THE EFFECT OF HORMONE REPLACEMENT THERAPY ON HDL-CARGO OF POSTMENOPAUSAL WOMEN

Special reference to microRNA content

Veera Jakoaho

Master's Thesis in Sports and Exercise Medicine
Faculty of Sport and Health Sciences
University of Jyväskylä
Spring 2020

TIIVISTELMÄ

Jakoaho, V. 2020. Hormonikorvaushoidon vaikutukset HDL:n kuljettamiin mikro-RNA-molekyyleihin postmenopausaalisilla naisilla. Liikuntatieteellinen tiedekunta, Jyväskylän yliopisto, Liikuntalääketieteen pro gradu -tutkielma, 60 s., 6 liitettä.

Ikääntyminen lisää sydän- ja verisuonitautien riskiä. Myös vaihdevuosien aikaiset hormonimuutokset altistavat naiset epäedullisille aineenvaihdunnan muutoksille, jotka tulisi erottaa ikääntymiseen liittyvistä muutoksista. Suuri korkean tiheyden lipoproteiinin kolesterolin (HDL-C) määrä on yhteydessä pienempään sydän- ja verisuonitautiriskiin. HDL-C:n määrä näyttäisi kasvavan vaihdevuosien aikana, mutta tällöin se ei anna suojaa sydän- ja verisuonitaudeilta. Vaihdevuosien aikana korkean tiheyden lipoproteiinin (HDL) rakenteessa saattaakin tapahtua muutoksia, jotka vaikuttavat sen toimintaan. Proteiinien ja rasvojen lisäksi HDL kuljettaa lyhyitä RNA-molekyylejä, mikro-RNA:ita (miRNA), jotka säätelevät solujen toimintaa. Systeemisen estradiolitason (E_2) on todettu vaikuttavan tiettyihin verenkierrossa kulkeviin miRNA:ihin, kuten miR-21, -126 ja -146a, joilla on yhteys myös sydän- ja verisuonitauteihin sekä kestävyyskuntoon. Estradiolipitoisuuden vaikutusta HDL:n kuljettamiin miRNA:ihin ei ole kuitenkaan tutkittu. Tämän tutkimuksen tarkoituksena oli selvittää vaihdevuosien hormonimuutosten vaikutusta HDL:n kuljettamiin miRNA:ihin tutkimalla hormonikorvaushoidon (HRT) vaikutusta miR-21, -126 ja -146a -tasoihin, sekä selvittää kyseisten miRNA:iden ja kestävyyskunnon välistä yhteyttä.

Tämä tutkielma oli osa ”Keski-ikäisten naisten metabolisten toimintahäiriöiden riski: systeeminen ja intrakriininen estrogeeni sekä mikro-RNA:t välittävinä tekijöinä” (EsmiRs) -tutkimusta. Tutkimusjoukko koostui terveistä vaihdevuodet ohittaneista HRT-käyttäjistä (n = 5) sekä ei-käyttäjistä (n = 5). Aineenvaihduntasairauksien puuttuminen mahdollisti HRT:n vaikutusten tutkimisen ilman tautirasitetta. Tutkittavien kestävyyskuntoa mitattiin maksimaalisella polkupyöräergometritestillä ja se kuvattiin kuormituksessa saavutetun hapenkulutuksen huippuarvon (VO_{2peak}) avulla. HDL eristettiin ennen testiä otetuista seeruminäytteistä ja edellä mainitut miRNA:t mitattiin käyttäen reaaliaikaista polymeraasiketjureaktioita (qPCR).

Ryhmien välillä ei ollut merkitsevää eroa HDL-C määrässä eikä HDL:n kuljettamissa miR-21, -126 ja -146a -tasoissa. miR-126 -tason ja E_2 välillä havaittiin positiivinen yhteys ($p = 0.029$). Tutkituista miRNA:ista ainoastaan miR-146a korreloi negatiivisesti VO_{2peak} -arvojen kanssa HRT-käyttäjillä ($p = 0.037$) sekä kiihtyvyysmittarilla mitatun vapaa-ajan liikunnan kanssa ei-käyttäjillä ($p = 0.037$).

Tämän tutkimuksen perusteella HRT:n käyttö ei vaikuta HDL-C:n määrään eikä HDL:n miR-21, -126 ja -146a tasoihin. Pienestä otoskoosta huolimatta miR-126 ja E_2 välillä havaittiin yhteys ja tutkimustulos tukee aiemmin havaittua yhteyttä miR-146a ja liikunnan välillä. Jatkossa tulisi tutkia tarkemmin vaihdevuosien hormonimuutosten vaikutusta HDL:n toimintaan.

Asiasanat: mikro-RNA, vaihdevuodet, estradioli, sydän- ja verisuonitaudit, kestävyyskunto

ABSTRACT

Jakoaho, V. 2020. The effects of hormone replacement therapy on HDL-cargo of postmenopausal women: special reference to microRNA content. Faculty of Sport and Health Sciences, University of Jyväskylä, Master's thesis in Sports and Exercise Medicine, 60 pp., 6 appendices.

Cardiovascular disease (CVD) risk increases with age. Also, menopause, a period when women undergo significant hormonal changes, predisposes to several unfavourable metabolic alterations which increase CVD risk and should be separated from age-related changes. Even though several studies show that high-density lipoprotein cholesterol (HDL-C) increases during menopause, it does not protect from CVD. Hence, high HDL-C levels may not always reduce CVD risk. High-density lipoprotein (HDL) may undergo compositional changes during menopause that affect its function. In addition to the protein and lipid cargo, HDL transports short RNA molecules, microRNAs (miRNAs), that regulate cell functions. Systemic estradiol (E_2) level has previously been reported to affect specific circulating miRNAs, such as miR-21, -126 and -146a, which also associate with CVD and cardiorespiratory fitness (CRF). However, no study has examined the effects of serum E_2 on HDL-carried miRNAs. The aim of this study was to examine the effects of menopausal hormonal changes on HDL-carried miRNAs by investigating the effects of hormone replacement therapy (HRT) on miR-21, -126 and 146a content of HDL and to examine the association between these miRNAs and CRF.

This study was part of "the Systemic and Intracrine Estrogen and MicroRNAs mediate the risk of metabolic dysfunction in middle-aged women" (EsmiRs) study. Participants comprised of healthy postmenopausal HRT using (n = 5) and non-using (n = 5) women without any notable metabolic diseases which enabled examining the effects of HRT without a disease burden. CRF level was measured with maximal graded exercise test and described as peak oxygen uptake (VO_{2peak}). Pre-exercise test serum samples were used to isolate HDL and the foregoing miRNAs were measured using quantitative polymerase chain reaction (qPCR).

The studied groups did not differ in HDL-C concentration nor HDL-carried mir-21, -126 and -146a levels. However, there was a positive correlation between miR-126 and serum E_2 ($p = 0.029$). From the studied miRNAs, only miR-146a correlated negatively with VO_{2peak} in HRT using women ($p = 0.037$) and positively with accelerometer measured leisure time physical activity in non-using women ($p = 0.037$).

Based on this study the use of HRT does not affect HDL-C nor HDL-carried miR-21, -126 and -146a levels. However, despite of the small sample size, association was found between miR-126 and E_2 and the present study supports previously observed association of miR-146a with physical activity. Future studies should focus on examining the effects of menopausal hormonal changes on HDL function.

Key words: microRNA, menopause, estradiol, cardiovascular disease, cardiorespiratory fitness

ABBREVIATIONS

AGO2	argonaute 2
apoA-I	apolipoprotein A-I
AU	arbitrary unit
BF	body fat
BMI	body mass index
CAD	coronary artery disease
cDNA	complementary DNA
CI	confidence interval
ci-miRNA	circulating microRNA
CRF	cardiorespiratory fitness
Ct	cycle threshold
CV	cardiovascular
CVD	cardiovascular disease
DGUC	density-gradient ultracentrifugation
E_2	estradiol
EC	endothelial cell
ERMA	the Estrogenic Regulation of Muscle Apoptosis – Study
EsmiRs	the Systemic and Intracrine Estrogen and MicroRNAs mediate the risk of metabolic dysfunction in middle-aged women – Study
ET	exercise training
EV	extracellular vesicle
FMP	final menstrual period
FSH	follicle-stimulating hormone
g	g-force
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HRT	hormone replacement therapy
H_2O	water

KBr	potassium bromide
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
LTPA	leisure time physical activity
MET	metabolic equivalent
MetS	metabolic syndrome
miR	microRNA
miRNA	microRNA
mRNA	messenger RNA
NGS	next generation sequencing
nm	nanometre
NPM1	nucleophosmin 1
PA	physical activity
PBS	phosphate-buffered saline
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RCT	reverse cholesterol transport
RER	respiratory exchange ratio
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
STRAW + 10	The Stages of Reproductive Aging Workshop + 10
TBS-T	tris-buffered saline-tween
TC	total cholesterol
TG	triglyceride
T2D	type 2 diabetes
VO_{2max}	maximal oxygen consumption
VO_{2peak}	peak oxygen uptake

W	watt
WB	western blot
WHO	World Health Organization
y	year
ΔCt	mean cycle threshold

TABLE OF CONTENTS

Appendix 2. Qiagen miRNeasy protocol (manual).

Appendix 3. Qiagen miScript protocol (manual).

Appendix 4. Questions for assessing self-reported leisure time physical activity (EsmiRs).

Appendix 5. miR-21, -126 and -146a content of EVs in HRT using and non-using women.

Appendix 6. NGS read counts of miRNA content of HDL of a premenopausal woman.

1 INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in women (Roth et al. 2018). Aging leads to several factors predisposing for CVD, yet certain metabolic alterations, such as a shift towards more atherogenic lipid profile, has been shown to associate with menopause even independent of chronological aging (Karvinen et al. 2019). Therefore, reduction in systemic estrogen level has been suggested to contribute to the increased CVD risk (Carr 2003).

Physical activity (PA) improves cardiorespiratory fitness (CRF) which in turn is associated with reduced CVD risk (Ross et al. 2016). Moreover, PA seems to associate with increased high-density lipoprotein (HDL-C) levels (Kodama et al. 2007). High HDL-C levels are generally considered to protect from CVD (von Eckardstein et al. 2001a). Increasing body of literature suggests that HDL-C may increase when women transition through menopause El Khoudary 2017). However, as CVD risk increases in postmenopause, increased HDL-C concentration may not provide cardioprotection after menopause (El Khoudary 2018). It has been proposed that high-density lipoprotein (HDL) particles may undergo compositional changes reducing the protective capacity of HDL in postmenopause (Woodard et al. 2011). However, it is not explicit which compositional factors may underlie the potential changes in HDL function.

During recent years short non-coding RNAs, microRNAs (miRNAs), have been extensively studied as candidates for involvement in various diseases (Mendell & Olson 2012). miRNAs are post-transcriptional regulators of gene expression, present in all cell types (Mori et al. 2019). They are also secreted into circulation and transported in distinct carriers, such as extracellular vesicles (EVs) and HDLs, hence participating in intercellular communication (Turchinovich et al. 2013). Circulating miRNA (ci-miRNA) signatures in blood have been reported to be altered in different physiological and pathological conditions, such as CVD, which makes them potential blood-based biomarkers (Mori et al. 2019). Specific serum ci-miRNAs, including miR-21, -126 and -146a, have been shown to differ between postmenopausal women using hormone replacement therapy (HRT) and postmenopausal non-using women (Kangas et al. 2014; Kangas et al. 2017). Hence systemic estrogen level affects ci-miRNA profile. However, whether this association is carrier specific, is not known.

Distribution of ci-miRNAs have been shown to differ between different carriers in health and disease (Vickers et al. 2011). Hence, a proper isolation of these carrier molecules is essential to determine the effects of systemic estrogen on ci-miRNAs. Serum contains significantly more HDL particles compared to EVs (Simonsen 2017 (Suppl.)). Therefore, the purpose of this Master's thesis was to isolate HDL from other serum miRNA carriers and to investigate the effects of HRT on the content of miR-21, -126 and -146a in HDL. Moreover, this study aimed to determine the association of physical fitness with HDL-carried miRNA levels.

This Master's thesis is part of "the Systemic and Intracrine Estrogen and MicroRNAs mediate the risk of metabolic dysfunction in middle-aged women" (EsmiRs) research project conducted at the University of Jyväskylä in the Gerontology Research Center and funded by Academy of Finland (grant 309504 to EKL). As for data collection, author of this study was responsible for HDL isolation and RNA extraction and quantification from ready-harvested serum samples (n = 10) during November – December 2019. The rest of the data were provided ready for the author.

