had indicatively significant positive association and miR-10b-5p indicatively significant negative association with E_2 concentration (P = 0.025, FDR = 0.066 for both associations). Although only the six mentioned miRs possessed significant association with FDR < 0.05, it is clear from the heatmap that the miRs under investigation form two separate clusters the other cluster being indicative of positive and the other of negative health outcomes.

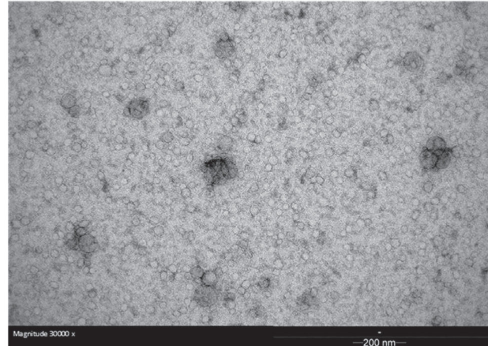


Figure 1. Electron microscope image of the extracted serum vesicles. Typical size of an exosome is less than 100 nm in diameter. A Tecnai G2 Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands).

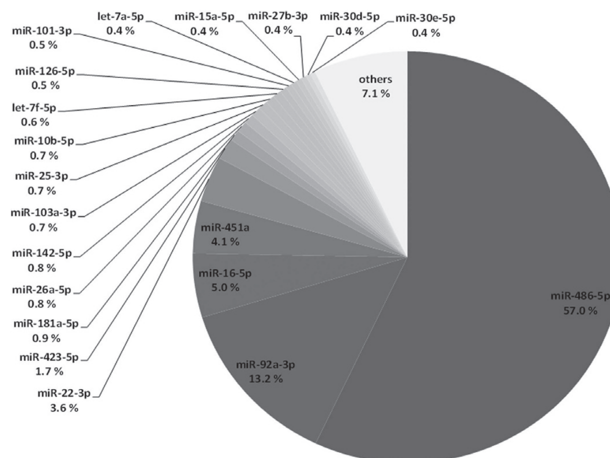


Figure 2. Relative serum exomiR content of all the samples.

The positive health outcome cluster miRs had positive associations with E_2 and the negative health outcome cluster miRs had negative associations with E_2 and concomitant negative and positive associations, respectively, with most of the adiposity and inflammation related variables except that with HDL and adiponectin which were clustered together with E_2 concentration. However, the associations with miRs and HDL count were only indicatively significant for miR-148a-3p and -27b-3p with HDL (P = 0.009, FDR = 0.126 and P = 0.012, FDR = 0.126, respectively) and for miR-27b-3p with adiponectin (P = 0.013, FDR = 0.273) while for the other miRs the associations were non-significant. The leading miRs for the negative health outcome cluster were miR-27b-3p, which had the highest number of significant associations and miR-148a-3p, which had very similar profile although some of the associations were only nominally significant. The higher NGS read count value of miR-27b-3p was significantly associated (FDR < 0.05) with greater adiposity (fat percentage, BMI, waist circumference, triglyceride concentration), with higher fasting glucose and insulin and with higher hsCRP, resistin and TNF-α (P = 0.014, FDR = 0.095) together with lower E_2 concentration. The positive health outcome cluster did not have as prominent leading miR, but the miRs having positive association with E_2, miR-106b-5p and miR-144-5p, had also negative association with TNF-α (P = 0.027, FDR = 0.099 and P = 0.008, FDR = 0.084, respectively). In addition, miR-106b-5p, -532-5p and -484 had statistically significant (FDR < 0.05) negative associations with resistin.

Validation of differentially expressed sequenced miRs. qPCR validations of the E_2-associated miRs are presented in Fig. 6. For miR-27b-3p, -148a-3p and -126-5p the levels were significantly lower in premenopausal women compared to postmenopausal No HRT group (P = 0.001, P = 0.001, P = 0.040, respectively) and

miR	Pre	Mean count No HRT	HRT	Overdispersion	Comparison (p-value, FDR) No HRT vs. Pre	HRT vs. Pre	HRT vs. No HRT
miR-126-5p	3942	5822	5601	0.002	<0.001↑	<0.001↑	<0.001↓
miR-142-5p	10591	6254	6669	0.007	<0.001↓	<0.001↓	<0.001↑
miR-484	2106	1201	1471	0.029	<0.001↓	<0.001↓	<0.001↑
miR-10b-5p	8535	6958	5323	0.007	<0.001↓	<0.001↓	<0.001↓
miR-532-5p	395	263	356	0.102	0.184	1	<0.001↑
miR-1285-3p	72	40	97	1.28	1	1	<0.001↑
miR-30a-5p	1121	1880	1485	0.101	0.065	1	0.001↓
miR-3688-3p	6	4	10	6.248	1	1	0.016↑
miR-29b-3p	0	6	17	6.436	0.934	0.494	0.029↑
miR-106b-5p	399	219	333	0.155	0.118	1	0.029↑
miR-29c-3p	174	154	209	0.201	1	1	0.033↑
miR-1306-5p	4	2	5	4.692	1	1	0.036↑
miR-148a-3p	2095	3863	2814	0.094	0.008↑	1	0.049↓
miR-301a-3p	12	38	22	2.061	1	1	0.049↓
miR-375	1056	629	329	0.482	1	0.045↓	0.115
miR-28-3p	319	607	443	0.103	0.009↑	1	0.275
miR-27b-3p	3307	8419	7025	0.034	<0.001↑	<0.001↑	0.325
miR-10a-5p	3958	7682	6252	0.029	<0.001↑	<0.001↑	0.671
miR-215-5p	190	1010	592	0.151	<0.001↑	<0.001↑	0.671
miR-144-5p	585	375	433	0.034	<0.001↓	<0.001↓	1
miR-186-5p	2056	2362	2598	0.026	0.063	<0.001↑	1

Table 2. Read counts of the differentially expressed miRs based on sequencing results. The average read counts of the studied groups are presented on the left (Pre: premenopausal women, HRT: postmenopausal hormone replacement therapy users, No HRT: postmenopausal non-users). FDR corrected group comparisons are presented on the right. Arrow head pointing up indicates upregulation and arrow head pointing down downregulation in the first group of the comparison. Overdispersion is a measure of a greater variability.

HRT group (P = 0.005, P = 0.040, P = 0.005, respectively). MiR-106b-5p was significantly lower in premenopausal women compared to postmenopausal HRT group (P = 0.040) and lower in No HRT women compared to their HRT co-twins (P = 0.043). No differences between any groups were detected for miR-28-3p and -30a-5p (data not shown).

Discussion

This is the first study showing that age and the use of HRT are associated with the miR contents of the circulating exosomes in women. Altogether, 241 distinct known serum exomiRs were detected by NGS among the studied groups from which 21 were differentially expressed. The NGS results showed that exomiR -levels differ between all of the three studied groups significantly in cases of four miRs. In addition, age-associated difference without HRT was detected in six miRs (postmenopausal No HRT vs premenopausal), and with HRT in ten miRs (postmenopausal HRT vs premenopausal) as well as treatment difference in ten miRs (postmenopausal HRT vs No HRT).

Menopause is recognized as a risk factor for the development of metabolic dysfunctions[16,17]. The loss of circulating E_2 is followed by androgen dominance and further by body fat accumulation around the abdominal area[18]. The excess amount of adipose tissue leads to unbalanced cytokine profile which is seen as increased levels of IL-6, TNF-α, IL-1β, resistin and leptin among other inflammatory molecules[19]. These circulating pro-inflammatory cytokines form a base for systemic low-grade inflammation also known to be associated with aging[20]. In addition to adipose tissue initiated release of pro-inflammatory cytokines, also issues in glucose metabolism, such as insulin resistance play a role in the systemic inflammation[21]. Furthermore, endothelic dysfunction and atherosclerosis are adding to the chronic inflammatory state[22]. In our study the premenopausal women had healthier profile in terms of metabolic measures such as waist circumference and plasma glucose levels compared to postmenopausal women. In addition, fat percentage was significantly higher among the postmenopausal women who were not under HRT compared to their co-twins under HRT. We were able to see a slight increase in TNF-α levels due to age, whether under HRT or not, but no significant differences in other classical pro-inflammatory markers between any of the studied groups. However, across the measured inflammatory markers a trend towards the most unbeneficial state was detected among postmenopausal women, who were not under HRT. Clear expected differences were detected in systemic steroid hormone levels as well as gonadotropins, from which high FSH levels are indicators of postmenopausal state. In addition, interesting associations with serum E_2 concentrations and differentially expressed miRs, inflammation, glucose metabolism and adiposity -related markers were found.

The present study revealed six different miRs which were associated (FDR < 0.05) with serum E_2 levels either positively (miR-106b-5p) or negatively (miR-148a-3p, -27-3p, -126-5p, -28-3p and -30a-5p). In addition, two miRs had indicatively positive (miR-144-5p, P < 0.05) and negative (miR-10b-6p, P < 0.05) association with

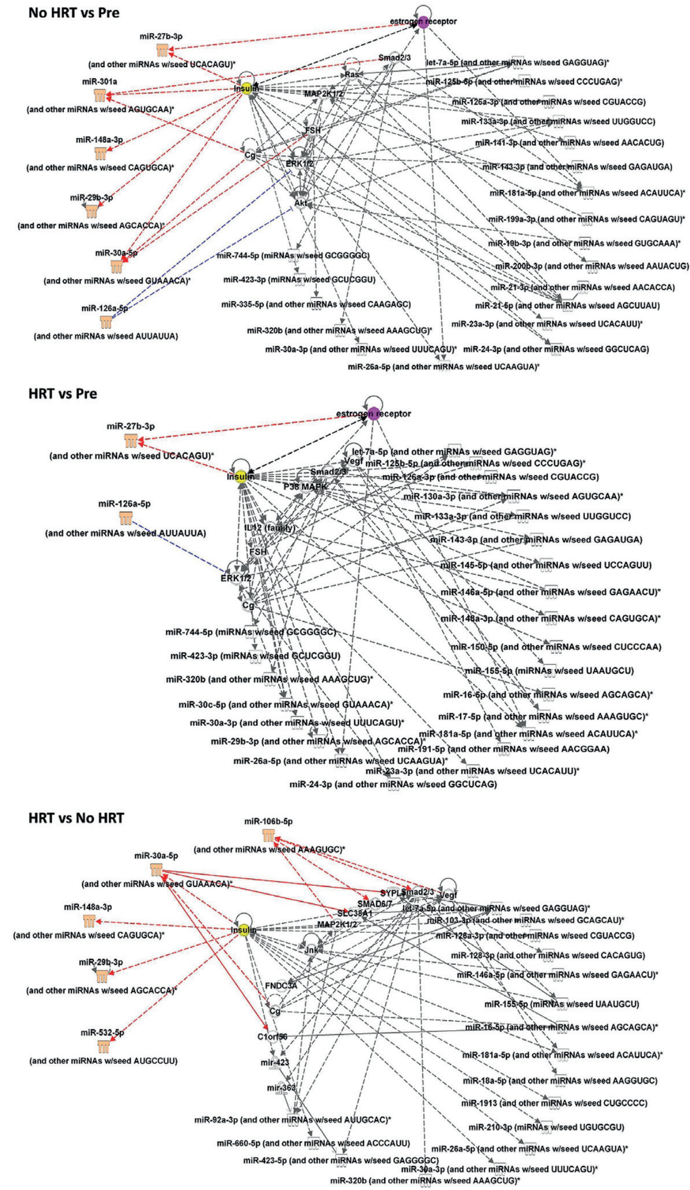
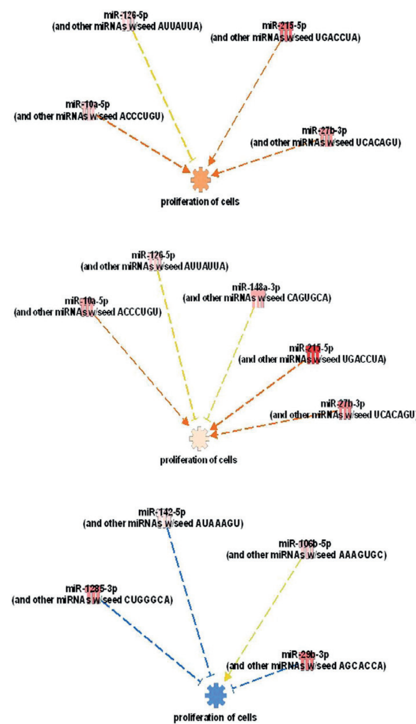


Figure 3. The most prominent network from IPA comparison analyses performed to all sequenced miRs. Differentially expressed miRs (FDR) are highlighted in the figure with coral colour. The arrow head of the red line tells the direction of the predicted affect. Blue line represents the predicted inhibition. Insulin is highlighted in yellow and ESR in pink. Smad2/3: Smad family member 2 or 3, Ras: protein superfamily of small GTPases, MAPK2K1/2: Mitogen activated protein kinase 2K1 or 2, FSH: Follicle stimulating hormone, Cg: Chorionic gonadotrophin, VEGF: Vascular endothelial growth factor, IL12: Interleukin 12, ERK1/2: Extracellular signal regulated kinase 1 or 2, SYPL1: Synaptophysin like 1. SLC38A1: Solute carrier family 38 member 1, Clorf56: Chromosome 1 open reading frame 56 solute, Jnk: Jun kinase, FNDC3A: fibronectin type III domain containing 3A.