I thank Adjunct Professor Eija Laakkonen for providing the data for this study. I would like to thank Postdoctoral Researcher Sira Karvinen for her contribution with respect to data collection and analysis, and guidance and support during this Master's thesis. I would also like to thank Laboratory Manager Maarit Lehti for her advice regarding data collection as well as Chief Laboratory Technician Petri Papponen for his assistance during laboratory visits at the Department of Biological and Environmental Sciences. Lastly, I would like to thank EsmiRs research group for assistance and laboratory staff for advice during my laboratory work at the Sports and health laboratory.

2 MENOPAUSE

Menopause is a natural part of women's aging defined as permanent cessation of menstrual periods due to the loss of ovarian function (WHO 1996). Hence, it is the time point when woman experiences her final menstrual period (FMP) and consequently can only be determined retrospectively (Soules et al. 2001). Menopause can be ascertained if amenorrhea has continued over 12 continuous months given that it is not associated with any other evident physiological or pathological reason (WHO 1996). On average, women in Finland become menopausal at the age of 51 (Mikkola 2019). The onset of menopause appears to be affected by socioeconomical status, genetic factor, racial and geographic differences and lifestyle factors, such as smoking (Soules et al. 2001; Gold 2011).

The Stages of Reproductive Aging Workshop + 10 (STRAW + 10) has defined a staging system of the reproductive aging (Harlow et al. 2012). The stages can also be described as three broad phases: pre-, peri- and postmenopause (Nelson 2008). Premenopause refers to the reproductive period before perimenopause (Nelson 2008) whereas perimenopause describes the transitional years commencing from the first symptoms of the oncoming menopause and continuing until one year after FMP (WHO 1996). Postmenopause indicates the time commencing from the FMP (Davis et al. 2015). Postmenopause can be further divided into early postmenopause which describes the first five to eight years after FMP and late postmenopause which begins thereafter and continues until demise (Harlow et al. 2012).

Menopausal hormonal changes are a combined result of aging of the hypothalamus and the ovaries (Davis et al. 2015). Already years before menopause, at the beginning of menopausal transition, a decrease in follicular mass results in reduction in inhibin, a product of ovarian granulosa cells, affecting pituitary gland and leading to an increase in gonadotrophin-releasing hormone production (Mikkola 2019). An increase in follicle-stimulating hormone (FSH) and impaired surge of luteinizing hormone release together with the simultaneous ovarian aging leads to a decrease in estradiol (E_2) (Davis et al. 2015) which is the most important form of estrogen during the reproductive years (Gold 2011). After a marked decline of ovarian follicles, E_2 has decreased significantly and menstruation ceases (Mikkola 2019). Due to cessation of

ovarian cyclic activity also production of progesterone, ovarian hormone produced by corpus luteum, declines (WHO 1996). After menopause, FSH levels continue to rise whereas E_2 levels decrease approximately up to two years after which the concentrations stabilize (Harlow et al. 2012). Due to menopause approximately one third of women's lives is spent in an estrogen-deficient state and it may therefore have significant health consequences (Gold 2011).

Menopause associates with various symptoms of which vasomotor symptoms, i.e. hot flushes and night sweats, as well as vaginal dryness are the most prominent ones (Davis et al. 2015). Some of the commonly experienced symptoms such as insomnia, urinary incontinence, sexual dysfunction and mood symptoms may rather be secondary to other symptoms (Nelson 2008). The presence and severity of symptoms can vary considerably between women (Davis et al. 2015). In addition, estrogen deficiency has been associated with several long-term consequences, such as bone loss (Greendale et al. 2012) possible impairments in cognitive functions (Karlamangla et al. 2017) and decline in physical performance (Cooper et al. 2008). Moreover, menopause associates with several metabolic alterations, such as increased central fat accumulation, decreased lean body mass, reduced glucose tolerance, unfavorable alterations in lipid profile and altered inflammatory markers, all of which are risk factors for CVD (Carr 2003). HRT is used to treat menopausal symptoms (Nelson 2008) and it may partially oppose many of the unfavorable changes induced by menopause (Salpeter et al. 2006a).

2.1 Menopause and cardiovascular disease risk

CVDs refer to all disorders influencing the heart and blood vessels and are commonly associated with a pathological process, atherosclerosis, wherein lipids accumulate inside artery wall formulating plaques and thus, constrain oxygen-rich blood flow (Mendis et al. 2011). Several metabolic factors including total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), HDL-C, triglycerides (TGs) and body fat (BF) as well as lifestyle factors, such as regular PA, play an important role in the etiology of CVD (Yusuf et al. 2004; Mendis et al. 2011). CVD is the leading cause of mortality worldwide (Roth et al. 2018). The incidence of CVD is rare among women below 50 years of age but by the age of 70, women develop CVD at equal rate as men (Carr 2003). Women experiencing menopause prematurely due to surgical

bilateral oophorectomy, have been shown to have increased risk for CVD compared to premenopausal women of the same age (Gohlke-Bärwolf 2000). Moreover, women who have undergone hysterectomy, with or without oophorectomy, may have higher risk of developing CVD compared to women without hysterectomy status (Howard et al. 2005). Hence, estrogen depletion has been suggested to account for the increased disease risk (Carr 2003).

Decline in systemic estrogen level at menopause associates with alterations in metabolic profile and cardiovascular (CV) function increasing the risk for CVD (Rosano et al. 2007). Estrogen deficiency has detrimental effects on arteries by various mechanisms (Mikkola 2019). Estrogen depletion promotes vascular inflammation, oxidative stress and endothelial dysfunction, which is the initial step of atherosclerosis (Rosano et al. 2007; Auro et al. 2014). In addition, menopause associates with proatherogenic changes in blood lipids such as increased total cholesterol, LDL-C and TG levels (Ambikairajah et al. 2019). Instead, most studies have reported that HDL-C concentration decreases or remains unchanged (Carr 2003). Furthermore, menopause increases central adiposity (Carr 2003) which is also an important component of metabolic syndrome (MetS), an accumulation of metabolic disorders, which is a risk factor for CVD (Pu et al. 2017). Increased body fat also affects blood lipid concentrations through which it can promote progression of CVD (Romero-Corral et al. 2010). Hence, menopause is linked to various metabolic changes through which it can act as an independent risk factor for CVD (Gohlke-Bärwolf 2000). However, a distinct relationship between postmenopausal status and CVD has not been established (Atsma et al. 2006).

2.2 Hormone replacement therapy and cardiovascular disease risk

HRT has proven effective in alleviating menopausal symptoms and therefore, it may improve the quality of life (Mikkola 2019). It has been approved for prevention of osteoporosis or fractures in women at risk (de Villiers et al. 2016). The most effective hormone therapy is estrogen therapy either alone or combined with progestogen, i.e. progesterone or synthetic progestin (Davis et al. 2015). Women who have undergone hysterectomy can be treated solely with estrogen whereas the combined regimen is used for women with a uterus for endometrial protection (de Villiers et al. 2016). Treatment can be administered orally, transdermally or

intervaginally (Nelson 2008). Administration route may produce different effects, especially on lipid concentrations, due to hepatic metabolism which affects orally administered HRT (Mikkola 2019). Treatment should be adjusted to the lowest possible yet effective dose and designed based on individual treatment goals and evaluation of benefit/risk profile (de Villiers et al. 2016).

HRT has been suggested to have beneficial effects on several MetS components and CVD risk factors including LDL-C, HDL-C, abdominal fat and insulin resistance (Salpeter et al. 2006a). Timing of initiation of HRT appears to play a significant role in CVD protection (Hodis et al. 2016). HRT seems to protect younger postmenopausal women from coronary heart disease events, a cut-off being 60 years of age or 10 years since menopause (Salpeter et al. 2006b). However, initiation of treatment after 10 or more years postmenopause does not seem to protect from atherosclerosis (Hodis et al. 2016). HRT may have primary preventive effect as estrogen appears to protect healthy vascular system, but it cannot reverse already progressed atherosclerosis (Mikkola 2019).

3 CARDIORESPIRATORY FITNESS

Cardiorespiratory fitness reflects the capacity of the respiratory and circulatory systems to transport oxygen to skeletal muscles in order to perform physical work (Ross et al. 2016). CRF can be described as maximal oxygen uptake (VO_{2max}) which is a direct measure indicating the maximum amount of oxygen consumed during maximal exercise, or as peak oxygen uptake (VO_{2peak}) which indicates the highest value of VO_2 achieved during exercise and is usually measured with maximal incremental exercise test (Bassett & Howley 2000; Ross et al. 2016). CRF is an important indicator of CV health (Ross et al. 2016). Low CRF is a risk factor for CVD (Kodama et al. 2009) and a strong predictor of CV mortality (Imboden et al. 2018).

Exercise training (ET) results in increases in VO_{2peak} (Ross et al. 2016) and it has been shown to have several beneficial effects on CVD risk factors, including lipid profile (Lin et al. 2015). ET-induced increase in CRF can also be seen in postmenopausal women (Blumenthal et al. 1991; Mandrup et al. 2017). However, some studies have suggested that postmenopausal women may have impaired cardiorespiratory capacity compared to pre- or perimenopausal women which may predispose to increased CVD risk (Lynch et al. 2002; Mercuro et al. 2006). Nevertheless, some studies have shown that leisure time physical activity (LTPA) and exercise training have similar benefits on CVD risk factors regardless of menopausal status (Mandrup et al. 2017; Karvinen et al. 2019). Higher levels of LTPA or higher CRF level seem to associate with greater health benefits and more favorable lipid profile, including HDL-C, during menopausal transition (Karvinen et al. 2019) and at postmenopause (Haddock et al. 1998). However, LTPA at similar level as in premenopause does not seem to completely compensate menopause-related changes in serum lipids and therefore higher levels of PA may be needed to gain clinically relevant reduction in serum cardiovascular risk factors (Karvinen et al. 2019).

4 HIGH-DENSITY LIPOPROTEIN

HDL is the smallest and densest of the five significant lipoproteins having a high protein/lipid ratio as approximately half of HDL mass comprises of proteins and half consists of lipids (Kontush et al. 2013). HDLs are a heterogenous group of lipoproteins comprising of several subpopulations into which HDL particles can be classified according to their size, charge, shape, density and apolipoprotein and lipid composition (Kontush et al. 2015). The particle density and size range from 1.063-1.21 g/ml and 7.2-12 nm (Stoke's diameter), respectively (von Eckardstein et al. 1994).

The main constituents of HDL are presented in figure 1. Compared to other lipoproteins, HDL contains a greater number of divergent proteins (> 80) from which apolipoproteins and enzymes are considered functionally most essential (Kontush et al. 2015). The most abundant protein constituent of HDL is apolipoprotein A-I (apoA-I) accounting for approximately 70 % of HDL protein content (Kontush & Chapman 2006). Almost all HDL particles appear to contain antiatherogenic apoA-I which has a great significance in lipid metabolism (Kontush et al. 2015). HDL lipidome (> 200 lipid species) comprise of different lipid classes which have distinct functions (Kontush et al. 2013). Free cholesterol accounts for 5-10 % of HDL lipid mass and is considered significant in CVD prediction in the form of HDL-C (Kajani et al. 2018).

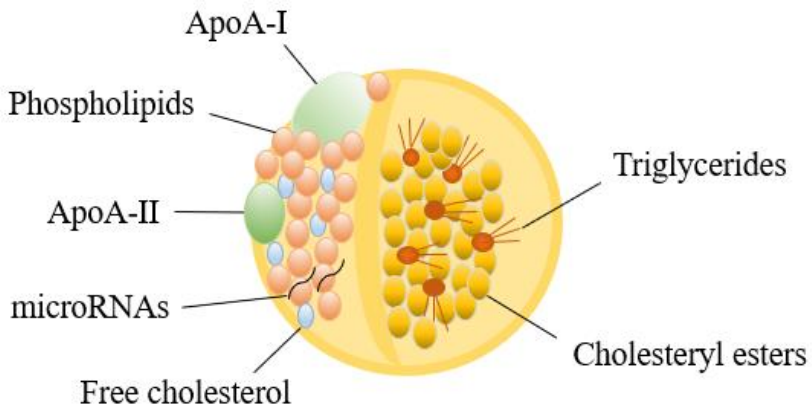


FIGURE 1. Author's view of the main constituents of HDL. ApoA-I = apolipoprotein A-I, ApoA-II = apolipoprotein A-II.

During last decades HDL has been recognized to have atheroprotective qualities (Rader & Hovingh 2014). Epidemiological and clinical studies have uniformly shown an inverse association between plasma HDL-C level and CVD risk (The Emerging Risk Factors Collaboration 2009). This has led to "HDL hypothesis" according to which increased plasma HDL-C concentration results in decreased CVD risk (Rader & Hovingh 2014). However, even though pharmacological studies have succeeded in increasing plasma HDL-C levels, they have consistently failed to reduce the risk of CVD events (Camont et al. 2011). Routinely measured plasma HDL-C is a rather crude measure and does not describe HDL function which seems to reflect the atheroprotective capacity of HDL (Rader & Hovingh 2014). Reverse cholesterol transport (RCT) is perhaps the most widely acknowledged mechanism by which HDL protects from atherosclerosis (Camont et al. 2011). The main characteristic of atherogenesis is abnormal lipid accumulation within macrophages in the arterial wall leading to foam cell formation which RCT may counteract by promoting HDL-mediated cholesterol removal (Favari et al. 2015). In RCT, HDL particles remove cholesterol from peripheral non-hepatic cells, particularly macrophages, and transport it to the liver and release it for excretion into bile (von Eckardstein et al. 2001a).

Beyond its role in RCT, HDL contributes to a vast number of biological activities by which it exerts antiatherogenic properties (Camont et al. 2011). The non-cholesterol cargo of HDL has been suggested to contribute to several of these functions (Vickers & Remaley 2014). In addition to lipids and proteins, HDL contains a variety of small metabolites and short regulatory RNAs, microRNAs (figure 1) (Kajani et al. 2018). miRNAs are secreted from cells and carried in HDL which delivers them to recipient cells enabling cell-to-cell communication and gene regulation (Vickers & Remaley 2012). Hence, HDL particles may rather be carrier molecules with multifaceted functions than solely transporters of cholesterol (Kuai et al. 2016).

4.1 Menopause and HDL

Atherogenic lipid concentrations seem to increase rapidly when women approach menopause (Auro et al. 2014). Menopause is consistently reported to associate with higher total cholesterol and LDL-C concentrations (Ambikairajah et al. 2019). However, results from studies reporting

postmenopausal HDL-C concentrations show more inconsistencies (El Khoudary 2017). Some cross-sectional studies have reported a decrease in HDL-C levels (Jensen et al. 1990; Cho et al. 2008) while others have detected no difference between pre- and postmenopausal groups (de Aloysio et al. 1999; Peters et al. 1999; Kuh et al. 2005; de Kat et al. 2017). However, increasing evidence from cross-sectional (Pansini et al. 1993; Mandrup et al. 2017) and longitudinal studies (Matthews et al. 2009; Abdulnour et al. 2012; Karvinen et al. 2019) suggests that HDL-C may increase when women transition through menopause.

Since some studies have shown that higher HDL-C levels associate with increased CVD risk in postmenopausal women, protective effects of HDL-C after menopause have been questioned (El Khoudary et al. 2018). However, menopause-related changes in HDL metabolism appear to be more intricate than HDL-C level measurements indicate (Carr 2003). It has been suggested that HDL may experience both compositional and functional changes during menopause potentially diminishing the atheroprotective capacity of HDL (Woodard et al. 2011). Therefore, increased levels of HDL-C in postmenopause may in fact not always be cardioprotective (El Khoudary et al. 2018).

4.2 Effects of hormone replacement therapy on HDL

HRT has been shown to affect HDL-C levels (Salpeter et al. 2006a). Orally administered estrogen has been reported to increase HDL-C levels by reducing hepatic lipase activity and stimulating apoA-I production in postmenopausal women (Tikkanen et al. 1982; Walsh et al. 1991). In contrast, progestogens seem to oppose the increase in HDL-C and therefore, combined treatment regimens may not lead to an increase in HDL-C (Godsland 2001). Indeed, some studies have even reported a decrease in HDL-C due to combined HRT (Taskinen et al. 1996; Kotecha et al. 2020).

However, it is essential to recognize that HDL subclasses differ according to their antiatherogenic properties (Camont et al. 2011). Studies have shown that HRT may also affect HDL particle size and composition (Tangney et al. 2001; Auro et al. 2014). Larger particles are considered particularly important in RCT (von Eckardstein et al. 2001a). Even though hormone

treatment may reduce HDL-C levels, it does not necessarily reduce cholesterol efflux capacity, a metric of HDL function, which is essential in terms of CVD protection (von Eckardstein et al. 2001b; Mikkola et al. 2002).

5 MicroRNAs

MicroRNAs are small endogenous, ~22-nucleotide, non-coding RNAs which regulate gene expression post-transcriptionally (Bartel 2004). miRNAs control gene expression, and thus modulate biological homeostasis, by base pairing to target messenger RNA (mRNA) and consequently either repress translation, degrade mRNA, or both (Friedman et al. 2009). Currently the human genome is known to contain over 2500 mature miRNAs (Kozomara et al. 2019).

miRNAs are suggested to participate in regulation of majority of the developmental, cellular and physiological processes (Bartel 2018). miRNAs can simultaneously target numerous different genes leading to intensified gene inhibition (Laffont & Rayner 2017). As a single miRNA can regulate hundreds of mRNAs and any given mRNA contains multiple miRNA binding sites, gene expression regulated by miRNAs is highly intricate and interconnected (Vickers & Remaley 2012). It has been estimated that miRNAs regulate the expression of over 60 % of human protein-coding genes (Friedman et al. 2009). Alterations in miRNA expression are observed, and have suggested to play a role, in various human pathologies including cancer and CVD (Mendell & Olson 2012). Furthermore, miRNAs have an essential role in regulating HDL and cholesterol metabolism (Laffont & Rayner 2017).

miRNAs are suggested to be expressed in all cell types, many of which typically contain a specific subset of miRNAs (Mori et al. 2019). In addition, miRNAs are present in human circulation as well as in various other body fluids making them potential biomarkers of disease (Chen et al. 2008). When miRNAs are in circulation, they are referred to ci-miRNAs (Cortez & Calin 2009).

5.1 Circulating miRNAs

Previously intercellular communication has been considered to restrict to cell-to-cell junctions or secreted signals, for instance neurotransmitters or hormones (Vickers et al. 2011). However, to date it has been shown that miRNAs can be secreted into circulation by various cell types,

and they can be taken up by other cells, and thereby mediate intercellular communication by controlling gene expression in recipient cells (Chen et al. 2012). Ci-miRNAs are linked to lipid- or protein-based carriers and are therefore protected from endogenous ribonuclease (RNase) activity increasing their stability and preventing their degradation (Vickers & Remaley 2012). Ci-miRNAs can be packed inside EVs, which are small membrane-bound vesicles secreted from cells, namely microvesicles, exosomes and apoptotic bodies (Chen et al. 2012). Most recently, lipoproteins, namely HDL and low-density lipoprotein (LDL), have been reported to transport miRNAs (Vickers et al. 2011). In addition, a significant portion of ci-miRNAs have been observed to be attached to vesicle-free RNA-binding protein complexes, such as Argonaute 2 (AGO2) (Arroyo et al. 2011) however, it is unclear if ci-miRNAs bound solely to proteins are actively secreted and taken up by cells (Turchinovich et al. 2013).

miRNAs have been demonstrated to be remarkably stable in serum and plasma, hence serving as potential blood-based biomarkers of health and disease (Mori et al. 2019). Since the discovery of ci-miRNAs, their role as biomarkers and moderators have been investigated in various pathological and physiological conditions (Silva et al. 2017). Systemic miRNA signature has been observed to be altered in several pathologies including CVD, cancer and type 2 diabetes (T2D) (Mori et al. 2019). Alterations in ci-miRNA profile has also been observed in response to exercise (Silva et al. 2017).

Ci-miRNAs can be measured either from plasma or serum (Cortez & Calin 2009). When ci-miRNAs are measured directly from serum or plasma, ci-miRNA signature comprises of various miRNA carrier subclasses (Boon & Vickers 2013). Comprehensiveness of isolation of different miRNA carriers in previous studies is variable (Mori et al. 2019). However, a proper isolation of different compounds is crucial in order to determine the biological properties of these particles and to better understand their contribution as carriers (Yuana et al. 2014). miRNA profile, including the content and top-ranked miRNAs, has been reported to vary among different carrier subclasses in both health and disease (Vickers et al. 2011; Florijn et al. 2019). Therefore, measuring miRNA content of different carriers, instead of merely serum miRNA profile, may improve biomarker sensitivity of miRNAs in different conditions and determine carrier specific miRNA functions in recipient cells (Florijn et al. 2019). Figure 2 illustrates the biophysical properties of lipid-based miRNA carriers.

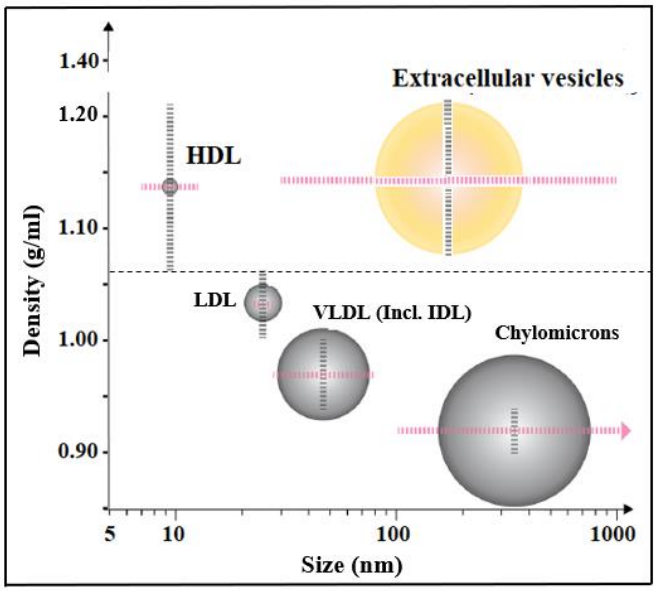


FIGURE 2. Biophysical properties of lipoproteins and extracellular vesicles (modified from Simonsen 2017). The density range of each particle is described with grey, vertical dashed line while the pink, horizontal dashed line illustrates the size range of each species. Black horizontal dashed line represents the density used for isolating HDL and EVs from other compounds. HDL = high-density lipoprotein, LDL = low-density lipoprotein, VLDL = very low-density lipoprotein, IDL = intermediate-density lipoprotein.

5.1.1 Circulating miRNAs and serum estradiol

The association of estradiol on miRNA expression has been demonstrated in several tissues (Pérez-Cremades et al. 2018). Furthermore, specific systemic miRNAs have also been shown to associate with serum E_2 (Kangas et al. 2014; Kangas et al. 2017). Kangas et al. (2014) observed that circulating serum miR-21 and miR-146a levels differed between postmenopausal twins discordant for the use of HRT. Furthermore, Kangas et al. (2017) reported a negative association between miR-148a-3p, -27-3p, -126-5p, -28-3p and -30a-5p levels and E_2 and a positive association of miR-106-5p with E_2 in their study of premenopausal women and postmenopausal HRT using and non-using twins. Ci-miRNA profile can potentially be used to evaluate the effects of menopausal hormonal changes as well as individual effects resulting from the use of HRT (Kangas et al. 2017).

5.1.2 Circulating miRNAs and aerobic exercise

Endurance exercise also alters the ci-miRNA profile (Silva et al. 2017). Certain systemic miRNAs associate with fitness and health parameters and therefore, as exercise training is known to produce substantial health benefits, ci-miRNAs may mediate adaptations to ET (Sapp et al. 2017). Alterations in systemic miRNA profile has been observed in both acute and chronic exercise training (Fernández-Sanjurjo et al. 2018). Furthermore, specific ci-miRNAs have been reported to correlate with CRF level and may therefore be potential biomarkers of aerobic exercise capacity (Baggish et al. 2011; Bye et al. 2013; Sapp et al. 2019). As low CRF is a major contributor to cardiovascular disease, specific miRNAs associating with low CRF could be used as potential early biomarkers of the disease (Bye et al. 2013).

5.2 HDL as miRNA carrier

Vickers et al. (2011) discovered that HDL carries endogenous miRNAs which it transports to recipient cells. Furthermore, HDL-delivered miRNAs have been demonstrated to be functional in recipient cells altering target gene expression (Vickers et al. 2011; Tabet et al. 2014). This export process is suggested to be selective and regulated by specific signaling pathways (Boon & Vickers 2013). The exact mechanism of how HDL carries miRNAs is currently unclear albeit extracellular miRNAs have been proposed to bind to phosphatidylcholine which is the principal phospholipid in HDL (Zhao et al. 2019). Due to several unique properties of HDL, such as small size, high tolerability and intrinsic targeting properties to several if not all tissues and cells, HDL may transport its cargo and deliver cellular messages effectively to recipient cells (Kuai et al. 2016). It has been suggested that HDL-carried miRNAs may potentially partly mediate or alter some of the biological functions of HDL (Vickers et al. 2011).