Comparison	Diseases and functions	RNA targets in the pathways
HRT vs Pre	Cancer, Organismal injury and abnormalities, Reproductive system diseases	ADAM9, ADORA2B, BIRC5, BRAF, CD44, DDX20, DHFR, DROSCHA, DTL, E2F2, EFNB2, EPHB6, ERK1/2, FOXO1, PHB, PMAIP1, PPP2R2C, ST14, TNFSF12, TP53, TYMS, WEE1, ZEB1, ZEB2
No HRT vs Pre	Cellular development, Cellular growth and proliferation, Cancer	ADAM9, BBC3, BIRC5, BRAF, CYP2A6, EFNB2, EPHB6, ERK1/2, IGF1, IGF1R, MMP13, ODC1, PDPK1, PHB, PPP2R2C, RPS6KA5, ST14, TNFSF12, TYMS, WEE1, ZEB1,
HRT vs No HRT	Cancer, Organismal injury and abnormalities, Reproductive system diseases	ACVR2A, CAV2, CCNE1, CDH1, COL15A1, COL4A1, COL5A2, DNMT3A, DUSP2, FAM3C, FBN1, GRHL3, LOXL2, MYBL2, MYC, NF2, PMP22, PPIC, PPM1D, PURA, Rb, TCL1A, TDG, TP53, Tpm1, ZFP36L1,

Figure 4. IPA comparison analysis of the 3 different comparisons for differentially expressed miRs. The figure shows miRs in each comparison predicted to regulate cell proliferation. Red color indicates activation, blue inhibition and yellow controversial findings of the specific miR. Younger age and the use of HRT were predicted to inhibit the cell proliferation. The table shows the main networks for differentially ex-pressed miRs identified by IPA with its annotation as the top diseases and functions and the interacting molecules i.e., potential miR targets as well as miRs included in the network. The underlined miR targets are also involved in cell proliferation (figure).

serum E_2. To our knowledge two of these miRs have been previously shown to have an association with E_2 in breast cancer cells. Nassa *et al.*[23] have demonstrated that miR-30a-5p is regulated by estrogen receptor β (ESRβ) whereas Tao *et al.*[24] demonstrated that miR-148a is downregulated by E_2 through GPER in breast cancer cells. In addition, 17-β-estradiol–ESRα–miR-27b -connection has been demonstrated on human leukemia cell line[25]. Our NGS data indicated significant differences between postmenopausal twins for miR-30a-5p and between all the studied groups for miR-148a-3p and miR-27b-3p. All miRs had a negative association with serum E_2 which is in line with the mentioned studies.

Based on recent publications, some of the E_2 associated miRs identified in the present study have connections to aging. MiR-10b-5p has been shown to be associated with the motor onset in both Parkinson's and Huntington's diseases[26] indicating its function in the development of age-associated neurodegenerative disorders. Also, the role of circulating miR-10b-5p in osteogenic differentiation after fracture at postmenopausal age has been recognized[27]. In addition, changes in miR-28-3p levels during early senescence were observed in endothelial cells, thus, indicating miR's possible regulatory role in the aging of vascular endothelium. Even though no direct effect of E_2 has been stated in the mentioned studies, it has been well demonstrated that estrogen has a role as a neuro-, osteo- and vascular protector[28–31]. Our earlier studies have also shown beneficial effects of postmenopausal HRT on body composition measures, including fat, muscle and bone properties[32–36]. The connection between lipoproteins, vascular inflammation and exosomes has also been recognized by others[37]. MiR-27b-3p has been shown to regulate fat metabolism and inflammation by targeting RXRα and PPARγ[38,39]. In the present study, mir-27b-3p was one of the most interesting single miRs belonging to the negative outcome cluster. The NGS data indicated higher levels of mir-27b-3p in the circulating exosomes of the postmenopausal twins compared to premenopausal

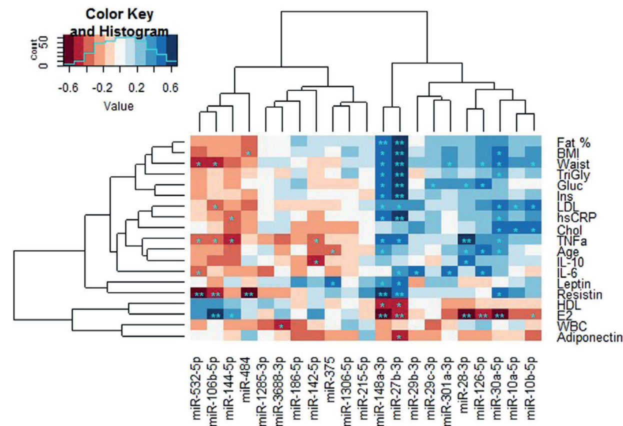


Figure 5. Clustered heatmap of the selected miRs and other measured variables. Blue indicates positive and red negative associations. Spearman correlation coefficient was used for the analyses. *P < 0.05 (nominal), **P < 0.05 (FDR corrected). Positive health outcome cluster is on the left side with positive associations with E2 and negative health outcome cluster on the right side with negative E2 associations. BMI: body mass index, TriGly: triglycerides Gluc: plasma glucose, Ins: serum insulin, hsCRP: high sensitive c-reactive protein, IL-6: interleukin 6, LDL: low densi-ty lipoprotein, TNFa: tumour necrosis factor alpha, IL-10: interleukin 10, Chol: cholesterol, WBC: white blood cell count, HDL: high density lipoprotein, E2: 17β-estradiol. RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

women indicating an age association. This miR was negatively associated with serum E_2, HDL and adiponectin (last 2 nominal) whereas positive associations were detected with other adiposity markers such as fat percentage and triglycerides and nominally with LDL. In addition, the levels of miR-27b-3p were positively associated with serum hsCRP and resistin as well as insulin and plasma glucose concentrations, all of these markers indicative of unbeneficial metabolic and inflammatory status. The associations for miR-148a-3p were similar to miR-27b-3p but only nominally significant in some cases. Altogether, these results support the function of miR-27b-3p (and miR-148a-3p) in the regulation of lipid and glucose metabolism and further reveal its possible negative role in women's aging with hormonal changes.

Different miR carrier systems share a bunch of common miRs but they also seem to have a unique content which differs from the other miR vehicles and, also, from the cell of origin[40]. Exosomes have been shown to deliver gene-based intercellular messages adding to the complexity of cell-cell communication. According to Cheng *et al.*[41], exosomes provide a protected and enriched environment for miRs compared to the intracellular and other cell free miRs. In their study, exosome derived miRs were associated with neuronal signaling whereas the main responsibility of the cell free miRs was the signaling related to the cellular homeostasis, emphasizing the functional differences between cell free miRs and exomiRs. In our previous studies, we have shown that the circulating cell free levels of miR-21 and miR-146a differ between the HRT users and their non-using co-twins[14]. In the present study, we were able to detect the same miRs in the exosomes by NGS, however, significant differences were not obtained. That may be considered as a supporting indicator of the differential roles of the two types of miR transfer. The mechanism how the exomiRs are sorted into the exported exosomes is an unanswered question. It is under active speculation whether the sorting takes place by the miR sequence or whether the exomiR content is a description of the whole miR pool of the exosome forming cell. It is important to recognize that the sequencing data can be interpreted by several ways: one could either focus on the profile as a whole and compare the relative amounts and changes in the relative patterns, or the other option is to focus on specific miRs. Validation of specific miRs is not inevitably the whole truth as very often miRs work as a group fine-tuning each other's function. In the present study, qPCR validation of the six E_2-associated miRs confirmed the NGS results fully for miR-27b-3p and miR-126-5p and partly for miR-148a-3p and miR-106b-3p, however, no differences for miR-28-3p and -30a-5p were detected.

According to the functional network analyses obtained by IPA, the most prominent predicted upstream regulators of the studied miRs include insulin as well as estrogen receptors. These results support the link between the systemic E_2 levels, insulin signaling and the studied miRs. In the comparison analyses performed using only the differentially expressed miRs, the cell proliferation processes were predicted to be inactivated at younger age as well as due to the use of HRT. Interestingly, different set of miRs seem to regulate cell proliferation processes depending on whether comparing only postmenopausal twins or premenopausal and postmenopausal women. Cell proliferation is usually considered to be more active at younger age. However, as we do not know the cell origin or destination of the exomiRs, we cannot say much about the specific processes and cells under the influence. Based on the present findings, the predicted activation of cell proliferation especially among the postmenopausal

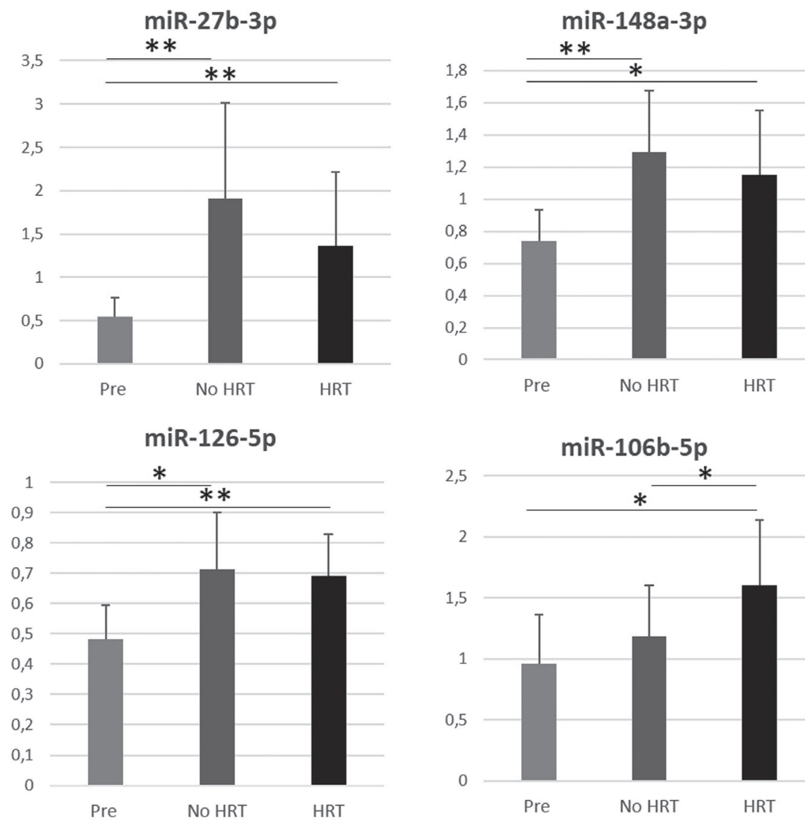


Figure 6. qPCR validation of the miRs associated with systemic E2. Pre: Premenopausal women, No HRT: Postmenopausal women without hormone replacement therapy, HRT: Postmenopausal women using HRT. *P < 0.05, **P < 0.01. Results are presented as mean relative ex-pressions +SD.

women without HRT, could be related to increasing amounts of adiposity, inflammatory cells or possibly tumor cells, all related to natural aging. These results suggest that the postmenopausal HRT users are more similar to premenopausal women in terms of miR regulated cell proliferation than postmenopausal non-users. Based on this genetically controlled study, these effects are achieved to most part by the HRT usage. The study has been performed with a small unique population and thus cannot be generalized directly to female population as a whole. However, as a MZ twin study, it provides a valuable genetically controlled design. Further cell culture experiments are needed in order to say more about the specific functions of HRT sensitive exomiRs on specific cell types.

The recent achieved technologies in molecular biology, including NGS, provide promising tools to discover the broad functions of miRs in both physiological and pathological conditions. To our knowledge, the present study was the first to show a detailed serum exomiR profile of women with different age and hormonal status. We demonstrated that the differential expression patterns were emphasized among exomiRs interplaying with cellular homeostasis, glucose- and lipid metabolism as well as inflammation. Furthermore, we were able to identify miR groups related to more positive or negative health outcomes in which the systemic E_2 concentration played a significant role as a divider. Our findings suggest that the serum exomiRs are sensitive to hormonal changes among women and carry important regulatory messages between cells. The results can be used as a directional starting point for the usage of miR signature, obtained by NGS, as a medical tool for prognostics and diagnostics in aging women. In addition, the miR signature can potentially be applied when evaluating the benefits of HRT usage and its individual suitability in personalized medicine.

Materials and Methods

Study design. The present 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)". A

more detailed design and the recruitment process of the SAWEs- study has been described previously[42,43]. Briefly, the study participants were recruited from the Finnish Twin Cohort (n = 13888 pairs)[44]. The invitation was sent only to women born in 1943-1952 (n = 537 pairs). To be able to take part in the study, both co-sisters needed to participate. This study included 15 MZ female twin pairs who had no contraindications, were discordant for the use of HRT (mean duration of HRT use 6.9 ± 4.1) and were willing to participate to the study. Five of the HRT users were using preparations containing only E_2 (1-2 mg), six used estrogenic (1–2 mg)+ progestogenic compounds and four tibolone (2.5 mg) based treatment. Since the aim of the present study was to investigate the effects of E_2 based HRT, tibolone based HRT users and their co-twins were excluded. Finally, 11 MZ twin pairs were included in the present study, however, the NGS read quality of one sample was not sufficient enough thus, this twin pair was excluded from the analysis. The number of participants is relatively small but comparable with other MZ co-twin studies[45–47] and has enough statistical power to obtain clinically relevant results. No significant differences in physical activity levels (slightly modified Grimby scale[48]), daily energy intake (5-day diary) or smoking habits between the twins were identified. In addition, eight women aged 30 to 40 years with no use of hormonal contraceptives during the last 5 years, were included for the present study representing a premenopausal group. Contraindications for participation were chronic musculoskeletal diseases, type 1 or 2 diabetes, mental disorders, asthma with oral glucocorticosteroid treatment, cancer, drug or alcohol abuse, and Crohn's disease. High blood pressure was the only condition to which daily medication was used (HRT: n = 3, No HRT: n = 4). Other occasional medication included antihistamines, paracetamol and ibuprofein. Overall, the participants were considered as healthy women.

The study was conducted according to the guidelines of the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Central Finland Hospital District (E0606/06). Written informed consent was provided by the study participants prior the laboratory measurements.

Serum analyses. Whole blood samples were collected under standard fasting conditions from antecubital vein in a supine position. The samples of the premenopausal women were collected during the first five days of the menstrual cycle representing the lowest E_2 concentrations. The blood was allowed to clot for 30 mins in room temperature followed by serum separation by centrifugation at 4000 rpm. All the samples were snap frozen and stored in −70 °C in 0.5 ml aliquots.

Exosome isolation, imaging and RNA extraction. Serum exosomes were isolated from 450 μl of sample by using Exoquick Exosome Precipitation Solution according to manufacturer's protocol (#EXOQ5A-1, System Biosciences).

Electron microscopy was used for checking the size of the extracted vesicles. Briefly, the isolated exosomes were deposited on Formvar carbon-coated, glow-discharged grids. After 20 minutes, the grids were washed with PBS and exosomes were fixed in 1% glutaraldehyde for 5 minutes. After washing with distilled water the grids were stained with neutral uranylacetate and embedded in methylcellulose/uranyl acetate and examined in a Tecnai G2 Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands). Images were captured by Quemesa CCD camera using iTEM software (Olympus Soft Imaging Solutions GMBH, Munster, Germany). FLUOROCET Ultrasensitive Exosome Quantitation Assay Kit (System BioSciences), measuring exosome esterase activity, was used to assess the exosome quantity in samples (4 per each studied group) according to manufacturer's protocol. Briefly, the protein concentration of exosome-PBS -solution was measured using BCA protein method by Nanodrop (1000) using wavelength 562 nm. Equal volume of exosome preparation was loaded into each 96-well plate. Promega Glomax Multi + Detection system with fluorescence module was used for the measurement (excitation = 525 nm, emission = 580 to 640 nm). Results are presented as relative fluorescence unit (RFU) per total protein content of the sample (RFU/μg).

Total RNA extraction was performed by using Trisure reagent (Bioline) according to manufacturers' instructions with slight modifications. Additional step was added to the homogenization step where 7 μl of synthetic cel-miR-39 miR mimic (1,6 × 10^8 copies/μl, Qiagen cat. no 219610) was added to each sample to serve as a spike-in control for monitoring the miR purification and amplification. Chloroform was used for the phase separation and 1 μl of nuclease free glycogen (Glycogen RNA Grade, 20 mg/ml, Fermentas) for enhancing the RNA precipitation. RNA concentration was measured by Nanodrop 1000 (Thermo Scientific). Prior the library preparation RNA quality and recovery was checked by qPCR according to manufacturer's protocol (Qiagen miScript Primer assays and II RT kit for cDNA synthesis and MiScript SYBR Green PCR Kit for RT-qPCR) from which the recovery of cel-miR-39 spike-in control (Ct_{mean} = 24.9 ± 1.0) and miR-21-5p (Ct_{mean} = 27.3 ± 1.3) was verified.

cDNA library preparation and small RNA sequencing. The small RNA libraries were prepared using TruSeq Small RNA Sample Preparation Kit (Illumina, USA) with multiplexing adapters. Following the TruSeq Small RNA Sample Preparation Kit user guide (Rev. E), the total RNA, including the small RNA fractions, were ligated to 5′ and 3′ adaptors sequentially before converted to cDNA by reverse transcription. cDNAs were amplified with PCR by using primers containing unique six base index sequences distinguishing different samples from one another. Finally, the samples were subjected to 6% (w/v) non-denaturing polyacrylamide gel electrophoresis (PAGE). cDNA library fragments between 145 and 160 bp corresponding the miR libraries were excised from the gel, purified and eluted. The final miR library pellet was air dried and resuspended in 10 μL nuclease-free water and quantity of the libraries was measured with Qubit fluorometer. Ready miR library pools (8–16 samples on single pool) were loaded to MiSeq V3 flow cell in 12 pM concentrations. To increase signal integrity, 10% of PhiX was spiked in the library pool. MiR libraries were sequenced with MiSeq reagent kit V3 150 cycles using 36 bp reads with single-end chemistry. Three replicates were used in each NGS run.

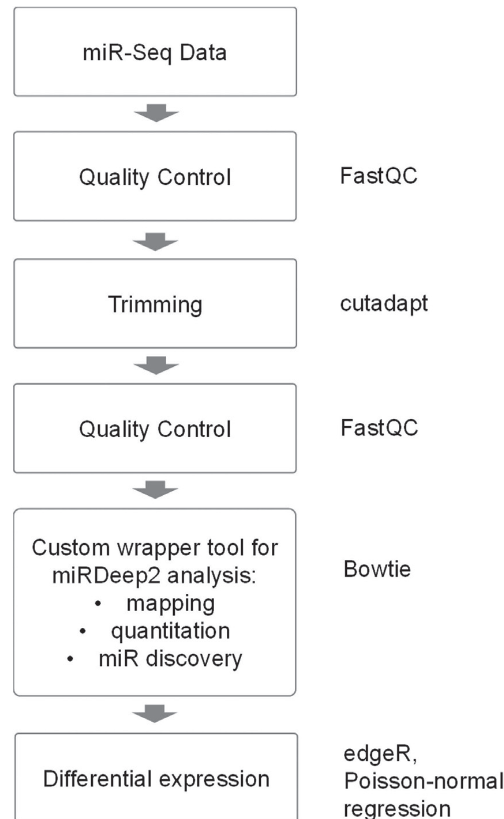


Figure 7. Workflow of the handling of the sequencing data. All the methods and R packages used in the analyses are presented in the figure.

miR validation. The miRs having differential expression in NGS results and significant association with systemic E_2 (FDR < 0.05, Fig. 5) were validated with RT-qPCR. These miRs included miR-27b-3p, -148a-3p, -126-5p, -28-3p, -30a-5p and -106b-5p. Validation was performed using samples from five postmenopausal twin pairs and ten premenopausal women. MiScript II RT Kit was used for cDNA synthesis (Qiagen) according to manufacturer's protocol. cDNA was diluted 1:3 for the RT-qPCR which was performed using miScript SYBR Green PCR Kit and miScript Primer assays (Qiagen: Cat. No. MS00031668, MS00003556, MS00006636, MS00009254, MS00007350, MS00003402, MS00019789) according to manufacturer's protocol. Ct-values less than 36 were included in the analyses. Results were normalized to spike-in cel-miR-39 values of each sample (Ct(av) = 25.1 ± 1.0). A pooled calibrator sample was used across the different plates to obtain 2^{-dCt} (=RQ) results. Subsequently, normalized relative quantities (NRQ) were calculated for each miR of each sample (NRQ = RQ/NF), where normalization factor (NF) presented the geometric mean of RQs of all expressed miRNAs per sample[49].

Bioinformatics and statistical analyses. The workflow for cleaning and analyzing the sequencing results is presented in the Fig. 7. Briefly, quality of the raw reads was assessed with FastQC and reads were trimmed using cutadapt based on the FastQC 'Overrepresented sequences' module output and using a minimum read length filter of nine bases. Trimmed read files were analyzed with miRDeep2, a comprehensive computational tool for miR analysis and discovery which uses a probabilistic model of miR biogenesis to score compatibility of the position and frequency of sequenced RNA with the secondary structure of the miR precursor[50]. Mapping to the reference genome (hg38) was performed using bowtie (version 1.0.1) and miRbase version 18 was used for retrieving miR information. Differential expression analysis was performed on miRDeep2 output data utilizing a custom R script adapted from the differential expression module in the CAP-miRSEQ tool[50,51]. The tools employed in different steps rely on various programming languages. To simplify the workflow a set of R functions was created to carry out QC, trimming, miRDeep analysis and assessment of differential expression in a pipeline-like fashion run from one wrapper script. In addition, Poisson-normal regression model was created to analyze the related

participants (unpublished manuscript). Briefly, read counts were modeled as Poisson distributed variables with over distribution modeled through a normally distributed random variable. The advantage of this model over e.g. the negative-binomial model is that it allows flexible modeling of dependency among related subjects through the random effect correlation matrix. The model was applied on each miR on MPlus version 7 and FDR approach was used to adjust for multiple testing. All other statistical computations were done in R software (versions 3.1.3–3.3.1). R packages used in the bioinformatics workflow are mentioned in Fig. 7.

The group analyses are based on three groups: (1) premenopausal women ($n = 8$), (2) postmenopausal No HRT ($n = 10$) and (3) postmenopausal HRT users ($n = 10$), the latter two forming MZ twin pairs. Based on the normal distribution of the studied variables, tested by Shapiro-Wilk test, the group comparisons were performed either with Independent samples T test and paired samples T test (twins), in case of parametric variables, or with Mann Whitney U test and Wilcoxon signed-rank test (twins) in case of non-parametric variables. Two-sided tests were used. Ingenuity pathway analyses (IPA) tool was used for pathway and comparison analyses of all sequenced miRs and differentially expressed miRs. $P < 0.05$ was considered significant. Spearman's rank correlation coefficient was used for correlation analyses and R packages "gplots" and "RColorBrewer" were used for creating the clustered heatmap (Fig. 5). Data analyses and visualizations were carried out using Eclipse IDE Luna (4.4.2) and the StatET plugin for R (3.4.2) with RJ 2.0, IBM SPSS Statistics (version 23.0, Chicago, IL) and R Studio (R Studio Team 2015, Boston, MA).

Data availability. The processed read counts are provided in the Supplemetary data (S1). The raw sequence read files are open at ArrayExpress database (www.ebi.ac.uk/arrayexpress) upon publication under access number E-MTAB-5245. Computer code availability is provided on request.

Limitations. The methods of sample collection, RNA extraction and further analyses are not yet standardized in NGS. The enzymatic steps in small RNA cDNA library preparation might favor certain miRs over others due to sequence-specific biases. However, same protocol was used across the study, therefore such bias would occur similarly in each sample and would not affect the results of the group comparisons. Also exosome extraction methods lack standardization.

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Author Contributions

R.K. is the corresponding author and has mainly performed all the laboratory work. E.K.L. and V.K. have jointly supervised the work and contributed to the writing. T.T. and V.F. have contributed equally to the statistical analyses and writing the manuscript. A.-M.S., J.P., S.S., M.A., J.K. and I.M. have contributed to the writing. All authors reviewed the manuscript.

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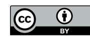
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II

MENOPAUSAL STATUS ASSOCIATES WITH SPECIFIC MI-CRORNA AND TARGET EXPRESSIONS IN SUBCUTANEOUS AD-IPOSE TISSUE

by

Kangas R, Morsiani C, Pizza G, Lanzarini C, Aukee P, Kaprio J, Sipilä S, Franceschi C,
Kovanen V, Laakkonen EK & Capri M. 2017

Submitted manuscript

III

CIRCULATING MIR-21, MIR-146A AND FAS LIGAND RESPOND TO POSTMENOPAUSAL ESTROGEN-BASED HORMONE REPLACEMENT THERAPY – A STUDY WITH MONOZYGOTIC TWIN PAIRS

by

Kangas R, Pöllänen E, Rippo MR, Lanzarini C, Prattichizzo F, Niskala P, Jylhävä J, Sipilä S, Kaprio J, Procopio AD, Capri M, Franceschi C, Olivieri F & Kovanen V. 2014

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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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Biological aging is associated with physiological deteriorations, which are partly due to changes in the hormonal profile. MicroRNAs regulate various processes associated with cell senescence; differentiation, replication and apoptosis. Serum microRNAs have 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 biopsy samples from 54 to 62 year-old postmenopausal monozygotic twins ($n = 11$ pairs) discordant for HRT usage. Premenopausal 30–35 year-old women ($n = 8$) were used as young controls. We focused on the hormonal aging and on the interaction between HRT use and the modulation of miR-21, miR-146a and classical inflammation markers. Fas-ligand was analysed since it functions in both apoptosis and inflammation.