Vickers et al. (2011) observed that miRNA profile of HDL is rather consistent among healthy individuals, yet it was altered in hypercholesterolemia and atherosclerosis suggesting that HDL cargo may be altered in disease state. Furthermore, Niculescu et al. (2015) observed alterations in miRNA content of HDL in coronary artery disease (CAD). Wagner et al. (2013) validated

several miRNAs to be present in HDL yet they observed only slight alterations in HDL-carried miRNA profile in CAD patients.

In order to determine miRNA content and function of HDL and other carriers, a proper isolation is essential (Florijn et al. 2019). HDL can be isolated from other lipoproteins according to its density using density-gradient ultracentrifugation (DGUC) (Yuana et al. 2014). However, when isolating either HDLs or EVs from plasma or serum, they readily co-isolate if no further purification measure is employed as the density of EVs and HDLs overlaps explicitly (figure 2) (Simonsen 2017). To minimize the co-isolation, a combination of isolation methods can be applied (Mori et al. 2019). As figure 2 shows, HDLs and EVs differ significantly according to their size and consequently, size-exclusion chromatography (SEC) is an appropriate method to avoid co-isolation of these entities (Simonsen 2017).

5.2.1 miR-21

Figure 3 illustrates systemic miRNA response to specific physiological conditions regarding miRNAs chosen for this study. miR-21 is highly expressed in endothelial cells (ECs) and has been suggested to be a modulator of vascular disease (Urbich et al. 2008). miR-21 regulates inflammation (Olivieri et al. 2013) and contributes to neointima lesion formation after vascular injury (Laffont & Rayner 2017). Circulating miR-21 levels have been shown to be upregulated in CVD patients (Olivieri et al. 2013; Pereira-da-Silva et al. 2018). Kangas et al. (2014) observed an association between systemic miR-21 levels and serum E_2 concentration. More specifically, postmenopausal women not using HRT had higher ci-miR-21 levels compared to their HRT using twin sisters and premenopausal women (Kangas et al. 2014). Moreover, systemic miR-21 levels have been shown to be upregulated in response to acute and chronic endurance training (Baggish et al. 2011; Wardle et al. 2015). Ci-miR-21 levels may also be an indicator of cardiorespiratory fitness as they have been shown to be lower in participants with higher VO_{2max} (Bye et al. 2013; Sapp et al. 2019).

5.2.2 miR-126

miR-126 is one of the most abundantly expressed miRNAs in ECs (Feinberg & Moore 2016). miR-126 affects vascular integrity and it is involved in vascular angiogenesis and has a role in preventing atherosclerotic lesion formation (Li et al. 2017). Circulating miR-126 levels have been reported to be downregulated in different atherosclerotic diseases (Pereira-da-Silva et al. 2018). In addition, miR-126 has been shown to bound rather efficiently to HDL (Wagner et al. 2013). Kangas et al. (2017) observed that serum miR-126 associates with serum E_2 level. Postmenopausal women not using HRT had higher ci-miR-126 levels compared to premenopausal and postmenopausal HRT using women (Kangas et al. 2017). In addition, ci-miR-126 levels have been shown to be increased after acute and sustained exercise training (Uhlemann et al. 2014; Barber et al. 2019) and ci-miR-126 level has been shown to negatively correlate with CRF in postmenopausal sedentary women with MetS (Sapp et al. 2019).

5.2.3 miR-146a

miR-146a has an essential role in regulating inflammation and dysregulation of miR-146a may contribute to low-level chronic inflammation associating with various age-related disorders with inflammatory background, such as CVD (Olivieri et al. 2013). However, miR-146a may also confer protective functions in the vessel wall by dampening EC inflammation via a negative feedback loop (Feinberg & Moore 2016) and decreasing lipid uptake in macrophages (Laffont & Rayner 2017). Systemic miR-146a levels have been shown to be increased in cardiovascular disease (Olivieri et al. 2013; Feinberg & Moore 2016). Kangas et al. (2014) demonstrated that serum miR-146a levels differed when comparing postmenopausal HRT using women and their non-using twin sisters indicating a response to HRT. In addition, ci-miR-146a is one of the most common miRNAs demonstrated to respond to exercise training (Sapp et al. 2017). Systemic miR-146a levels have been shown to be upregulated after acute bout of exercise (Baggish et al. 2011), prolonged aerobic exercise (Baggish et al. 2014) and sustained ET (Baggish et al. 2011; Barber et al. 2019). Moreover, Baggish et al. (2011) observed a linear correlation between peak exercise miR-146a level and VO_{2max} indicating a potential role of miR-146a as a marker of CRF.

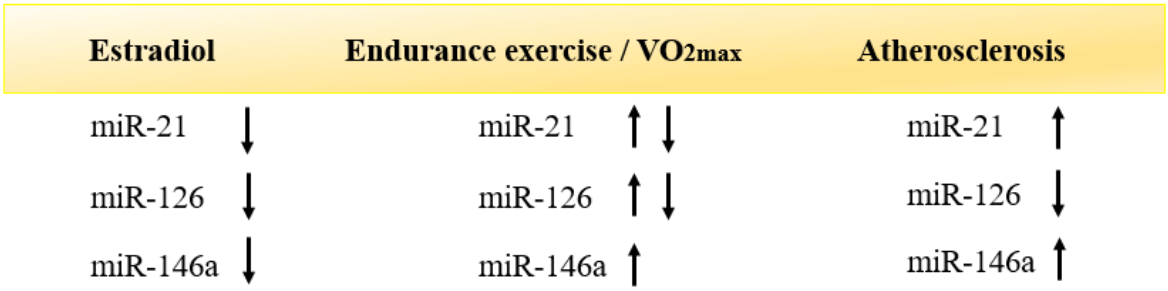


FIGURE 3. Serum ci-miR-21, -126 and -146a response to HRT, endurance exercise and cardiorespiratory fitness and atherosclerosis. Upward arrow describes ci-miRNA upregulation in response to described condition while downward arrow represents downregulation of specific ci-miRNA. Arrows going both ways indicate evidence of both up- and downregulation.

6 AIM OF THE STUDY

The aim of this Master's thesis is to examine whether the use of HRT affects HDL-C concentration and miRNA content of HDL in postmenopausal women. Specific serum ci-miRNAs have previously been identified to associate with serum estradiol (Kangas et al. 2014; Kangas et al. 2017). However, it is not known if this association is carrier specific. Hence, this study investigates whether miRNA content of HDL differs in postmenopausal HRT users compared to non-users. Furthermore, the purpose is to examine the association of cardiorespiratory fitness level and specific CVD risk factors with miRNA levels.

Specific research questions:

1. Does the use of HRT affect serum HDL-C level in postmenopausal women?
2. Does the use of HRT alter miR-21, -126 and -146a content of HDL in postmenopausal women?
3. Does cardiorespiratory fitness level or CVD risk factors associate with miR-21, -126 and -146a content of HDL in postmenopausal women?

7 MATERIALS AND METHODS

This Master's thesis is part of "the Systemic and Intracrine Estrogen and MicroRNAs mediate the risk of metabolic dysfunction in middle-aged women" (EsmiRs) study conducted at the University of Jyväskylä in the Gerontology Research Center. EsmiRs study examines the biological mechanisms leading to unfavorable health alterations induced by menopause. EsmiRs study is a 4-year follow-up study based on "the Estrogenic Regulation of Muscle Apoptosis" (ERMA) study population. The collection of EsmiRs data proceeds in three phases: a survey (1st phase), laboratory visit repeating ERMA measurements (2nd phase) and new metabolic measurements (3rd phase).

7.1 Participants and study design

Participants of this study (n = 10) are a sub-population of EsmiRs study subjects (aim n = 60) who were chosen to participate in metabolic measurements conducted at the third phase of EsmiRs study. To be included into third phase participants needed to have completed the second phase and be either pre- or perimenopausal, postmenopausal (no HRT) or postmenopausal HRT users with verified menopausal status and intact ovaries and uterus. Other inclusion criteria were body mass index (BMI) < 30 or >18,5, no hormone preparations in use (except for HRT using women), no notable metabolic diseases affecting metabolism nor medication in constant use for a metabolic disease, no continuous use of other medication affecting metabolism, no contraindications for maximal aerobic exercise test and no daily smoking. 20 women who first met the inclusion criteria were chosen into each group. Each group contained four clusters which were set according to BMI and PA level. Maximum number of women in each cluster was 5 and as the cluster was fulfilled, no more participants were included into that cluster.

Participants of the present study comprise of postmenopausal HRT using women (n = 5) and postmenopausal non-using women (n = 5) chosen from subjects who had participated in maximal aerobic exercise test by the end of October 2019 and hence had their VO_{2peak} values and pre-test serum samples available. By then 6 HRT users and 18 non-users had undergone maximal aerobic exercise test. One HRT user was excluded due to terminated exercise test.

Therefore, 5 HRT using women were included into the final study design. Each HRT using woman was matched with a non-using pair according to VO_{2peak}. Furthermore, study groups (HRT/no HRT) were matched based on body fat percentage. HRT users were required to be present users and have used hormone preparation for at least 3 months. All HRT using participants were using orally administered combined treatment regimen, containing both estrogen and progestogen and had commenced the treatment during the ongoing year (2019).

EsmiRs study was approved by the Ethics Committee of the Central Finland Health Care District (KSSHP Dnro 9U/2018). A written informed consent was obtained from all participants before starting the study. The study was conducted in conformity with the Declaration of Helsinki following good clinical and scientific practice.

7.2 Blood sampling

To retrieve blood lipid and hormone levels from the participants, blood samples were harvested after overnight fasting between 7:00 and 10:00 a.m. Blood was drawn from the antecubital vein in a supine position during the first laboratory visit. To separate the serum, whole blood was left to clot for 30 minutes at room temperature after which it was centrifuged at 2.200 × g and the sera were aliquoted and stored at – 80 °C as described previously (Kovanen et al. 2018). E_2 and FSH levels were measured from serum using IMMULITE® 2000 XPi (Siemens Healthcare Diagnostics, UK) following manufacturer's instructions. TC, HDL-C, LDL-C and TGs were determined using KONELAB 20 XTi analyzer (Thermo Fischer Scientific, Finland) according to manufacturer's instructions.

At the second laboratory visit, as participants performed maximal aerobic exercise test, blood samples were harvested after overnight fasting at 7:15 a.m. before the test. A total amount of 18 ml of peripheral blood was collected into two 9 ml Vacuette® EDTA K3 tubes at three different time points: pre, post and 1-hour post exercise test. The whole blood was mixed with 18 ml of RPMI-medium and plasma containing HDL and white blood cells were separated through a density gradient centrifugation with Ficoll-Paque™ PLUS medium (GE healthcare).

In this study, pre-exercise test serum samples were used to isolate HDL and extract RNA to determine miR-21, miR-126 and miR-146a content of HDL.

7.3 HDL isolation

HDL was isolated from serum using density-gradient ultracentrifugation with potassium bromide (KBr) density being 1.063 g/ml. Density adjusted phosphate-buffered saline (PBS) PBS-1.063 (0.0834 g KBr/ml) and PBS-1.21 (0.3184 g KBr/ml) were prepared. 1.668 g of KBr was weight into tubes (Beckman Coulter 355618) for the serum samples, for the volume used for centrifugation (20 ml). 1.175 g of KBr was weight into HDL sample tubes. Workflow of HDL isolation is presented in figure 4.

Serum samples were defrosted overnight, and each sample was transferred into a tube with KBr (1.668 g) (figure 4, step 1). PBS (pH 7.4) was added to reach 20 ml if needed. The mixture was centrifuged (60 000 rpm, 4 ℃, Beckman, 70 Ti rotor) for 20 hours. LDL (5 ml) was collected from the top of each tube and stored at – 80 ℃ for later use (figure 4, step 2). To purify HDL at the right density, HDL (5 ml) was collected from the bottom of each tube and transferred into tubes with KBr (1.175 g). Tubes were filled with density adjusted PBS (1.21) until 20 ml. Mixture was centrifuged (60 000 rpm, 4 ℃) for 48 hours. HDL (5 ml) was collected from the top of each tube (figure 4, step 3). To reduce the volume of collected HDL samples before EV purification, samples were concentrated with Amicon ultra Centrifugal filters (Cat no. UFC510024, Merck) (figure 4, step 4). 4 ml of each sample was used for concentration. Samples were centrifuged (14 000 × g, 4 ℃, Heraeus Fresco 17 centrifuge) for 10 minutes to concentrate HDL. Concentrated HDL was further centrifuged (1000 × g, 4 ℃) for 2 minutes to collect HDL (60 µl) into tubes (figure 4, step 5).