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

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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

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(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 (Pacifici et al., 1989, 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,b), 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-kβ-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-κB-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-1β) 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 signalling 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 E_2 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 = 13,888 pairs) (Kaprio and Koskenvuo, 2002). Invitations were sent to all postmenopausal MZ twin sister pairs born from 1943 to 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 $\pm$ 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 and 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 $\leq$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 E_2 (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 E_2 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.

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

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 μl 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 μl, containing 3.35 μl of sample RNA, 2 μl of primers, 1.26 μl of RNase inhibitor (diluted 1:10), 1 μl of 10× buffer, 1 μl of 10 mM dNTP's, 0.66 μl of reverse transcriptase and 0.73 μl nuclease free H_2O. 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 μl, containing 0.5 μl 20× Taqman MicroRNA Assay (5′-FAM PCR primers and probes), 5 μl of Taqman® Universal Master Mix 2× (Applied Biosystems) and 1.33 μl 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 μl, which contained 1.67 μl of sample RNA, 1 μl of each stem-loop primer, 0.6 μl of RNA inhibitor (diluted 1:10), 0.5 μl of 10× buffer, 0.4 μl of 10 mM dNTP's, 0.3 μl reverse transcriptase and 0.5 μl H_2O. 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 μl and contained 0.25 μl of the 20x Taqman MicroRNA Assay (5′-FAM PCR primers and probes), 2.75 μl of the TaqMan Fast Universal PCR Master Mix (2×) (Applied Biosystems) and 2.25 μl 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 $\Delta Ct = \text{mean } Ct_{miR\ X} - \text{mean } Ct_{miR\ 17}$ and $\Delta Ct = \text{mean } Ct_{miR\ X} - \text{mean } Ct_{RNU44}$ 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® 1000 (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 Jylhava et al. (2012). Serum 17β-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

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.
Statistically significant results are marked as bold.

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 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, clear 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 ($p = 0.030$). 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

Fig. 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) (Fig. 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 ($r = 0.709$; $p = 0.022$).

4. Discussion

The purpose of this study was to investigate if estrogen or estrogen and progesterone containing postmenopausal HRT interplays with

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.
Statistically significant results are marked as bold.

Table 4
Serum (s-) and tissue miR relative expressions in arbitrary units (AU) and serum FasL concentrations and tissue transcript levels.

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**$^{\mathbf{M}}$	**0.001**$^{\mathbf{M}}$	**0.018**$^{\mathbf{W}}$	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**$^{\mathbf{W}}$	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**$^{\mathbf{M}}$	**0.021**$^{\mathbf{W}}$	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.
Statistically significant results are marked as bold.

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 E_2 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 E_2 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". 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

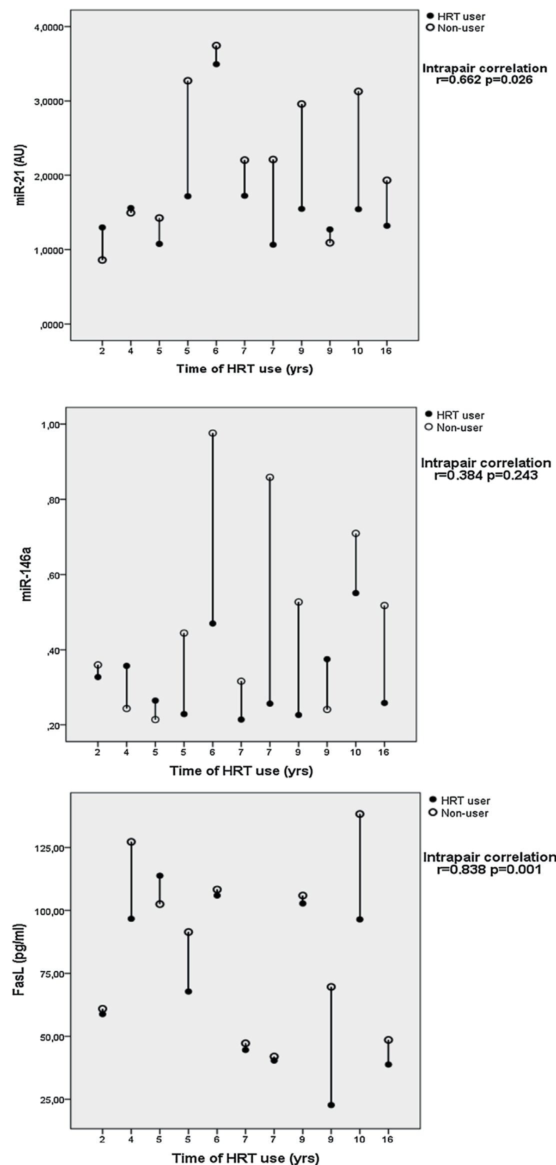

Fig. 1. Pairwise values in relation to the time of HRT use in years, and overall intrapair correlations of circulating miR-21, miR-146a transcripts and FasL serum concentrations.

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 sgb130, were lower in HRT users than the non-user co-twins, suggesting the E_2 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-1B, IL-6, TNFα 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 E_2 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

Table 5
Spearman correlations of circulating miR-21 and miR-146a transcript levels and FasL concentrations with measured inflammation parameters.

	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[a]	0.519
sgbp130 (ng/ml)	0.393	0.257	0.055	0.881	0.295[a]	0.243
cfDNA (μg/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						
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[a]	0.109
sgbp130 (ng/ml)	0.287	0.422	−0.018	0.960	0.154[a]	0.496
cfDNA (μg/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						
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[a]	0.907
sgbp130 (ng/ml)	0.098	0.789	**0.709**	**0.022**	**0.525**[a]	**0.040**
cfDNA (μg/ml)	−0.009	0.979	−0.064	0.853	0.033	0.894

Statistically significant results are marked as bold.
[a] $n = 10$ pairs.

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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2014.11.001.

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IV

DECLINING PHYSICAL PERFORMANCE ASSOCIATES WITH SERUM FASL, MIR-21, AND MIR-146A IN AGING SPRINTERS

by

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Research Article

Declining Physical Performance Associates with Serum FasL, miR-21, and miR-146a in Aging Sprinters

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Aging is associated with systemic inflammation and cellular apoptosis accelerating physiological dysfunctions. Whether physically active way of life affects these associations is unclear. This study measured the levels of serum inflammatory and apoptotic molecules, their change over 10 years, and their associations with physical performance in sprint-trained male athletes. HsCRP, cell counts, HGB, FasL, miR-21, and miR-146a were measured cross-sectionally ($n = 67$, 18–90 yrs) and serum FasL, miR-21, and miR-146a and their aging-related associations with physical performance were assessed over a 10-year follow-up ($n = 49$, 50–90 yrs). The cross-sectional study showed positive age correlations for neutrophils and negative for lymphocytes, red blood cells, HGB, FasL, and miR-146a. During the 10-year follow-up, FasL decreased ($P = 0.017$) and miR-21 ($P < 0.001$) and miR-146a ($P = 0.005$) levels increased. When combining the molecule levels, aging, and physical performance, FasL associated with countermovement jump and bench press ($P < 0.001$), miR-21 and miR-146a with knee flexion ($P = 0.023$; $P < 0.001$), and bench press ($P = 0.004$; $P < 0.001$) and miR-146a with sprint performance ($P < 0.001$). The studied serum molecules changed in an age-dependent manner and were associated with declining physical performance. They have potential as biomarkers of aging-related processes influencing the development of physiological dysfunctions. Further research is needed focusing on the origins and targets of circulating microRNAs to clarify their function in various tissues with aging.

1. Introduction

Physical exercise affects inflammatory state. The common understanding is that an acute bout of exercise results in a temporal inflammatory response, while regular training has a protective anti-inflammatory effect [1–4]. In addition to immune cells, skeletal muscle tissue contributes to the inflammatory response by releasing inflammatory molecules, such as TNF-α and IL-6, following of acute exercise [5]. During inflammation, whether induced by age, disease, or acute exercise, controlling the cellular balance of the inflammatory cells is important. Apoptosis, or programmed cell destruction, is a crucial mechanism for maintaining cellular balance in all tissues. Disruptions in the cellular homeostasis lead to either an accumulation of poorly functioning cells or a deficit of important cells, both recognized in aging. One way to regulate immune cell homeostasis, and subsequent immune response, is through Fas ligand - Fas receptor (FasL-Fas) interaction on the cell surfaces, which leads to target cell destruction [6]. There are indications that habitual training influences the apoptotic processes of the immune system. According to Mooren et al. [7], the basal levels of leukocyte apoptosis as well as the levels of exercise-induced apoptosis are distinct between highly trained and poorly trained men, suggesting training-induced adaptation to leukocyte homeostasis.

Traces from inflammation and cellular apoptosis can be detected from the circulation by measuring specific molecules from the blood. In addition to the classical inflammation (e.g., CRP, IL6, and TNF-α) and apoptotic markers

Cross-sectional 2012	*Group A*	*Group B*	*Group C*	*Group D*
Age (yrs)	18 to 39	50 to 66	66 to 79	79 to 90
N	18	16	18	15

Follow-up baseline in 2002, end-point in 2012	Baseline	End-point	Baseline	End-point	Baseline	End-point
Age (yrs)	40 to 56	50 to 66	56 to 69	66 to 79	69 to 80	79 to 90
N	16	16	18	18	15	15

Figure 1: Descriptions of the study designs needed in the current study.

(cytochrome c, Fas, and FasL), the small RNA molecules called microRNAs (miRs) are novel and potentially even more sensitive tools for screening these physiological processes. miRs are noncoding RNAs regulating gene expression by blocking the translation of specific target mRNA into proteins. miRs are released, either actively or passively, from different cell types into the blood stream, where they reflect the gene regulation changes in their cells of origin. Their role in intercellular communication has also been recognized [8]. In healthy conditions, circulating miRs most likely originate from blood or epithelial cells or from organs with high vascularization, including skeletal muscle cells [9]. Owing to trauma, cancer, cardiovascular disease, or other conditions influencing metabolism, the miR signature in the circulation changes.

mir-21 and miR-146a are among the miRs that seem to be responsive to various physiological stimuli. These miRs are associated with aging-related processes such as senescence and inflammation, miR-21 having proinflammatory and miR-146a having anti-inflammatory effects [10, 11]. Both miRs induce apoptosis by targeting FasL-Fas signaling, which strengthens the interplay of these molecules with each other and their role as regulators of immune cell homeostasis [12, 13]. Circulating miR-21 has also been shown to promote cachexia-related apoptosis in skeletal muscle cells [14]. Physical exercise creates a whole body adaptive responses that also affect miR regulation and expression in different cell types and subsequently in the circulation [15–17]. miR-21 and miR-146a have also been shown to be highly responsive to physical exercise. The pioneering work by Baggish et al. [15] demonstrated that these miRs were associated with cardiovascular/musculoskeletal adaptations and low-grade inflammation and changed in response to acute exercise as well as sustained training. Circulating miR-146a levels were upregulated by acute cycling exercise before and after 90 days of sustained row training, whereas miR-21 was only upregulated by acute exercise prior to the sustained training period. In addition, the highest levels of miR-146a during exercise were found to correlate with peak VO_{2max} [15], whereas miR-21 levels have been shown to be upregulated in males with low VO_2 max, indicating the contrasting roles of these two miRs [18]. Nielsen et al. [17] demonstrated that miR-146a decreased immediately after a bout of acute exercise, whereas the basal levels of miR-21 were downregulated after 12 weeks of endurance training. Both miR-21 and miR-146a have also been shown to exist at different levels in the plasma of young male endurance and strength trained athletes [19]. The above-mentioned studies demonstrate that these specific miRs detected in the circulation are affected differently by exercise type and duration and have potential as indicators of physiological changes [15, 16, 19, 20].

In order to evaluate if circulating FasL, miR-21, and miR-146a have the potential as biomarkers of training adaptations in aging athletes, longitudinal studies are needed. The aim of this study was to determine whether circulating FasL, miR-21, and miR-146a levels change over 10-year period among competitive male masters sprinters with a long-term training background and to assess their associations with physical performance measures and aging.

2. Methods

2.1. Study Design and Ethics. This study is part of an ongoing Athlete Aging Study (ATHLAS) [21–25] on young adult athletes and masters athletes from different sport disciplines. The participants were recruited from the memberships of Finnish athletic organizations. The present study comprised male sprinters. The sprinters had a long-term background in sprint training and had been successful in national or international 100–400 m sprinting events. Both cross-sectional and longitudinal study designs were used (Figure 1). In the cross-sectional analysis (using data from follow-up measurements conducted in 2012), the sprinters were divided into four age groups: (A) 18 to 39 years ($n = 18$), (B) 50 to 66 years ($n = 16$),

(C) 66 to 79 years (n = 18), and (D) 79 to 90 years (n = 15). In addition, baseline measurements (conducted in 2002) were available for 49 older masters sprinters (aged 50 to 90 years at follow-up) belonging to age groups B, C, and D, thereby allowing longitudinal analysis. Both the baseline and the follow-up measurements consisted of similar standardized two-day measurements.

Prior to the measurements, training and health history were elicited and evaluated using questionnaires. The average number of training years among the masters athletes at baseline was 34.1 ± 15.1 years. More detailed self-reported training histories (training frequency, sprint-specific training hours, and other training hours) and their changes over 10 years are presented in Tables 1 and 3. The sprint-specific training hours per week included sprints, jumps, and strength training, while the other training hours included all other notably strenuous physical exercises. Subjects over age 55 underwent a medical examination. Current ability to participate in physically demanding measurement was assessed ad hoc, individually by the study physician. The health of all the participants was in general good with no acute conditions (infections, traumas) or functionally limiting chronic neurological, cardiovascular, endocrinological, or musculoskeletal conditions. In a few cases, some physical tests were not performed due to local musculoskeletal pain.

The study was conducted according to the guidelines of the Declaration of Helsinki. All participants were informed a priori about the possible risks and discomfort of the physical and clinical measurements. Written informed consent, including permission for the use of the gathered data for research purposes only, was provided by the study subjects. The study protocol was approved by the ethics committees of the University of Jyväskylä (in 2002 for baseline) and the Central Finland Healthcare District (in 2012 for follow-up).

2.2. Physical Performance and Body Composition Measurements. Participants were instructed to rest (no heavy training or competition) two days before the measurements. On the first measurement day, the participants performed a maximal 60 m sprint twice on an indoor running track with spiked running shoes. Explosive force production of the lower limbs was measured by a vertical countermovement jump (CMJ). Isometric knee flexion force and isometric upper limb force were measured by bench press performance using a David 200 dynamometer (David Fitness and Medical Ltd., Outokumpu, Finland). The best of 2 or 3 trials was recorded in the subsequent analyses. In addition, the assessment of total body fat and lean mass (LBM) was performed with bioelectrical impedance (Spectrum II, RJL System, Detroit, MI). More detailed descriptions of the measurements are given elsewhere [22–25]. Participants' meals during the measurement days were arranged by the research organizers.

2.3. Serum Analyses. To obtain serum, venous blood was collected on the second measurement day under standard fasting conditions at least 12 h after exercise. The serum samples were stored at −80°C until analyzed. Serum FasL concentration was measured using a Human Fas Ligand/TNFSF6 Quantikine® ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Reactions were performed as duplicates and a common sample was added to each plate in order to observe whether controlling for interassay variation was necessary. HsCRP measurements at baseline and follow-up were performed with an Immulite 1000 Immunoassay System. Leukocyte counts were part of the standard medical complete blood count analysis.

2.4. RNA Extraction and miR Analyses. The RNA extraction methods and miR-21 and miR-146a analyses have been described previously [26]. Briefly, total RNA was isolated from 100 μl of serum with a total RNA purification kit (Norgen Biotek Corporation, Thorold, ON, Canada) according to the manufacturer's protocol. Synthetic *C. elegans* Cel-miR-39 (5′-UCA CCG GGU GUA AAU CAG CUU G-3′, Invitrogen) (25 fM, concentration determined by dilution series) was added at the lysis step to all of the samples as a spike-in control in order to monitor the efficiency and uniformity of the RNA extraction and qPCR procedure. RNA was reverse-transcribed to cDNA (V_{tot} = 10 μl) by a Taqman reverse transcription kit and the qPCR (V_{tot} = 10 μl) was performed with a Taqman Universal Mastermix II NO Ung using Taqman miR assays (hsa-miR-21: 5′-UAGCUUAUCAGACUGAUGUUGA-3′, hsa-miR-146a: 5′-UGAGAACUGAAUUCCAUGGGUU-3′) (Device: Applied Biosystems, ABI 7300).

Ct values less than 35 were accepted for the analysis. All the samples were normalized to a reference sample of an average sprint athlete across the plates. ΔCt values were calculated as $\Delta Ct = \text{mean } Ct_{miR\text{-}X} - \text{mean } Ct_{miR\,(reference)}$. Each reaction was performed in duplicate and the relative expressions were calculated by using the $2^{-\Delta Ct}$ method. To observe the possible contaminations and primer-dimers, no template controls (NTCs) were used in either the RT or qPCR reactions.

2.5. Statistical Analyses. In the age-correlation analyses of the cross-sectional design, Pearson's correlation coefficient was used for continuous variables and Spearman's correlation coefficient for ordinal variables. The paired sample t-test for parametric variables and Wilcoxon signed rank test for nonparametric variables were used in the longitudinal comparison in the 10-year follow-up design. In addition, Generalized Estimating Equations (GEE) models were constructed to study the association of FasL, miR-21, and miR-146a with the performance measures over age. The model was based on the two measurements points (2002, 2012) used in the longitudinal study with unstructured working correlation matrix specification and athletes' age as the descriptive metric of time. As the effect of age is generally nonlinear we used polynomial terms (quadratic and cubic) of age to include curvature in modeling the age-related effect of the FasL and the miRs on the outcomes. The results were adjusted for LBM. More detailed prediction equations based on GEE-models are presented in the supplementary data (S1 in Supplementary Material available online at https://doi.org/10.1155/2017/8468469).

TABLE 1: Self-reported training history and physical performance measures in different age groups and correlations of the variables with age.

	A 18–39 yrs (n = 18)	B 50–66 yrs (n = 16)	C 66–79 yrs (n = 18)	D 79–90 yrs (n = 15)	Correlation with age (all groups)/P value	95% CI/coefficient of determination	Correlation with age (only B, C, D)/P value	95% CI/coefficient of determination
Self-reported training								
Frequency (times/wk)	6.8 ± 2.3 (n = 15)	3.6 ± 1.6 (n = 14)	3.5 ± 1.3	3.1 ± 1.1 (n = 14)	−0.659 (n = 61) **P < 0.001**	−0.8 to −0.5 0.434	−0.176 (n = 46) P = 0.241	−0.4 to 0.1 0.031
Sprint training (h/wk)	7.0 ± 5.5	2.6 ± 1.7 (n = 14)	3.2 ± 3.2	2.7 ± 2.3 (n = 14)	−0.380^S(n = 64) **P = 0.002**	−0.6 to −0.1 0.144	−0.085^S(n = 46) P = 0.573	−0.3 to 0.2 0.007
Other training (h/wk)	2.5 ± 4.2	0.4 ± 0.6 (n = 14)	1.3 ± 2.0	2.0 ± 2.5 (n = 14)	0.179^S(n = 64) P = 0.157	−0.1 to 0.4 0.032	0.310^S(n = 46) **P = 0.036**	0.0 to 0.6 0.096
Physical performance								
Sprint 60 m (s)	7.52 ± 0.36 (n = 12)	8.43 ± 0.63 (n = 13)	9.29 ± 0.61 (n = 13)	10.81±1.30 (n = 10)	0.898^S(n = 48) **P < 0.001**	0.8 to 0.9 0.806	0.841^S(n = 36) **P < 0.001**	0.7 to 0.9 0.707
CMJ (cm)	45.7 ± 11.8 (n = 15)	32.0 ± 4.2 (n = 12)	25.8 ± 5.0 (n = 12)	18.9 ± 4.7 (n = 11)	−0.810 (n = 50) **P < 0.001**	−0.9 to −0.7 0.656	−0.782 (n = 35) **P < 0.001**	−0.9 to −0.6 0.612
Isometric knee flexion (N)	326 ± 86 (n = 17)	253 ± 57 (n = 15)	227 ± 34 (n = 14)	190 ± 49 (n = 12)	−0.647 (n = 58) **P < 0.001**	−0.8 to −0.5 0.419	−0.469 (n = 41) **P = 0.002**	−0.7 to −0.2 0.220
Isometric bench press (N)	1307 ± 347 (n = 17)	945 ± 159 (n = 14)	717 ± 175 (n = 13)	565 ± 126 (n = 11)	−0.777 (n = 55) **P < 0.001**	−0.9 to −0.6 0.604	−0.776 (n = 38) **P < 0.001**	−0.9 to −0.6 0.602

Table is formed based on the cross-sectional study design (2012) including all the athletes from ages 18 to 90 yrs. Results are presented as means ± SD. Age correlations are presented: (1) all athletes and (2) masters athletes only. CMJ: countermovement jump. ^{S}Spearman's correlation coefficient.

3. Results

3.1. Participant Characteristics and Serum FasL and miR Levels in Different Age Groups. Participants' characteristics in the different age groups and age correlations of the variables are presented in Tables 1 and 2. The age correlations are presented in two different ways: (1) inclusive of all the participants (ages 18 to 90) and (2) inclusive only of the masters athletes (ages 50 to 90). A modest negative correlation was observed between age and training frequency when all the athletes were included in the analyses ($r = -0.659$, 95% CI = −0.8 to −0.5, $P < 0.001$), with age accounting for 43.3% of the variation in training frequency. The same correlation was very low when only the masters athletes were included ($r = -0.176$, 95% CI = −0.4 to 0.1, $P = 0.241$), with age accounting only for 3.1% of the variation. Among the physical performance measures, a high positive correlation was found between age and 60 m sprint time among all the athletes ($r = 0.898$, 95% CI = 0.8 to 0.9, $P < 0.001$), with age accounting for 80.6% of the variation in sprint time. The pattern was similar when only the masters athletes were included in the analyses ($r = 0.841$, 95% CI = 0.7 to 0.9, $P < 0.001$), with age accounting for 70.7% of the variation in sprint time. The other physical performance measures showed a modest to high negative correlation with age when all the athletes were included (CMJ: $r = -0.81$, 95% CI = −0.9 to −0.7, $P < 0.001$, 65.6%; knee flexion: $r = -0.647$, 95% CI = −0.8 to −0.5, $P < 0.001$, 41.9%; and bench press: $r = -0.777$, 95% CI = −0.9 to −0.6, $P < 0.001$, 60.4%). When only the masters athletes were included, high negative correlations were observed between age and CMJ and age and bench press (CMJ: $r = -0.782$, 95% CI = −0.9 to −0.6, $P < 0.001$; bench press: $r = -0.776$, 95% CI = −0.9 to −0.6, $P < 0.001$), with age accounting for 61.2% and 60.2% of the variation, respectively. Between age and knee flexion, only a modest negative correlation was detected ($r = -0.469$, 95% CI = −0.7 to −0.2, $P = 0.002$), with age accounting for 22% of the variation in knee flexion strength.

Among the body anthropometric variables, modest negative correlations were observed between age and height, age and weight, and age and LBM when all the athletes were included (height: $r = -0.512$, 95% CI = 0.7 to −0.3, $P < 0.001$; weight: $r = -0.419$, 95% CI = −0.6 to −0.2, $P < 0.001$; LBM: $r = -0.525$, 95% CI = −0.7 to −0.3, $P < 0.001$), with age accounting for 26.2%, 17.6%, and 27.6% of the variation, respectively. Of the blood cell counts, low to modest negative correlations were detected between age and lymphocyte percentage and age and RBC when all the athletes were included (LYM%: $r = -0.384$, 95% CI = −0.6 to −0.2, $P = 0.002$; RBC: $r = -0.433$, 95% CI = −0.6 to −0.2, $P < 0.001$), with age accounting for 14.7% and 18.7% of the variation, respectively. In addition, a modest positive correlation was detected between age and neutrophil percentage ($r = 0.411$, 95% CI = 0.2 to 0.6, $P = 0.001$), with age accounting for 16.9% of the variation in neutrophils. When only the masters athletes were included, the pattern was similar in slightly weaker age correlations (LYM%: $r = -0.315$, 95% CI = −0.6 to 0.0, $P = 0.031$; RBC: $r = -0.370$, 95% CI = −0.6 to −0.1, $P = 0.010$; and NEUT%: $r = 0.326$, 95% CI = 0.0 to 0.6, $P = 0.025$), with age accounting for 9.9%, 13.7%, and 10.6% of the variation, respectively. A low negative correlation was observed between age and HGB both when all the athletes were included ($r = -0.383$, 95% CI = −0.6 to −0.2, $P = 0.002$) and when only the masters athletes were included ($r = -0.370$, 95% CI = −0.6 to −0.1, $P = 0.010$), with age accounting for 14.7% and 13.7% of the variation in HGB, respectively. Of the studied serum molecules, a modest negative correlation between age and FasL was detected when all the athletes were included in the analyses ($r = -0.596$, 95% CI = −0.7 to −0.4, $P < 0.001$), with age accounting for 35.5% of the variation in FasL concentration. A modest positive correlation was detected between age and miR-146a when all the athletes were included ($r = -0.611$, 95% CI = −0.7 to −0.4, $P < 0.001$), with age accounting for 37.3% of the miR-146a variation. No significant correlations between age and the serum molecules were detected when only masters athletes were included in the analyses.