HDL was purified from EVs using SEC (IZON qEVoriginal, 35 nm, SP5). Concentrated samples (60 µl) were taken to room temperature to thaw on ice. SEC column was taken to room temperature and positioned into upright position. 440 µl filtered PBS was added into thawed samples in order to reach the 500 µl sample volume required for SEC. Buffer was drained from the column and the column was flushed with 10 ml of filtered PBS. Each sample (500 µl) was

loaded into the column and void volume (3 ml) was collected immediately after the sample began to run. Thereafter, EV and HDL zone volumes were collected into 15 fractions, 0.5 ml in each fraction. After each sample, the column was flushed with 15 ml of filtered PBS before loading the next sample into the column. EV zone volume was collected into fractions 1-3 as informed by the manufacturer. To determine the zone of HDL, total protein was measured from SEC fractions (figure 4, step 6). Total protein content was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) with an automated KoneLab instrument (Thermo Scientific, Vantaa, Finland). Based on total protein measurement, majority of HDL was verified to locate between fractions 7-11. HDL was concentrated from these fractions as described above to condense the volume for RNA extraction (figure 4, step 7).

7.4 Western blot analysis

Western blot (WB) analysis was performed to validate the enrichment of HDL in SEC fractions 7-11 (figure 4, step 6) (appendix 1). For this purpose, antibody against apoA-I, a protein derived from HDL, was used. For the validation purpose, HDL samples from whole blood received from blood bank were used. HDL was isolated as described previously followed by SEC. 15 µl samples from the harvested fractions were solubilized in Laemmli sample buffer. In order to denature proteins, samples were heated at 95°C for 10 min after which they were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 60–90 min at 270 V using 4–20% gradient gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes in a Turbo blotter (Trans-Blot Turbo Blotting System, 170-4155, Bio-Rad Laboratories). Membranes were blocked in commercial blocking buffer (Odyssey Blocking Buffer (PBS), LI-COR) for 2 h and then incubated overnight at 4°C with commercially available primary antibody (1:10 000; ab52945, Abcam). After the primary antibody incubation membranes were washed in tris-buffered saline-tween (TBS-T), incubated with suitable secondary antibody (1:10 000) diluted in 1:1 Pierce blocking buffer and TBS-T for 1 h followed by washing in TBS-T. Protein was visualized by fluorescence using ChemiDoc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories) (appendix 1).

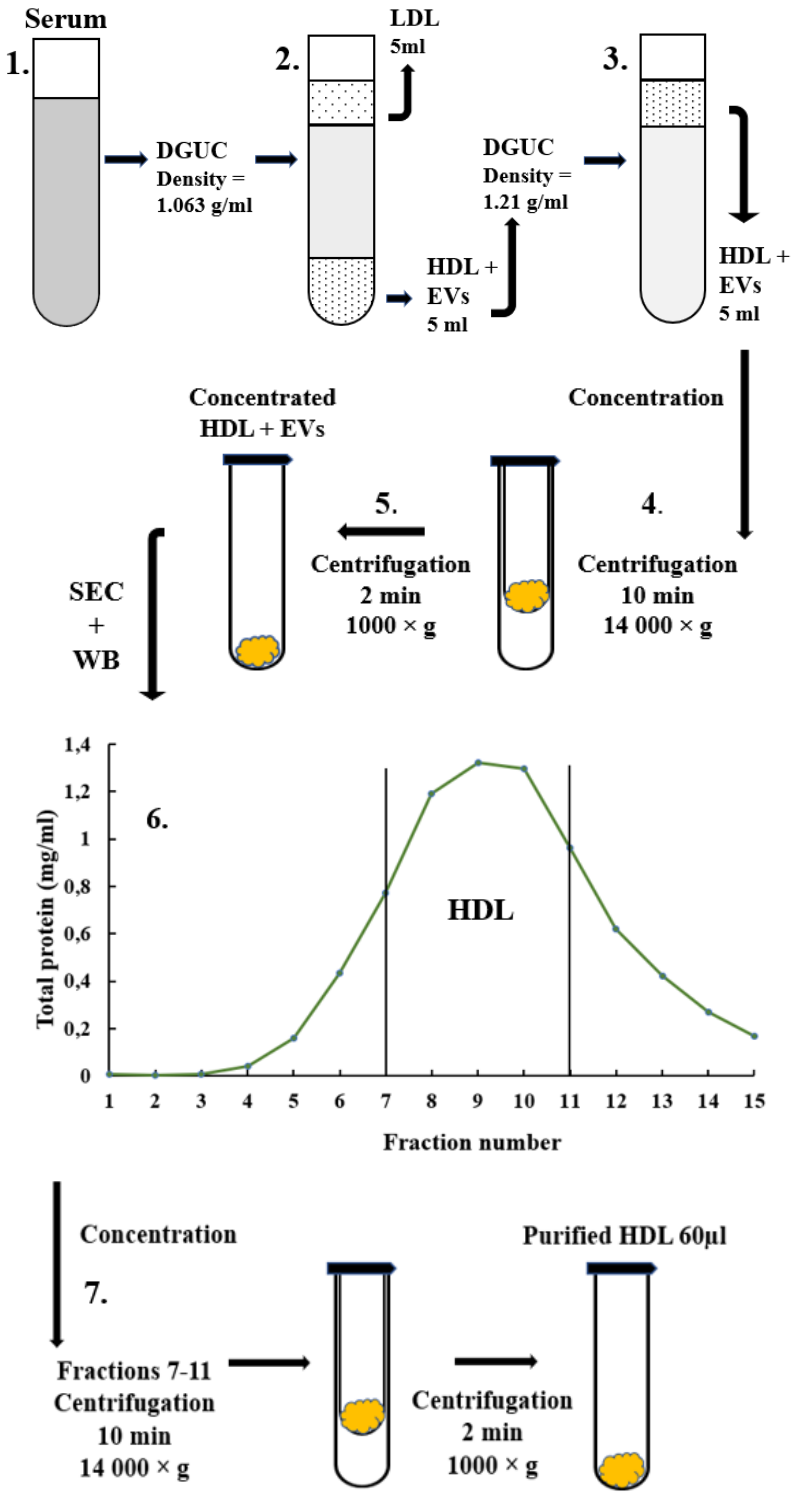


FIGURE 4. Workflow of HDL isolation process. DGUC= density-gradient ultracentrifugation, SEC= size exclusion chromatography, WB = western blot. Numbers (1-7) indicate a specific step in HDL isolation process.

7.5 RNA extraction and complementary DNA synthesis

Total RNA was extracted from 50 µl of purified HDL using miRNeasy serum/plasma kit (QIAGEN, cat. no. 217184) complying with manufacturer's instructions (appendix 2). 150 µl of PBS was added to the samples to achieve similar volume as used in the protocol (200 µl). Synthetic *Caenorhabditis elegans* miR-39 spike-in control (3.5 µl) was added to the samples in order to detect the recovery of RNA. Final centrifugation was performed at full speed (14 000 × g, 4 ℃, Heraeus Fresco 17 centrifuge) for 1 minute.

RNA was reverse transcribed into complementary DNA (cDNA) using miScript II RT Kit (QIAGEN, cat. no 218161). cDNA synthesis is a routine method to create a stable template of the RNA-sample for further analysis. miScript HiFlex buffer was used to prepare cDNA. cDNA synthesis was done according to manufacturer's instructions (appendix 3). Reverse-transcription master mix was done for total volume of 88 µl and the total reaction volume for each sample was 20 µl (table 1). Rest of each RNA samples were stored for later use. As for one sample, only 10 µl of RNA was extracted and therefore 2 µl of RNase-free water (H_2O) was added to the mixture. Incubation was done at 37 ℃ for 60 minutes (Eppendorf AG 22331, Hamburg). To inactivate miScript Reverse Transcriptase Mix, samples were further incubated at 95 ℃ for 5 minutes. Samples were stored overnight at 4 ℃.

TABLE 1. Reverse transcription of RNA samples.

cDNA synthesis	Each sample (µl)	Master mix (µl)
Hiflex-buffer	4	44
10 x nucleic mix	2	22
Reverse transcriptase	2	22
RNA	12	
Total volume	20	88

7.6 quantitative PCR

For quantitative polymerase chain reaction (qPCR), miScript Primer Assay (QIAGEN) and miScript SYRB Green PCR kit (QIAGEN miScript SYBR Green PCR Kit, cat no. 218073) were used to measure miR-21-5p (MS00009079), miR-126-3p (MS00003430) and miR-146a-5p (MS00003535) levels. Synthetic *Caenorhabditis elegans* miR-39 (Qiagen, 219610) level was measured from each sample to serve as a reference gene, describing the efficiency of RNA extraction. The PCR kit contained miScript Universal primer and QuantiTect SYBR Green PCR Master mix. The total reaction volume was 10 µl, containing 5 µl of QuantiTect SYBR Green PCR Master mix, 1 µl of miScript Universal primer, 1µl of primer assay (target primer), 2 µl of H_2O and 1 µl of template (cDNA). Each sample was performed as a duplicate in 384-well plate. Acceptable deviation between duplicates was < 5 % of standard deviation (SD). Samples were centrifuged (1000 × g, Jouan C3i multifunction centrifuge, Thermo, USA) for 1 minute at room temperature prior to qPCR run (Bio-Rad CFX384 Real-Time System, C1000 Touch Thermal Cycler). Reaction protocol for qPCR is presented in table 2. Melting curve analysis was performed after qPCR run to verify the specificity of the qPCR products.

TABLE 2. Reaction protocol for qPCR.

Cycle number	Step	Time	Temperature
1	Initial activation	15 min	95 °C
40	Denaturation	15 s	94 °C
40	Annealing	30 s	55 °C
40	Extension	30 s	70 °C

Threshold level was determined according to default settings provided by the software (Bio-Rad CFX Maestro 1.1). The samples were normalized using miR-39 as a reference. Mean cycle threshold (ΔCt) values were calculated as ΔCt = mean Ct_{miRX} – mean $Ct_{miR39,}$ Ct being cycle threshold and X being the measured miRNA. Relative expression of each miRNA for each sample was calculated using $2^{-\Delta Ct}$ formula.

7.7 Physical fitness and physical activity measurements

VO_{2peak} was measured during maximal graded incremental bicycle ergometer test (Ergoselect 200, Ergoline GmbH, Bitz, Germany). Test was performed after overnight fasting. Participants were instructed to abstain from alcohol intake as well as strenuous exercise for 48-hours prior to testing. The test comprised of both submaximal and maximal phases starting with a resting phase lasting for 3-minutes during which participants were sitting on the bicycle ergometer. Thereafter participants cycled for 4-minutes at intensity of 20 watts (W) after which the workload was increased by 20 W every 4-minutes until respiratory exchange ratio (RER) of 1.0 was attained. After reaching the desired RER, intensity was increased every minute by 20 W. This was proceeded until volitional exhaustion after which there was a 5-minute recovery phase at intensity of 50 W. Two highest successive 30-second VO_2-measurements were used to determine VO_{2peak}. VO_{2peak} was described as VO_{2peak} relative to body weight (ml/kg/min).

LTPA was assessed from self-reports as well as measured using accelerometers. Self-reported LTPA activity pattern was assessed using a questionnaire. The questionnaire is described elsewhere (Kujala et al. 1998). LTPA was assessed based on three questions (appendix 4) describing monthly frequency, mean duration and mean intensity of LTPA to distinguish participants' opinion of their overall LTPA level. Metabolic equivalent (MET) values were assigned for each activity from which the total volume of LTPA was calculated as a product of duration, intensity and frequency. The total LTPA was described as MET hours per day.

LTPA was measured using GT3X+ and wGT3X+ ActiGraph accelerometers (Pensacola, Florida, USA). Accelerometers were instructed to be placed on the right hip and used for 7 consecutive days during waking hours, excluding bathing or while doing other water-based activities. In addition, participants were asked to record their wake-up time and working hours into a diary as well as record if they had removed the monitor for longer than 30 minutes. Raw data from the accelerometers were collected at 60 Hz and filtered after which it was converted into 60-s epoch counts. Further data analysis was done by using a customized Excel-based program as described previously (Kovanen et al. 2018). Data normalization was done for 10-

hour wake time. Entries about working hours in activity diaries were used to distinguish LTPA from total daily PA. Activity was described as total counts for 10-hour LTPA.

7.8 Anthropometrics and body composition

BMI and BF percentage were measured after overnight fasting between 7:00 and 10:00 a.m. with participants wearing only undergarments. BMI was calculated from measured weight and height (kg/m^2). For weight and height measurements, a beam scale and a stadiometer were used, respectively (Kovanen et al. 2018). To assess BF percentage, a multifrequency bioelectrical impedance analyzer (InBody^{TM} 720; Biospace, Seoul, Korea) was used. BMI and BF percentage are reported based on measurements done at the second laboratory visit.