3.2. Changes in the Physical Performance of Masters Athletes over 10 Years. Changes in the training frequencies and the change percentage of the specific physical performance measures in 10 years are presented in Table 3. The results are shown for masters sprinters as a whole and divided into 3 age groups (B: 50–66 yrs, C: 66–79 yrs, and D: 79–90 yrs presenting the end-point ages). The self-reported weekly training frequency, sprint, and other training hours decreased when all the masters athletes were involved in the analyses ($P < 0.001$, $P < 0.001$, and $P = 0.003$, resp.). When grouping the athletes, the declines in all the self-reported training activities remained significant for the group B ($P = 0.011$, $P = 0.009$, and $P = 0.008$, resp.). For group C, no significant changes in the training activities over 10 years were reported. For the group D, weekly training frequency and sprint training hours decreased significantly ($P = 0.004$, $P = 0.023$, resp.). From the physical performance measures 60 m sprint, CMJ and isometric bench press changed during the 10 years' follow-up period when all the masters sprinters were included ($P < 0.001$ for all measures). When the masters sprinters were divided into 3 age groups the changes remained for all groups B ($P = 0.001$, $P = 0.001$, and $P = 0.036$, resp.), C ($P = 0.002$, $P = 0.001$, and $P = 0.008$, resp.), and D ($P = 0.005$, $P < 0.001$, and $P < 0.001$, resp.), with the highest deterioration seen among the oldest athletes (group D). There were no significant changes in the knee flexion strength in 10 years.

3.3. Changes in the Serum hsCRP, FasL, miR-21, and miR-146a Levels of Masters Athletes over 10 Years. The change percentages of the blood parameters, hsCRP, FasL, miR-21, and miR-146a over 10 years are presented in Table 3. The results are shown for the masters sprinters both as a whole and divided into 3 age groups (B: 50–66 yrs, C: 66–79 yrs, and D: 79–90 yrs presenting the end-point ages). Serum hsCRP did not change over the 10-year period among the athletes. The serum FasL concentrations decreased ($P = 0.017$) and serum miR-21 and miR-146a levels increased ($P < 0.001$, $P < 0.005$, resp.) among the masters sprinters as a single group during the 10 years. At the age-group level, the changes were

Table 2: Participant characteristics and blood parameters in different age groups and correlations of the variables with age.

	A 18–39 yrs (n = 18)	B 50–66 yrs (n = 16)	C 66–79 yrs (n = 18)	D 79–90 yrs (n = 15)	Correlation with age (all groups)/P value	95% CI/coefficient of determination	Correlation with age (only B, C, D)/P value	95% CI/coefficient of determination
Anthropometrics and body composition								
Height (cm)	180.1 ± 5.0	178.2 ± 8.1	172.8 ± 4.7	172.4 ± 4.9	−0.512 (n = 67) **P < 0.001**	−0.7 to −0.3 0.262	−0.464 (n = 49) **P = 0.001**	−0.7 to −0.2 0.215
Weight (kg)	78.3 ± 6.8	80.2 ± 9.7	71.0 ± 6.2	69.4 ± 6.4	−0.419 (n = 67) **P < 0.001**	−0.6 to −0.2 0.176	−0.568 (n = 49) **P < 0.001**	−0.7 to −0.3 0.323
LBM (kg)	67.2 ± 5.7	66.3 ± 7.0	60.2 ± 4.1	58.3 ± 4.8	−0.525 (n = 67) **P < 0.001**	−0.7 to −0.3 0.276	−0.564 (n = 49) **P < 0.001**	−0.7 to −0.3 0.318
Body fat mass (kg)	11.2 ± 4.1	13.9 ± 6.1	11.1 ± 3.7	11.2 ± 3.6	−0.028 (n = 67) P = 0.857	−0.3 to 0.2 0.001	−0.301 (n = 49) **P = 0.035**	−0.5 to 0.0 0.091
Blood cell count								
WBC	5.6 ± 1.4	5.6 ± 1.0	5.7 ± 2.2 (n = 17)	5.4 ± 0.8 (n = 14)	−0.057 (n = 65) P = 0.652	−0.3 to 0.2 0.003	0.099 (n = 47) P = 0.509	−0.2 to 0.4 0.010
LYM%	39.2 ± 7.2	35.9 ± 6.2	34.2 ± 5.6 (n = 17)	31.0 ± 9.4 (n = 14)	−0.384 (n = 65) **P = 0.002**	−0.6 to −0.2 0.147	−0.315 (n = 47) **P = 0.031**	−0.6 to 0.0 0.099
MXD%	12.3 ± 4.8	11.5 ± 3.0	11.5 ± 1.9 (n = 17)	10.9 ± 2.4 (n = 14)	−0.174 (n = 65) P = 0.165	−0.4 to 0.1 0.030	−0.112 (n = 47) P = 0.455	−0.4 to 0.2 0.013
NEUT%	48.6 ± 8.5	52.6 ± 6.9	54.3 ± 6.6 (n = 17)	58.1 ± 9.5 (n = 14)	0.411 (n = 65) **P = 0.001**	0.2 to 0.6 0.169	0.326 (n = 47) **P = 0.025**	0.0 to 0.6 0.106
RBC	5.1 ± 0.4	4.9 ± 0.4	4.9 ± 0.4 (n = 17)	4.4 ± 0.3 (n = 14)	−0.443 (n = 65) **P < 0.000**	−0.6 to −0.2 0.187	−0.370 (n = 47) **P = 0.010**	−0.6 to −0.1 0.137
PLT	211.9 ± 44.0	242.3 ± 44.3	210.0 ± 83.6 (n = 17)	199.7 ± 42.9 (n = 14)	−0.100 (n = 65) P = 0.429	−0.3 to 0.1 0.010	−0.375 (n = 47) **P = 0.009**	−0.6 to −0.1 0.141
HGB	154.3 ± 9.3	152.9 ± 12.4	149.4 ± 9.3 (n = 17)	140.3 ± 8.0 (n = 14)	−0.383 (n = 65) **P = 0.002**	−0.6 to −0.2 0.147	−0.443 (n = 47) **P = 0.002**	−0.6 to −0.2 0.187
Serum molecules								
hsCRP (mg/L)	1.6 ± 5.1	1.7 ± 3.3	2.3 ± 6.0 (n = 17)	1.3 ± 1.2 (n = 14)	−0.035 (n = 65) 0.784	−0.3 to 0.2 0.001	−0.102 (n = 47) P = 0.999	−0.4 to 0.2 0.010
FasL (pg/ml)	92.3 ± 24.7	56.8 ± 21.9 (n = 14)	56.0 ± 18.2 (n = 14)	52.3 ± 23.4 (n = 13)	−0.596 (n = 59) **P < 0.001**	−0.7 to −0.4 0.355	−0.181 (n = 41) P = 0.258	−0.5 to 0.1 0.033
miR-21 (RE)	1.81 ± 0.75	2.22 ± 1.41	1.74 ± 1.05	1.65 ± 0.62	-0.095^{S} (n = 67) P = 0.444	−0.3 to 0.1 0.009	-0.118^{S} (n = 49) P = 0.419	−0.4 to 0.2 0.014
miR-146a (RE)	5.82 ± 2.66	1.83 ± 1.16	1.50 ± 0.71	1.51 ± 0.96	-0.611^{S} (n = 67) **P < 0.001**	−0.7 to −0.4 0.373	-0.105^{S} (n = 49) P = 0.473	−0.4 to 0.2 0.011

Table is formed based on the cross-sectional study design (2012) including all the athletes from ages 18 to 90 yrs. Results are presented as means ± SD. Age correlations are presented in two ways: (1) one including all the athletes and (2) one including only masters athletes. LBM: lean body mass, WBC: white blood cells, LYM: lymphocytes, MXD: mixed leukocytes, NEUT: neutrophils, RBC: red blood cells, PLT: platelet, HGB: hemoglobin, hsCRP: high sensitivity c-reactive protein, FasL: Fas-ligand, and RE: relative expression. ^{S}Spearman's correlation coefficient.

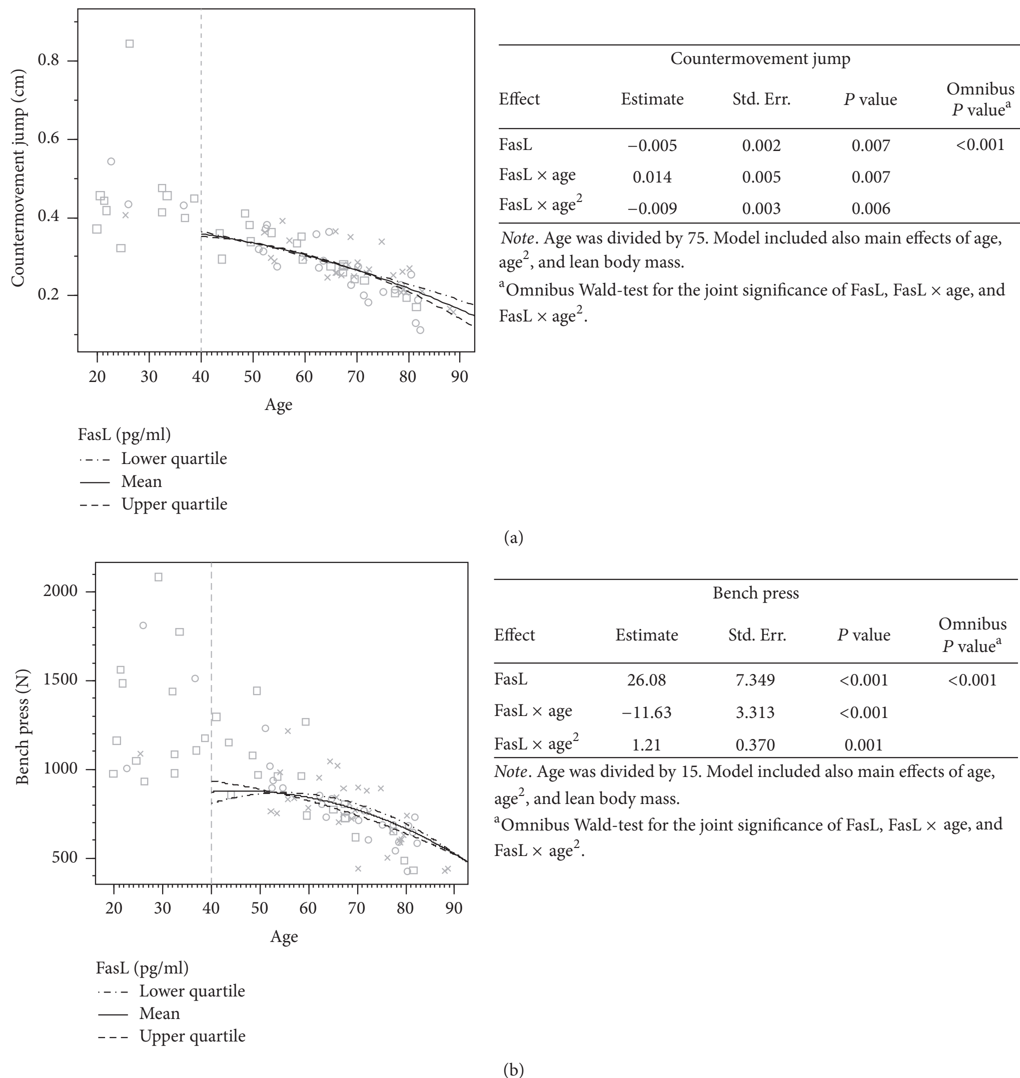
Table 3: The change in physical performance measures, self-reported training amounts, and serum molecule levels among all and age-grouped masters sprinters in the 10-year follow-up.