7.9 Statistical analyses

All statistical analyses were performed using IBM SPSS for Windows 24 statistical software. Data are presented using means and SDs. To determine the normality of variables, the Shapiro-Wilk test was applied and skewness and kurtosis of the distributions of variables were interpreted. In all analyses p-value < 0.05 was considered to indicate statistical significance.

To compare miRNA levels and HDL-C concentration between the studied groups, Independent Samples T-test was used. To determine differences between HRT users and non-users regarding other variables, Independent Samples T-test was used for normally distributed variables and Mann-Whitney U test was used for non-normally distributed variables.

To investigate the statistical relationship between miRNAs and cardiorespiratory fitness level, Spearman's rank correlations coefficient was used. To examine correlations between miRNAs and other measured variables and correlations between fitness and activity measures, Pearson correlation coefficient and Spearman correlations coefficient was used for normally distributed variables and non-normally distributed variables, respectively. Correlation coefficient has a value between −1 and 1 indicating the strength and direction of correlation where value 0 denotes that there is no linear relationship between variables (Uhari & Nieminen 2012, 202).

8 RESULTS

Baseline characteristics and differences between HRT using and non-using women are shown in table 3. Values are presented as mean ± SD and as variable value range. There was no difference between the studied groups in age, BMI, BF percentage and VO_{2peak}.

TABLE 3. Characteristics of the participants and differences between the studied groups.

Variable	Post + HRT (n = 5)	Post (n = 5)	p-value
Age (y)[a]	55.3 ± 1.6	55.4 ± 1.7	0.948
Age range (y)	53-57	53-57	
BMI (kg/m^2)[a]	24.3 ± 2.8	24.6 ± 2.0	0.842
BMI range (kg/m^2)	22.1-28.3	22.9-27.8	
BF (%)[a]	32.8 ± 2.1	33.7 ± 5.9	0.761
BF range (%)	30.1-35.9	24.6-41.0	
VO_{2peak} (ml/kg/min)[b]	29.1 ± 3.2	29.1 ± 3.9	0.841
VO_{2peak} range (ml/kg/min)	25.8-34.3	26.9-36	

Values are presented as mean ± SD. Value range is described below. P-values were obtained using [a]Independent Samples T-test for normally distributed variables and [b]Mann-Whitney U test for non-normally distributed variables. Post + HRT = postmenopausal women using hormone replacement therapy, Post = postmenopausal women not using hormone replacement therapy, y = years, BMI = body mass index, kg/m^2 = kilograms per metres squared, BF = body fat, VO_{2peak} = peak oxygen uptake, ml/kg/min = milliliters per kilogram per minute.

8.1 Blood lipid concentrations, hormone levels, physical activity and miRNA levels

Table 4 presents the comparison of blood lipid concentrations, hormone levels and physical activity measures in HRT using and non-using women. There was no difference in HDL-C concentration between the studied groups ($t(8) = -1.036$, $p = 0.331$, 95% CI [−0.87621 – 0.33301]). The groups did not differ from each other, neither according to other measured blood lipids, E_2, FSH, nor to self-reported or accelerometer measured LTPA.

TABLE 4. Blood lipids, hormone levels and physical activity of HRT using and non-using participants.

Variable	Post + HRT (n = 5)	Post (n = 5)	p-value
HDL-C (mmol/l)[a]	2.03 ± 0.46	2.30 ± 0.37	0.331
LDL-C (mmol/l)[a]	2.79 ± 0.58	2.98 ± 0.84	0.694
TC (mmol/l)[a]	5.14 ± 0.55	5.68 ± 0.62	0.189
TG (mmol/l)[a]	0.97 ± 0.24	1.03 ± 0.40	0.780
E_2 (nmol/l)[b]	0.39 ± 0.25	0.18 ± 0.12	0.095
FSH (IU/l)[a]	49.1 ± 25.0	97.9 ± 54.5	0.106
Self-reported LTPA (MET h/day)[b]	3.47 ± 2.81	3.20 ± 3.79	0.794
Measured LTPA (total counts/day)[a]	$4.1 \times 10^5 \pm 1.6 \times 10^5$	$3.6 \times 10^5 \pm 2.1 \times 10^4$	0.486

Values are presented as mean ± SD. [a]For normally distributed variables, Independent Samples T-test was used to obtain p-values. [b]For non-normally distributed variables, non-parametric Mann-Whitney U test was used to compare differences between the groups. Post + HRT = postmenopausal women using hormone replacement therapy, Post = postmenopausal women not using hormone replacement therapy, HDL-C = high-density lipoprotein cholesterol, LDL-C = low-density lipoprotein cholesterol, TC = total cholesterol, TG = triglycerides, E_2 = estradiol, FSH = follicle-stimulating hormone, LTPA = leisure time physical activity, MET = metabolic equivalent, mmol/l = millimoles per liter, nmol/l = nanomoles per liter, IU/l = international units per liter, h = hours.

Figure 5 shows the comparison of relative expression levels of the studied miRNAs in HRT users and non-users. There was no difference between the groups in miR-21 ($t(8) = 0.507$, $p = 0.626$, 95% CI [−0.04766 – 0.07451]), miR-126 ($t(8) = 1.119$, $p = 0.295$, 95% CI [−0.03638 − 0.10500]) and miR-146a ($t(8) = 0.844$, $p = 0.423$, 95% CI [−0.00693 − 0.01493]) levels.

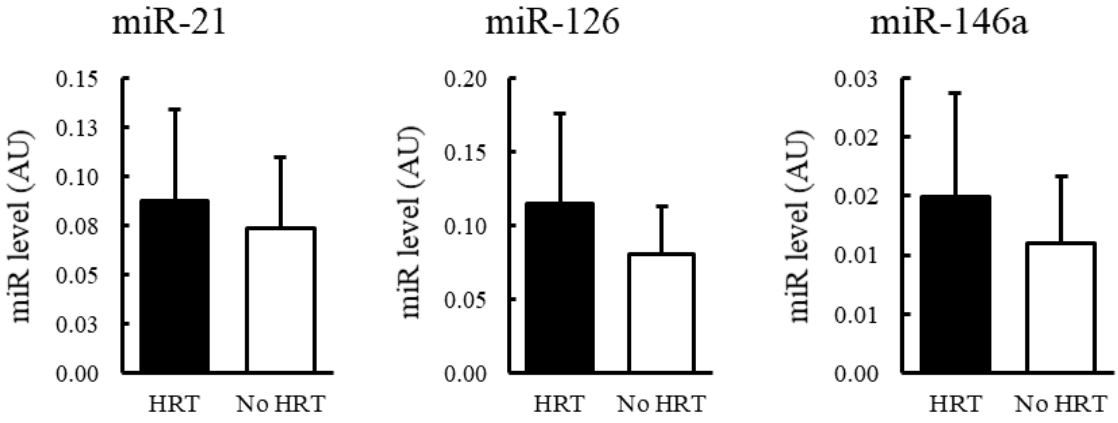


FIGURE 5. The relative expression level of miR-21, -126 and -146a in HRT using and non-using women in arbitrary units (AU). Values are presented as mean. SD bars describe SD. HRT = postmenopausal women using hormone replacement therapy (n = 5), No HRT = postmenopausal women not using hormone replacement therapy (n = 5).

8.2 Associations of miRNAs with serum estradiol and CVD risk markers

The miRNAs selected for this study are linked to E_2 and CVD, hence the associations of these miRNAs on E_2 and CVD associated blood lipids as well as body composition were studied. Correlations of miR-21, -126 and -146a levels with participants' serum E_2 concentration, blood characteristics and body composition are presented in table 5. When examining all participants together (n = 10), there was a positive correlation between miR-126 and E_2 ($r = 0.685$, $p = 0.029$). However, when examining the groups separately, a positive correlation was only observed in non-using women ($r = 0.900$, $p = 0.037$) but not in HRT using women. Additionally, there was a negative correlation between participants' miR-146a levels and total cholesterol ($r = −0.651$, $p = 0.042$), yet when observing the groups separately the correlation was only significant in HRT using women ($r = −0.903$, $p = 0.036$). Furthermore, miR-126 correlated

positively with TGs (r = 0.926, p = 0.024) in non-using women and negatively with BF percentage (r = −0.957, p = 0.011) in HRT using women. Other variables did not correlate significantly with any of the studied miRNAs.

TABLE 5. Correlations of miR-21, -126 and -146a levels with serum estradiol, blood lipids and body composition.

	Post + HRT (n = 5)		Post (n = 5)		All (n = 10)	
	r	p	r	p	r	p
miR-21correlations						
E_2 (nmol/l)*	−0.600	0.285	0.600	0.295	0.248	0.489
HDL-C (mmol/l)	0.022	0.972	−0.149	0.811	−0.101	0.781
LDL-C (mmol/l)	−0.581	0.304	−0.004	0.995	−0.283	0.428
TC (mmol/l)	−0.605	0.280	0.107	0.863	−0.317	0.373
TG (mmol/l)	0.253	0.681	0.812	0.095	0.498	0.143
BF (%)	−0.293	0.633	0.162	0.795	−0.007	0.985
miR-126 correlations						
E_2 (nmol/l)*	0.600	0.286	**0.900**	**0.037**	**0.685**	**0.029**
HDL-C (mmol/l)	0.789	0.113	−0.517	0.372	0.219	0.543
LDL-C (mmol/l)	−0.727	0.164	0.560	0.327	−0.196	0.588
TC (mmol/l)	−0.345	0.570	0.572	0.314	−0.171	0.636
TG (mmol/l)	−0.622	0.263	**0.926**	**0.024**	0.039	0.916
BF (%)	**−0.957**	**0.011**	0.496	0.395	−0.111	0.760
miR-146a correlations						
E_2 (nmol/l)*	0.700	0.188	0.300	0.624	0.467	0.174
HDL-C (mmol/l)	0.094	0.880	0.058	0.926	−0.029	0.937
LDL-C (mmol/l)	−0.727	0.164	−0.426	0.475	−0.514	0.128
TC (mmol/l)	**−0.903**	**0.036**	−0.337	0.579	**−0.651**	**0.042**
TG (mmol/l)	−0.737	0.156	0.461	0.434	−0.177	0.626
BF (%)	−0.564	0.322	0.054	0.931	−0.160	0.659

Statistically significant values are bolded. Pearson correlation and *Spearman correlation was used to obtain results for normally distributed variables and non-normally distributed variables, respectively. Post + HRT = postmenopausal women using hormone replacement therapy, Post = postmenopausal women not using hormone replacement therapy, All = both studied groups together, r = correlation coefficient, HDL-C = high-density lipoprotein cholesterol, LDL-C = low-density lipoprotein cholesterol, TC = total cholesterol, TG = triglycerides, E_2 = estradiol, BF = body fat, mmol/l = millimoles per liter, nmol/l = nanomoles per liter.

8.3 Associations of miRNAs with cardiorespiratory fitness and physical activity

As especially miR-146a has previously been associated with PA parameters, the correlations of the studied miRNAs with CRF and LTPA was examined (table 6). When observing all participants together (n = 10), there was no association between VO_{2peak} and miR-21 (r = −0.139, p = 0.701), miR-126 (r = −0.455, p = 0.187) and miR-146a (r = −0.527, p = 0.117) levels. However, when observing the groups separately, miR-146a correlated negatively with VO_{2peak} in HRT using women (r = −0.900, p = 0.037) but not in non-using women. Interestingly, miR-146a correlated positively with accelerometer measured LTPA in non-using women (r = 0.900, p = 0.037) but not in HRT using women.

TABLE 6. Correlations of miR-21, -126 and -146a levels with fitness and activity parameters.

	Post + HRT (n = 5)		Post (n = 5)		All (n = 10)	
	r	p	r	p	r	p
miR-21correlations						
VO_{2peak}	0.300	0.624	−0.500	0.391	−0.139	0.701
Self-reported LTPA	−0.359	0.553	−0.200	0.747	−0.292	0.413
Measured LTPA	0.200	0.747	0.800	0.104	0.309	0.385
miR-126 correlations						
VO_{2peak}	−0.800	0.104	−0.100	0.873	−0.455	0.187
Self-reported LTPA	−0.667	0.219	−0.700	0.188	−0.529	0.116
Measured LTPA	−0.200	0.747	0.200	0.747	0.067	0.855
miR-146a correlations						
VO_{2peak}	**−0.900**	**0.037**	−0.200	0.747	−0.527	0.117
Self-reported LTPA	−0.821	0.089	0.100	0.873	−0.298	0.403
Measured LTPA	−0.500	0.391	**0.900**	**0.037**	0.103	0.777

Statistically significant results are bolded. Spearman correlation was used to measure associations between variables. r = correlation coefficient, Post + HRT = postmenopausal women using hormone replacement therapy, Post = postmenopausal women not using hormone replacement therapy, All = both studied groups together, Measured LTPA = accelerometer measured leisure time physical activity described as total counts per day, Self-reported LTPA = self-reported leisure time physical activity described as MET hours per day, VO_{2peak} = peak oxygen uptake described as VO_{2peak} relative to body weight (ml/kg/min).

Correlations between physical fitness and self-reported LTPA and accelerometer measured LTPA are shown in table 7. There was no association between any of the physical fitness and activity parameters.

TABLE 7. Correlations between participants' (n = 10) cardiorespiratory fitness and physical activity measurements.

	VO_{2peak}		Self-reported LTPA	
	r	p	r	p
Self-reported LTPA	0.541	0.106		
Measured LTPA	0.139	0.701	0.365	0.300

Spearman correlation was used to examine correlations between variables. r = correlation coefficient, Measured LTPA = accelerometer measured leisure time physical activity described as total counts per day, Self-reported LTPA = self-reported leisure time physical activity described as MET hours per day, VO_{2peak} = peak oxygen uptake described as VO_{2peak} relative to body weight (ml/kg/min).

9 DISCUSSION

The purpose of this Master's thesis was to examine if hormone replacement therapy has an effect on HDL-C level and miR-21, -126 and -146a content of HDL in postmenopausal women. This study also examined if miRNA content of HDL associates with cardiorespiratory fitness level and CVD risk markers in postmenopausal women. According to the present results, neither HDL-C concentration nor miR-21, -126 and -146a content of HDL differed between postmenopausal HRT using and non-using women. In contrast to previous findings with negative correlation between serum miR-126 and E_2 level, in this study miR-126 in HDL correlated positively with serum E_2. In addition, this study showed that miR-21, -126 and -146a content of HDL did not associate with VO_{2peak} in postmenopausal women when observing all women together. Nevertheless, there was a negative correlation between miR-146a and VO_{2peak} in postmenopausal HRT using women and a positive correlation between miR-146a and accelerometer measured LTPA in non-using women. Furthermore, results show a negative correlation of miR-146a with TC, a positive correlation of miR-126 with TGs in non-using women and a negative correlation of miR-126 with BF percentage in HRT using women.

9.1 HDL cholesterol in HRT using and non-using women

Examining the effect of HRT provides a useful way to study the effects of menopausal hormonal changes without age interference. However, the effect of HRT on HDL-C is somewhat inconsistent according to the existing literature. The effects seem to depend on the dosage, type of preparation and administration route (Godsland 2001). In the present study all HRT users were using orally administered continuous combined treatment regimen due to intact uterus. No difference was found in HDL-C concentration between HRT using and non-using women (table 4). A systematic review examining the effects of different HRT regimens reported that estrogen alone increased HDL-C whereas these effects were opposed when combined regimen of estrogen and progestogen was used and the magnitude of opposing effect depended on the type and dose of progestogen (Godsland 2001). As in this study, also some other cross-sectional studies have shown no effect on continuous combined HRT on HDL-C (Creatsas et

al. 2003; Kuh et al. 2005), whereas others have shown an increase (Nabulsi et al. 1993; Vadlamudi et al. 1998).

However, as above-mentioned cross-sectional studies, also this study inflicts several methodological challenges as it did not analyze the change of HDL-C according to HRT usage and the type of HRT preparation or dosage was not controlled. Therefore, although no difference in HDL-C was observed between the studied groups, further interpretation of these results is limited especially due to the small sample size. Moreover, the studied groups did not differ from each other according to E_2 concentration which may have a role in the result. To overcome the limitations of cross-sectional studies, several randomized clinical trials have been carried out, many of which have observed a reduction in HDL-C in continuous combined HRT users (Taskinen et al. 1996; Loh et al. 2002; Gregersen et al. 2019; Kotecha et al. 2020). However, also no difference (Tangney et al. 2001; Teede et al. 2001) and an increase (Writing Group for the Women's Health Initiative Investigators 2002; Manson et al. 2003) in HDL-C in continuous treatment group has been observed compared to a placebo group. Unfortunately, some interventions have been rather short (3 months) or parallel designs without a placebo group. Interestingly, some parallel group studies have shown that the non-reduction or slight beneficial net effect in HDL-C seems to be more often related to sequential treatment mode (Munk-Jensen et al. 1994; Ylikorkala et al. 2000) or to a lower dose (Loh et al. 2002).

The possible association of HDL-C with miRNA levels should also be considered when examining the effects of HRT. It is unclear whether potential E_2 associated changes in miRNA content of HDL could lead to alterations in HDL-C concentration or changes in individual HDL particles. However, no association was observed between HDL-C and HDL-carried miR-21, -126 or -146a levels (table 5). Yet, as the present study showed no difference between the studied groups in either miRNA levels, E_2 concentration or HDL-C level, and this study did not measure individual HDL particles, these results should be interpreted with caution.

9.2 HDL-carried miRNAs in HRT using and non-using women

As figure 5 shows, groups did not differ in miR-21, -126 or -146a content of HDL. These results are inconsistent with previous studies which reported a difference in systemic miR-21, miR-146a (Kangas et al. 2014) and miR-126 (Kangas et al. 2017) levels between HRT using and non-using women. The difference between the results may in part be due to differences in miRNA carrier isolation methods. In previous studies miRNA profile was measured from serum without further purification or isolation of different miRNA carriers. Ci-miRNA profile has been demonstrated to differ between different carriers (Vickers et al. 2011) and as ci-miRNA studies differ in isolation methods and miRNA assessment, it is not uncommon that results are inconsistent between studies (Mori et al. 2019).

As miRNA profile of HDL did not differ between the studied groups, EVs need to be considered as potential carriers of functionally relevant miRNAs which might explain the previously observed results. Hence, miR-21, -126 and 146a profile of EVs was analyzed from HRT using and non-using women. Interestingly, there were no difference between the studied groups either in miR-21, -126 or -146a content of EVs (appendix 5).

However, miRNAs associated with EVs or HDLs are only a fraction of all ci-miRNAs (Mori et al. 2019). Arroyo et al. (2011) reported that majority of ci-miRNAs are attached to protein complexes. Therefore, it is possible that the association with serum E_2 may be in AGO2 and nucleophosmin 1 (NPM1) -attached miRNAs. However, AGO2-bound miRNAs have rather been considered non-specific remnants resulting from cell death (Turchinovich et al. 2013). Currently, it is still under debate whether packaging and release of AGO2- or NPM1-bound miRNAs is an actively regulated process leading to alterations in target cell function as it seems to be with HDLs and EVs (Mori et al. 2019). There are only few indications that miRNAs attached to AGO2 have been internalized into cells and remained functional (Ferreira et al. 2014; Prud'homme et al. 2016). Therefore, if the association with E_2 were to be in protein-attached miRNAs, it is unclear if it has any functional relevance.

One reason for the similar findings from HDL and EV miRNAs may be that the studied groups did not differ from each other in E_2 concentration. In previous studies (Kangas et al. 2014; Kangas et al. 2017) HRT using and non-using women differed in E_2 concentration and the mean duration of HRT use was significantly longer (6.9 y.) compared to the usage time in the present study (average one y.). However, previous studies have shown that even rather short usage time (3 months) may affect HDL-C levels (Gregersen et al. 2019) and HDL particle distribution (Tangney et al. 2001).

Differences in ci-miRNA measurements should also be considered. Kangas et al. (2014) examined ci-miRNAs (miR-21 and miR-146a) using qPCR whereas Kangas et al. (2017) used next generation sequencing (NGS) to identify miRNAs associated with E_2. Interestingly, the association with miR-21 and miR-146a and E_2 was not replicated when using NGS even though the same cohort was used in both studies. Therefore, albeit ci-miRNAs chosen for this study have been demonstrated to be altered due to HRT usage, there have been inconsistent results regarding these miRNAs even when examining the same study population.

After finalizing the data analysis of the present study, also NGS was performed from HDL test sample of a premenopausal woman to examine the prevalence of chosen miRNAs in HDL (appendix 6). Read counts showed that miR-21, -126 and -146a were all included in the 15 most common miRNAs in HDL. Interestingly, no other previously E_2 associated ci-miRNAs were included in the top-ranked miRNAs. However, there may be differences in top-ranked miRNAs between pre- and postmenopausal women whereas NGS was performed from HDL sample of a premenopausal woman. Nevertheless, the NGS result suggests that miRNAs chosen for this study potentially have a physiological relevance when carried in HDL. Therefore, as HRT usage did not induce a change in these miRNAs, the effect of aging on HDL-carried mir-21, -126 and -146a should be considered. Indeed, differences in these miRNAs have also been observed between premenopausal women and postmenopausal HRT users when measured from serum (Kangas et al. 2014; Kangas et al. 2017) and miR-21 and -146a are known to be associated with age-associated inflammatory events (Olivieri et al. 2013).

Despite the similar miRNA levels between the studied groups, a positive correlation of miR-126 with E_2 was observed (table 5). This contradicts the negative correlation between serum ci-miR-126 and E_2 reported by Kangas et al. (2017). However, Li et al. (2017) observed a positive association between serum ci-miR-126 and E_2 when studying regularly menstruating women during different phases of menstrual cycle. In addition, Li et al. (2017) observed that premenopausal women had higher ci-miR-126 levels compared to postmenopausal women which is contradictory to results reported by Kangas et al. (2017). The mechanism by which E_2 seems to affect miR-126 level supports the positive association observed in the present study. E_2 appears to enhance miR-126 expression in human umbilical vein endothelial cells promoting endothelial function, which may in part explain the antiatherogenic effects of estrogen (Li et al. 2017). Indeed, multiple studies have reported vital functions of miR-126 on endothelium and vasculature and serum ci-miR-126 levels have been reported to decrease in atherosclerosis (Pérez-Cremades et al. 2018). However, results reported by Li et al. (2017) are not directly comparable to the present results as their study group comprised of younger regularly menstruating women. Nevertheless, it seems that E_2 may affect serum miR-126 level and potentially protect vasculature from atherosclerosis via miR-126 regulation.

9.3 HDL-carried miRNAs, aerobic fitness and physical activity

When examining all study subject together (n=10), there was no association between miR-21, -126 and -146a level and VO_{2peak} (table 6). Interestingly, when analyzing the groups separately, miR-146a correlated negatively with VO_{2peak} in HRT using women (table 6). This negative association is contrary to previously reported result. Baggish et al. (2011) reported that post-exercise ci-miR-146a levels correlated positively with absolute VO_{2max}. However, study design of the present study differs from above-mentioned as miRNA levels were measured from pre-exercise test serum samples without investigating effects of exercise intervention on VO_{2peak} or miRNA level. In addition, there are notable differences in study population as Baggish et al. (2011) examined 10 competitive male athletes whose training volume, VO_{2peak} and health status was rather different compared to our study subjects. Therefore, it should be considered that other health related factors may contribute to the negative association observed in the present study. Van Craenenbroeck et al. (2016) observed similar negative correlation between miR-146a and VO_{2peak} in subjects with chronic kidney disease albeit the association was not

independent of arterial stiffness. Even though women in the present study were considered healthy without any known indication of atherosclerosis, above results suggest that increased miR-146a levels with impairments in vasculature may associate negatively with VO_{2peak}.

In addition, miR-146a was found to correlate positively with accelerometer measured LTPA in non-using women (table 6). Interestingly, the association was opposite to association between miR-146a and VO_{2peak} in HRT using women. However, this may in part be explained by the non-existent correlation between VO_{2peak} and accelerometer measured PA (table 7). When observing individual values, measured PA seems higher than measured VO_{2peak} in some participants. Relative VO_{2peak} values are also affected by weight of an individual. In addition, improvements in CRF in response to exercise are partially genetically controlled (Williams et al. 2017). Moreover, rather short PA measuring time (7 days) may have encouraged to increase activity during this period, leading to overestimation of PA level.

The positive association between miR-146a and PA observed in the present study is in line with several previously reported results. Various studies have shown an increase in ci-miR-146a level in response to acute, prolonged and chronic exercise training (Baggish et al. 2011; Baggish et al. 2014; Wardle et al. 2015). However, study populations in these studies have comprised of either athletes or male participants. Nevertheless, Barber et al. (2019) observed an increase in ci-miR-146a level after 20-week training period in sedentary middle-aged men and women. Also, downregulation of miR-146a has been observed in obese subjects after exercise intervention albeit this opposite result may be due to other beneficial metabolic alterations (Russo et al. 2018).