	All 50–90 yrs	B 50–66 yrs	C 66–79 yrs	D 79–90 yrs
Training frequency				
Change (times/wk)	−0.89 ± 1.22 (n = 42)	−0.91 ± 1.15 (n = 14)	−0.73 ± 1.43 (n = 15)	−1.06 ± 1.09 (n = 13)
P value	**P < 0.001**	**P = 0.011**	P = 0.067	**P = 0.004**
Sprint training				
Change (h/wk)	−1.84 ± 3.00 (n = 42)	−1.80 ± 2.25 (n = 14)	−1.94 ± 4.08 (n = 15)	−1.77 ± 2.43 (n = 13)
P values	**P < 0.001**	**P = 0.009**	P = 0.078	**P = 0.023**
Other training				
Change (h/wk)	−1.02 ± 2.62 (n = 32)	−1.53 ± 1.61 (n = 11)	−0.93 ± 3.44 (n = 11)	−0.56 ± 2.49 (n = 10)
P value	**P = 0.006**	**P = 0.008**	P = 0.221	P = 0.424
Sprint 60 m (s)				
Change%	11.9 (6.7–16.5) (n = 35)	8.2 (5.7–10.0) (n = 13)	12.2 (5.7–16.4) (n = 12)	15.8 (12.1–22.8) (n = 10)
P value	**P < 0.001**^{W}	**P = 0.001**^{W}	**P = 0.002**^{W}	**P = 0.005**^{W}
CMJ (cm)				
Change%	−15.4 ± 10.2 (n = 32)	−9.2 ± 6.3 (n = 11)	−15.7 ± 10.0 (n = 11)	−22.0 ± 10.4 (n = 10)
P value	**P < 0.001**	**P = 0.001**	**P = 0.001**	**P < 0.001**
Knee flexion (N)				
Change%	−1.8 ± 23.6 (n = 40)	5.5 ± 29.0 (n = 15)	−3.6 ± 20.1 (n = 13)	−9.07 ± 18.0 (n = 12)
P value	P = 0.062	P = 0.896	P = 0.287	P = 0.111
Isometric bench press (N)				
Change%	−10.9 ± 11.9 (n = 36)	−5.3 ± 9.8 (n = 14)	−14.0 ± 14.7 (n = 12)	−14.9 ± 8.51 (n = 10)
P value	**P < 0.001**	**P = 0.036**	**P = 0.008**	**P < 0.001**
hsCRP				
Change%	112 ± 349 (n = 47)	203 ± 512 (n = 16)	71.5 ± 230 (n = 17)	56.8 ± 220 (n = 14)
P value	P = 0.841	P = 0.404	P = 0.868	P = 0.220
FasL				
Change%	−5.3 ± 26.3 (n = 41)	−18.3 ± 16.7 (n = 14)	1.22 ± 29.1 (n = 14)	1.78 ± 27.9 (n = 13)
P value	**P = 0.017**	**P = 0.001**	P = 0.746	P = 0.587
miR-21				
Change%	77.0 (1.1–689) (n = 49)	107 (21.0–662) (n = 16)	37.9 (−34.7–834) (n = 18)	136 (21.3–995) (n = 15)
P value	**P < 0.001**^{W}	**P = 0.007**^{W}	P = 0.267^{W}	**P = 0.017**^{W}
miR-146a				
Change%	95.4 (−7.4–631) (n = 49)	93.0 (−3.0–1101) (n = 16)	18.6 (−26.0–444) (n = 18)	143 (53.6–861) (n = 15)
P value	**P = 0.005**^{W}	P = 0.079^{W}	P = 0.500^{W}	**P = 0.011**^{W}

The age ranges of the groups (B, C, and D) represent the ages at follow-up. Data are presented as means ± SD for parametric variables and as median (IQR) for nonparametric variables. ^{W}P value was calculated with paired t-test for normally distributed variables and with Wilcoxon signed rank test for nonparametric variables. ^{W}Wilcoxon signed rank test for nonparametric variables. CMJ = countermovement jump, hsCRP = high sensitivity C-reactive protein, and FasL: Fas-ligand.

significant for FasL and for miR-21 among the youngest masters sprinters (group B; P = 0.001; P = 0.007, resp.) and for miR-146a among the oldest group (D; P = 0.011). The change was also significant for miR-21 in the oldest group (group D, P = 0.017). The original values for the blood parameters are presented in supplementary data (S2).

3.4. FasL, miR-21, and miR-146a Associations with Physical Performance Measures in the 10-Year Follow-Up. A GEE model was constructed to combine the effects of a specific serum molecule (FasL, miR-21, or miR-146a) and the physical performance measures (60 m sprint, CMJ, knee flexion, or bench press) over time. The association curves for the physical performance measures according to the different measured circulating molecule levels are presented in Figures 2, 3, and 4. Only significant or trending curves are presented. The results for the models were adjusted with the LBM. Figure 2 shows that the combination of the effects of FasL



Countermovement jump				
Effect	Estimate	Std. Err.	*P* value	Omnibus *P* value[a]
FasL	−0.005	0.002	0.007	<0.001
FasL × age	0.014	0.005	0.007	
FasL × age^2	−0.009	0.003	0.006	

Note. Age was divided by 75. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of FasL, FasL × age, and FasL × age^2.

Bench press				
Effect	Estimate	Std. Err.	*P* value	Omnibus *P* value[a]
FasL	26.08	7.349	<0.001	<0.001
FasL × age	−11.63	3.313	<0.001	
FasL × age^2	1.21	0.370	0.001	

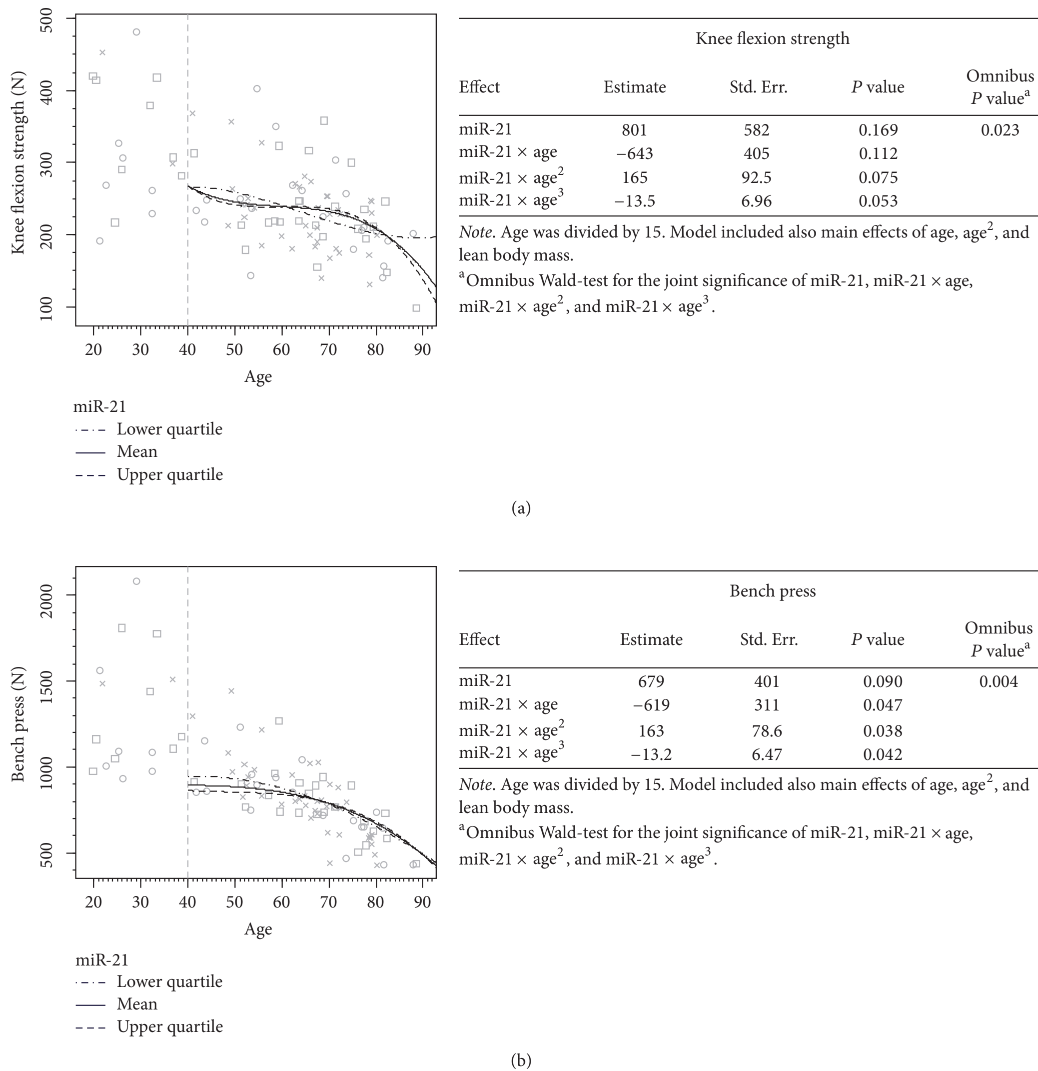
Note. Age was divided by 15. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of FasL, FasL × age, and FasL × age^2.

Figure 2: The association of serum FasL concentration with physical performance over time. Values for the younger participants (<40 years) without follow-up measures are presented on the left side of the images. The associations are based on the follow-up design (n = 49, >40 yrs). Cross (×) indicates that case is located within 0–37.5%, circle (○) within 37.5–67.5%, and square (□) within 67.5–100% of the cumulative share of the FasL distribution. The 3 different lines present the associations of the different serum marker levels with the physical performance measures over time. The tables next to the curves present the model used in forming the prediction curves, in greater detail, including statistics on the main effects of the studied serum marker and the possible quadratic and cubic effects.

and its interactions with age was statistically significant for the CMJ ($P < 0.001$) and bench press ($P < 0.001$). Also, all coefficient estimates for these outcomes were statistically significant indicating both a significant linear and quadratic curvature term. The model for the CMJ, when the serum FasL concentration was taken into account (Figure 2(a)), predicted a steeper decline in performance after age of 70 with slightly higher FasL serum concentrations than with lower FasL levels. For bench press, higher FasL levels at the younger ages predicted a steadier decline in performance while lower FasL values predicted better sustained performance at the older ages (Figure 2(b)).

Significant effects of miR-21 and age combined (Figure 3) were detected for knee flexion (P = 0.023) and bench press strength (P = 0.004). Statistically significant coefficient estimates relate mainly to miR-21 interaction with quadratic of



Knee flexion strength				
Effect	Estimate	Std. Err.	P value	Omnibus P value[a]
miR-21	801	582	0.169	0.023
miR-21 × age	−643	405	0.112	
miR-21 × age^2	165	92.5	0.075	
miR-21 × age^3	−13.5	6.96	0.053	

Note. Age was divided by 15. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of miR-21, miR-21 × age, miR-21 × age^2, and miR-21 × age^3.

(a)

Bench press				
Effect	Estimate	Std. Err.	P value	Omnibus P value[a]
miR-21	679	401	0.090	0.004
miR-21 × age	−619	311	0.047	
miR-21 × age^2	163	78.6	0.038	
miR-21 × age^3	−13.2	6.47	0.042	

Note. Age was divided by 15. Model included also main effects of age, age^2, and lean body mass.

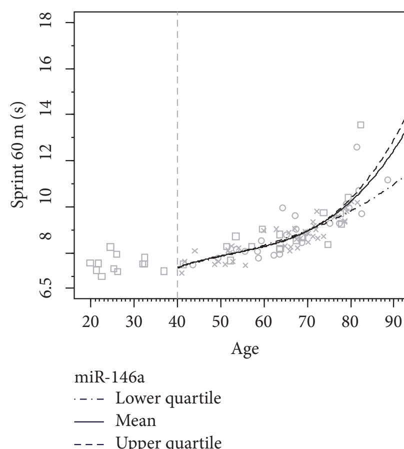
[a]Omnibus Wald-test for the joint significance of miR-21, miR-21 × age, miR-21 × age^2, and miR-21 × age^3.

(b)

Figure 3: The association of serum miR-21 level with physical performance over time. Values for the younger participants (<40 yrs) without follow-up measures are presented on the left side of the images. The predictions are based on the follow-up design ($n = 49$, >40 yrs). Cross (×) indicates that case is located within 0–37.5%, circle (○) within 37.5–67.5%, and square (□) within 67.5–100% of the cumulative share of the miR-21 -distribution. The 3 different lines present the associations of the different serum marker levels with the physical performance measures over time. The tables next to the curves present the model used in forming the prediction curves, in greater detail, including statistics on the main effects of the studied serum marker and the possible quadratic and cubic effects.

cubic terms of age indicating that curvature has a stronger role in the prediction. For knee flexion strength, when serum miR-21 levels were taken into account, low miR-21 levels predicted best performance prior to age 60 and high levels best performance between ages 60 and 80 (Figure 3(a)). Low miR-21 levels predicted highest performance in the bench press until age 65, after which the prediction curves were very similar to each other (Figure 3(b)).

Significant combination effects of miR-146a and age (Figure 4) were detected for sprint ($P < 0.001$), knee flexion ($P < 0.001$), and bench press strength ($P < 0.001$). The miR-146a levels predicted the largest differences in 60 m sprint performance after the age of 70, after which the lowest values predicted the best sprint performance (Figure 4(a)). For knee flexion strength, the lowest miR-146a levels predicted the best performance until age 60, after which, until age

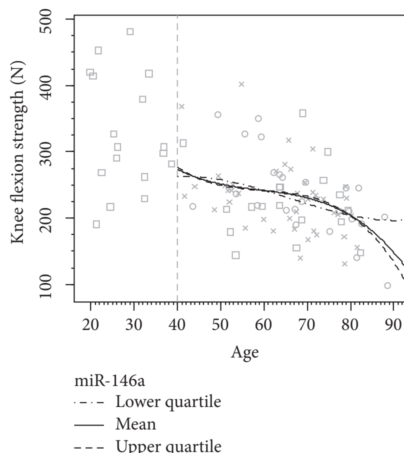


Sprint				
Effect	Estimate	Std. Err.	*P* value	Omnibus *P* value[a]
miR-146a	−7.43	2.24	0.001	<0.001
miR-146a × age	8.35	2.49	0.001	
miR-146a × age ^{2}	−3.06	0.908	0.001	
miR-146a × age ^{3}	0.367	0.108	0.001	

Note. Age was divided by 20. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of miR-146a, miR-146a × age, miR-146a × age ^{2}, and miR-146a × age ^{3}.

(a)

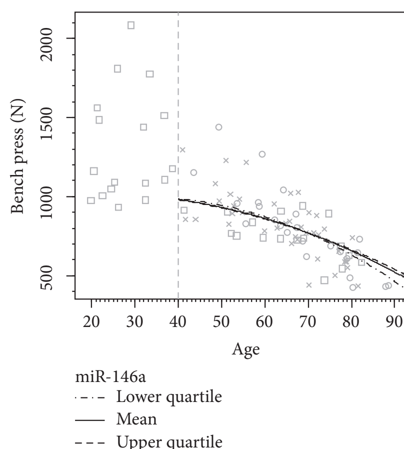


Knee flexion strength				
Effect	Estimate	Std. Err.	*P* value	Omnibus *P* value[a]
miR-146a	759	202	<0.001	<0.001
miR-146a × age	−788	197	<0.001	
miR-146a × age ^{2}	264	63.3	<0.001	
miR-146a × age ^{3}	−28.7	6.72	<0.001	

Note. Age was divided by 20. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of miR-146a, miR-146a × age, miR-146a × age ^{2}, and miR-146a × age ^{3}.

(b)



Bench press				
Effect	Estimate	Std. Err.	*P* value	Omnibus *P* value[a]
miR-146a	137	44.5	0.002	<0.001
miR-146a × age	−115	31.5	<0.001	
miR-146a × age ^{2}	21.7	5.39	<0.001	

Note. Age was divided by 20. Model included also main effects of age, age^2, and lean body mass.

[a]Omnibus Wald-test for the joint significance of miR-146a, miR-146a × age, miR-146a × age ^{2}, and miR-146a × age ^{3}.

(c)

Figure 4: The association of serum miR-146a level with physical performance over time. Values for the younger participants (<40 years) without follow-up measures are presented on the left side of the images. The predictions are based on the follow-up design (n = 49, >40 yrs). Cross (×) indicates that case is located within 0–37.5%, circle (○) within 37.5–67.5%, and square (□) within 67.5–100% of the cumulative share of the miR-146a distribution. The 3 different lines present the associations of the different serum marker levels with the physical performance measures over time. The tables next to the curves present the model used in forming the prediction curves, in greater detail, including statistics on the main effects of the studied serum marker and the possible quadratic and cubic effects.

80, the highest serum levels predicted the best performance in the knee flexion strength (Figure 4(b)). For bench press strength, the lowest miR-146a values predicted the highest performance until age 65, after which the lowest miR-146a levels predicted the steepest decline (Figure 4(c)).

4. Discussion

This study investigated the associations of circulating levels of traditional (hsCRP, leukocyte count, and FasL) and novel (miR-21 and miR-146a) inflammation- and apoptosis-related molecules with physical performance in competitive male sprinters of different ages. In addition, the associations of serum FasL, miR-21, and miR-146a levels with specific physical performance measures and aging were determined. We used both cross-sectional and follow-up study designs, with an emphasis on the latter, which focused on older masters sprinters. In the cross-sectional analysis, which included sprinters from ages 18 to 90 years, anthropometrics, LBM, physical performance measures, RBC, and HGB were, as expected, negatively associated with aging. For the traditional inflammation markers, no age-association was observed with hsCRP; instead, the percentage of serum lymphocytes decreased and that of neutrophils increased, with age. Serum FasL concentration and miR-146a levels correlated negatively with age when all the sprinters were included. However, when only the masters sprinters were studied, the age correlation was not significant, indicating that the most radical changes in these molecules generally occur during the interval between being a young sprinter and becoming a masters sprinter. The 10-year follow-up study design, which concerned masters sprinters only, showed, as expected, a worsening of physical performance in parallel with the decrement in the serum FasL and increment in the serum miR-21 and miR-146a levels. Interestingly, when grouped into 3 different age groups we obtained novel information about the time frames of the changes. For FasL and miR-21 the changes were significant for the 50- to 66-year-old (end-point age) sprinters and for miR-21 and miR-146a for the 79- to 90-year-old sprinters. No significant changes were observed in the 66–79-year-old athletes. In addition, associations with serum molecules, physical performance and aging were determined. We found nonlinear associations of circulating FasL concentration with CMJ height and bench press strength. MiR-21 levels were associated with knee flexion and bench press strength and miR-146a levels with sprint time, knee flexion, and bench press strength. The associations were based on the 10-year-follow-up data and age was used as a continuous determinant. Based on the constructed model, it is possible to predict whether and how the different levels of the studied serum molecules explain the declining physical performance measures over time.

Aging is accompanied with declining skeletal muscle properties and increasing numbers of systemic classical inflammatory markers, which, in general, affect physical functioning [27]. Inflammation and apoptosis are two crucial processes known to be altered during the aging process having broad physiological or even pathological influences in the body [28, 29]. Prolonged physical training has been shown to improve the systemic inflammatory state, especially by lowering hsCRP and IL-6 levels, as well as preventing the loss of muscle mass [3]. In the present study, hsCRP neither differed between the studied age groups nor changed during the 10-year follow-up among the masters athletes. Therefore, we focused on the more specific circulating molecules, FasL, miR-21, and miR-146a, interplaying with aging, inflammation, apoptosis, and skeletal muscle tissue [6, 10–14].

4.1. FasL as a Potential Biomarker. Serum FasL contributes to cellular homeostasis by inducing apoptosis of the target cells, especially T lymphocytes [6]. Serum FasL concentrations have been shown to decrease with aging [29, 30], with higher serum FasL levels being associated with diseases related to imbalanced homeostasis of the immune cells [31–33]. As the follow-up results show, the most radical change in serum FasL levels in the present study had occurred by age 66, with the levels having decreased significantly by that age. This could be interpreted as a decrement in the apoptotic rate. Lower serum FasL level predicted better overall performances (CMJ, bench press). In light of both these studies and our findings, the natural decrement in FasL during aging could be beneficial for balancing the changing metabolism and inflammatory status. However, conflicting studies and theories exist. The shift towards reduced apoptosis, measured by decreased serum FasL levels, could be followed by an accumulation of immune cells, resulting in "inflammaging" or an accumulation of other cell types, thereby increasing the risk for cancer development (reviewed by Tower [34]). However, the traditional inflammation marker hsCRP levels of the oldest athletes in the present study would appear to be in the normal healthy range and show no indication of an increased inflammatory state. Instead, the measured higher count of circulating neutrophils among older sprinters could be an indication of slightly higher inflammatory status compared to younger athletes. However, the role of neutrophils as driving forces of tissue repair and regeneration has also recently been discussed (reviewed by Jones et al. [35]). Immune cell homeostasis has been shown to differ between men with opposite training background [7]. The authors showed that the basal level of lymphocyte apoptosis, controlled by Fas-FasL interaction, is distinctly different between high and low trained men, being higher among the former. However, right after a bout of acute exercise, the high-trained men seemed to be more resistant to exercise-induced apoptosis. It is possible that, through physiological adaptations induced by long-term training, the basal levels of serum FasL could be kept at low levels without adding to inflammatory status; instead the postexercise condition would function in its own, adapted, way. However, this notion needs to be addressed by a study that also includes nontrained sedentary people.

4.2. miR-21 as a Potential Biomarker. miRs are regulating several biological processes in cells including those associated with adaptation to exercise, inflammation, and apoptosis (see S3). miR-21 is widely known as an antiapoptotic-miR owing to its presence at high levels in several malignancies. Its

systemic levels have also been shown to be upregulated in elderly people and its possible role as an inflammatory marker has been discussed [10]. In the present study, miR-21 levels increased significantly in the earlier years (40+), leveled out through the middle years (56+) and again increased significantly in the later years (69+). The self-reported training histories showed that the most significant decline in training occurred among the 40+ group and 69+ group, showing the opposite pattern to that of the miR-21 levels in those age groups. However, the associations between the change in sprint-specific training and the change in miR-21 levels (data not shown) were analyzed and no significant correlations were found ($R^2 = 0.074$; $P = 0.081$), indicating that the decline in training did not explain the increments in serum miR-21. Our association analyses indicate that lower miR-21 levels are more beneficial for knee flexion and bench press performance. These findings support the idea that the higher the level of miR-21, the more unbeneficial it is for physiological status. Our results are also in line with the study by Wardle et al. [19], who reported that young male strength athletes have lower levels of plasma miR-21 than endurance athletes, which could be a beneficial result favoring strength training.

4.3. miR-146a as a Potential Biomarker. miR-146a has been proposed as an anti-inflammatory miR, negatively regulating the inflammatory response by targeting TNF receptor-associated factor 6 (TRAF-6) and IL-1R-associated kinase (IRAK-1) [11]. In the present study, miR-146a serum levels increased significantly among the oldest participants after age 69. This finding raises the question of whether circulating miR-146a is one of the regulators and a component of the training-induced adaptation system, needed to balance the inflammatory status in the elderly. In the sprint association, the miR-146a levels at the earlier ages did not seem to predict performance in the later years; however, with lower levels after age 70, better 60 m sprint time was obtained. In the knee flexion association, similar results were obtained for miR-21: with lower levels, better performance was obtained in the later years. In the bench press association, with higher miR-146a levels, slightly better results in bench press performance were obtained in the later years. These results for miR-146a and physical performance associations in aging showed a distinct pattern for the sprint versus bench press, with lower levels being more beneficial for sprint and higher levels for bench press in the later years.

Masters athletes demonstrate that, with an active, motivated, and healthy lifestyle, aging does not inevitably lead to physical frailty and disability [36]. In our study, even explosive strength and sprinting performances were preserved at relatively high levels into old age, even if some athletes reported a decline in their training activity over the 10-year follow-up. However, despite habitual training, after 80 years of age the hitherto modest decline in the performance assumes a more radical form. It has been suggested that in old age the curvilinear decline in physical performance may be explained by a concomitant deterioration in several physiological systems [37]. The role of circulating molecules delivering intercellular messages in these deteriorative events is evident, however, very complex. In the present study, the decline in the physical performance measures over time was partially explained by changes in the serum FasL, miR-21, and miR-146a levels, molecules associated with inflammation and cellular homeostasis. More detailed functional and tissue specific studies are thus needed to better understand the role and regulation of these potential biomarkers in aging and training adaptations.

5. Conclusions

The main focus of the study was to determine whether specific circulating inflammation- and apoptosis-related molecules, that is, FasL, miR-21, and miR-146a, are associated with physical performance and aging among masters sprinters. Previous studies have demonstrated distinct associations of the studied molecules with physical performance and with age, but longitudinal combined associations have not been reported. We showed that the systemic levels of these molecules change over 10-year period and that associations exist between the molecules, specific physical performance measures, and aging. In addition, the associations with physical performances were slightly different depending on the age of the masters sprinters. Lower levels of FasL and miR-21 seemed to have more beneficial association with the performance measures generally, whereas the associations between miR-146a and performance are more dependent on the specific type of physical performance measure used. Further research with well-controlled study designs and populations are needed to determine whether these molecules are useful as biomarkers in the prediction of successful aging or identification of individuals at high risk for deterioration in performance with older age. In addition, the origin of the circulating biomarkers remains to be clarified.

Additional Points

Limitations and Benefits. This study only concerned relatively healthy males with a regular training background. However, it has been emphasized that athletes themselves are the best controls in the study of inherent aging [37]. Small percentage of the masters sprinters, who had taken part in the baseline measurements in 2002, was not available for the study follow-up in 2012. It is likely, therefore, that the follow-up setting is based on the fittest sprinters; this should be considered when interpreting the results. It should also be noted that the greatest emphasis in the physical performance association curves is on the middle age range, where the number of the participants was the greatest. The predictions are the most reliable until the age of 85 years from which onwards the thinning of the subjects might have unwanted effects on the predictions. It is important to bear in mind that miRs are sensitive and easily affected by external and internal stimuli. It was not possible to control for all the potentially confounding stimuli that could have affected the circulating miR levels (such as personal diet, lack of sleep, and nondiagnosed conditions). Previous studies have reported that miR-146a

reacts acutely to exercise [17, 38]. However, the present study population represents relatively healthy, regularly training males with a long training history. With their bodies being well adapted to even rather heavy exercise, no surprising acute effects on blood parameters during the brief physical measurements on the previous day of blood sampling were expected. The participants had a similar background in their training and other living habits (no regular smokers, no heavy drinking). Also, the fact that the study design was similar in both years strengthens longitudinal comparability. When studying athletes, the motivation to reach one's personal peak in measurements of performance is likely to be higher than in nonathletes. One of the major strengths of the study is the 10-year follow-up design: it is unique and leaves no space for genetic variation, which is an issue in cross-sectional designs, especially when studying partly genetically regulated biomarkers, as shown in our previous study with monozygotic twins [26].

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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