9.4 HDL-carried miRNAs and CVD risk markers

This study also observed an association between miRNAs and parameters linked to CVD risk (table 5). There was a negative correlation between miR-126 and BF percentage in HRT using women. Previously systemic miR-126 levels have been shown to be downregulated in T2D (Zampetaki et al. 2010). However, Lopez et al. (2017) observed a positive association between serum miR-126 and BF percentage which indicates that higher miR-126 level associates with

negative health parameters and is in part contrast to downregulation of miR-126 in T2D. Interestingly, miR-126 association between both BF percentage and E_2 observed in the present study are consistent with each other indicating that higher miR-126 levels associate with more favorable health status. This can be considered consistent with downregulation of ci-miR-126 in metabolic and inflammatory diseases such as T2D and CVD.

A positive correlation between miR-126 and TGs was found in non-using women (table 5). Krause et al. (2015) also observed a positive association between miR-126 and TGs in children with or without MetS traits albeit Al-Kafaji et al. (2016) reported a negative association between ci-miR-126 and TGs in diabetic patients. However, as T2D and metabolic disorders seem to alter miR-126 level, association of miR-126 with lipids observed in these subjects cannot be generalized to healthy postmenopausal women. However, menopause does associate with various metabolic alterations and interestingly the association between miR-126 and TGs in the present study was only observed in postmenopausal non-users. Indeed, when examining the present correlation with miR-126 and blood lipids and BF percentage, HRT using women showed negative correlations between miR-126 and negative outcome variables and positive correlations with positive health outcome variable (HDL-C) whereas in non-using women these correlations were in opposite direction. However, no other variables besides BF percentage and TGs correlated significantly with miRNAs. In addition, when observing statistical relationship between TGs and E_2, a positive correlation was found (data not shown) and therefore the association between miR-126 and TGs may not be independent and could potentially be explained by the association with E_2.

Furthermore, a negative association between miR-146a and TC was observed (table 5). Russo et al. (2018) observed a similar correlation in obese subjects even after adjusting for age and BMI. miR-146a levels decreased due to an exercise intervention as did TC, hence this could indicate that miR-146a may associate with exercise induced effects on lipid metabolism (Russo et al. 2018). Indeed, miR-146a has been shown to decrease lipid uptake in macrophages (Laffont & Rayner 2017). Our findings support previous observations (Zhou et al. 2018) of the potential of miR-126 and miR-146a as potential biomarkers of CVD risk.

9.5 Methodological considerations

In the present study HDL was purified from other systemic miRNA carriers using both density- and size-based separation methods. DGUC using KBr is a common and well accepted method for lipoprotein isolation (Yuana et al. 2014). A single-spin ultracentrifugation method has been described feasible for isolation of major lipoprotein classes (Redgrave et al. 1975). However, if all lipoprotein classes are collected at once, impurities in collected fractions may be more inevitable. This could be an issue as LDL particles have been shown to carry miRNAs as well, yet to smaller extent (Vickers et al. 2011). Therefore, to ascertain that HDL was thoroughly purified, a two-step ultracentrifugation was performed, first to separate all lipoproteins less dense than HDL and thereafter to isolate HDL. SEC, which is based on separating particles according to their size, was performed as a further purification method for separating HDL and EVs. As HDL and EVs may share some similar surface proteins, antibody-based isolation methods do not necessary yield a pure preparation of certain carrier. Therefore, isolation based on size is an appropriate method for isolating these compounds. To verify HDL enrichment in study samples WB analysis was performed.

There are several methods for miRNA profiling including qPCR, microarrays and NGS all of which have their strengths and limitations (Mori et al. 2019). qPCR has high sensitivity, it is less expensive, and it provides relatively easy data analysis (Moldovan et al. 2014). However, miRNA quantification mandates proper standards or exogenous controls which currently lack consensus (Fernández-Sanjurjo et al. 2018; Mori et al. 2019). The present study examined three miRNAs selected based on previously observed ci-miRNA association with E_2. Therefore, qPCR was chosen as an appropriate method. However, NGS allows novel and combinatorial biomarker detection (Mori et al. 2019) and as no study has previously investigated effects of HRT on HDL-carried miRNAs, NGS could have provided additional information on potential novel miRNAs associating with E_2 as the method is not limited to studies of known miRNAs. However, NGS results are normally verified with qPCR regardless and NGS results from HDL test sample demonstrated that the miRNAs chosen were relevant and highly abundant in HDL (appendix 6).

When using qPCR, some limitations regarding miRNA detection and normalization strategy should be addressed. Normalization of ci-miRNAs is often performed using relative quantification (Fernández-Sanjurjo et al. 2018) as was also done in the present study. When isolating miRNAs from tissues or cells, endogenous invariant genes can be used as a control (Moldovan et al. 2014). However, miRNA quantification from serum has its challenges as currently none of the existing normalization methods have been validated in-depth so far, partly resulting from limited and heterogeneous results of ci-miRNAs gained from specific contexts (Fernández-Sanjurjo et al. 2018). As done in the present study, normalization using an exogenous miRNA (*Caenorhabditis elegans* miR-39) has been utilized in many studies (Fernández-Sanjurjo et al. 2018) which also allows estimating the efficiency of miRNA extraction (Moldovan et al. 2014).

9.6 Study limitations

One key limitation in the present study is the small sample size inflicting insufficient statistical power in the analysis. Therefore, direct generalization of these results to the Finnish postmenopausal female population cannot be done. In addition, with cross-sectional study design direct cause-effect relationships cannot be offered. As we are not measuring change of an individual but comparing groups of different backgrounds, also other factors besides HRT usage may contribute to miRNA results and generalization of the results should be done with caution.

The groups did not differ according to E_2 concentration which may have a role in the results. Information of used dosages was either not available or dosage varied between HRT users as did the type of progestogen in use which could have affected measured variables, especially HDL-C. Furthermore, hormone levels and blood lipids were measured during the first laboratory visit whereas serum samples for RNA extraction were taken at the second laboratory visit. Therefore, E_2 concentrations may have differed between these two measurement points. As this study did not measure HDL particle size distribution, interpretations are limited to effects of HRT on HDL-C levels alone. However, more comprehensive metabolomics analyses

could have increased the understanding of effects of HRT on HDL and the relation between HDL and miRNAs.

Participants were rather fit and had quite similar cardiorespiratory fitness level, consequently a larger range in VO_{2peak} values could have provided more adequate information of potential association between CRF and miRNA profile. As no variable describing PA and CRF correlated with each other, it may refer to bias regarding measurements or reporting. Accelerometers were only used for 7 days which may have caused a temporarily change in activity pattern due to the knowledge of being measured. Also, retrospective nature of LTPA questions may have caused under- or overestimation. As this study examined associations and not miRNA response to exercise, a direct comparison to several previously reported results is rather difficult.

Regarding limitations of the data analysis, a few should be mentioned. WB analysis was done to validate HDL in study samples (appendix 1). HDL was verified to locate in specific fractions (7-11) by using apoA-I. However, currently we do not have functioning antibody for EVs after SEC isolation. Therefore, it is impossible to demonstrate with complete certainty that EVs are in specific fractions (1-3), as assumed. However, SEC columns are specifically designed for EV purification and therefore, it is likely that EVs locate in fractions 1-3.

Even though isolating different ci-miRNA carriers is a major strength of this study as it provides more detailed picture of the biomarker capacity of systemic miRNAs, comparing the present results to previous literature is challenging. Existing ci-miRNA literature is extremely variable regarding miRNA carrier separation and used separation methods. Furthermore, as for cell-free samples there is no functioning housekeeping gene that can be used for normalization which may lead to heterogenous results. In general, this may affect the clinical relevance of results by reducing the generalization and reproducibility and affect the biomarker capacity of ci-miRNAs.

9.7 Ethical considerations

This study is part of EsmiRs study which has been approved by the Ethics Committee of the Central Finland Health Care District. Before signing a written informed consent, participants received information of the study. The consent was signed in two phases. At the first phase participants gave their consent to use their health-related information in research purposes. At the second phase, participants approved that information received from laboratory measurements can be used in research purposes. All potential risks and discomfort as well as personal benefits related to laboratory measurements were described for the participants. The effects of small sample size on anonymity was acknowledged and it has been ensured that no individual participant can be identified from this study. Data have been handled with care and with total anonymity at every stage of the study. Author of this Master's thesis signed a written contract where she committed to follow above mentioned objectives of Responsible Conduct of Research. Data collection in the laboratory was carried out with carefulness and respect to laboratory practices. References of this study comprise of international and national scientific articles and textbooks which have been appropriately referred to.

9.8 Future directions

During recent years research has focused on examining the paradox of increased HDL-C levels and the increased CVD risk during menopausal transition. However, as HDL-C concentration seems to vary greatly depending on the particle size, measuring merely HDL-C levels may not adequately reflect the functional capacity of HDL. Because of heterogeneity of HDL particles, they differ from each other according to their size and content, consequently having different functional roles. The present study suggests that specific miRNA content of HDL was not sensitive to HRT usage. In the future it would be essential to investigate other potential menopause-related compositional and functional changes of HDL as it seems that HDL function, rather than HDL-C, may have a causal relation to CVD protection. Examining the effects of HRT provides a favorable study design in examining menopausal hormonal changes as it controls the effect of age. However, to better understand the effects of HRT on clinical outcomes, the effects of HRT on HDL particle size distribution and the highly abundant protein

content of the particles should be investigated. Currently ion-mobility or nuclear magnetic resonance can be used to quantify the size and concentration of HDL particles and targeted proteomics to quantify proteins. In addition, cholesterol efflux capacity from macrophages can be studied to examine HDL function. These methods should be carried out using a larger sample size and controlling factors related to HRT usage such as dosage, administration route and treatment type.

9.9 Conclusions

In conclusion, according to this cross-sectional study of postmenopausal women, the use of HRT did not affect HDL-C concentration nor miR-21, -126 and -146a content of HDL. To my knowledge this is the first study to examine the effects of HRT on miRNA content of HDL. These results suggest that the cardiovascular-related miRNAs carried by HDL do not seem to be affected by HRT and therefore may not be the most prominent factor underlying the changes in HDL composition and/or function in postmenopause. However, despite of small sample size, a positive correlation was found between serum E_2 and miR-126. In addition, miR-146a was found to correlate negatively with VO_{2peak} in HRT using women and positively with accelerometer measured LTPA in non-using women. These findings indicate that miR-146a, which has commonly been shown to respond to exercise training at systemic level, may associate with PA and fitness parameters when carried in HDL. Future studies should focus on examining other factors potentially affecting HDL composition and functionality in postmenopause using a larger sample size.

REFERENCES

Abdulnour, J., Doucet, E., Brochu, M., Lavoie, J., Strychar, I., Rabasa-Lhoret, R. & Prud'homme, D. 2012. The effect of the menopausal transition on body composition and cardiometabolic risk factors: a Montreal-Ottawa New Emerging Team group study. Menopause 19 (7), 760-767.

Al-Kafaji, G., Al Mahroos, G., Al-Muhtaresh, H. A., Skrypnyk, C., Sabry, M. A. & Ramadan, A. R. 2016. Decreased expression of circulating microRNA-126 in patients with type 2 diabetic nephropathy: A potential blood-based biomarker. Experimental and therapeutic medicine 12 (2), 815-822.

Ambikairajah, A., Walsh, E. & Cherbuin, N. 2019. Lipid profile differences during menopause: a review with meta-analysis. Menopause 26 (11), 1327-1333.

Arroyo, J. D., Chevillet, J. R., Kroh, E. M., Ruf, I. K., Pritchard, C. C., Gibson, D. F., Mitchell, P. S., Bennett, C. F., Pogosova-Agadjanyan, E. L. & Stirewalt, D. L.. 2011. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proceedings of the National Academy of Sciences 108 (12), 5003-5008.

Atsma, F., Bartelink, M-L. E., Grobbee, D. E. & van der Schouw, Y. T. 2006. Postmenopausal status and early menopause as independent risk factors for cardiovascular disease: a meta-analysis. Menopause 13 (2), 265-279.

Auro, K., Joensuu, A., Fischer, K., Kettunen, J., Salo, P., Mattsson, H., Niironen, M., Kaprio, J., Eriksson, J. G. & Lehtimäki, T. 2014. A metabolic view on menopause and ageing. Nature communications 5, 4708. doi:10.1038/ncomms5708.

Baggish, A. L., Hale, A., Weiner, R. B., Lewis, G. D., Systrom, D., Wang, F., Wang, T. J. & Chan, S. Y. 2011. Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. The Journal of physiology 589 (16), 3983-3994.

Baggish, A. L., Park, J., Min, P., Isaacs, S., Parker, B. A., Thompson, P. D., Troyanos, C., D'Hemecourt, P., Dyer, S. & Thiel, M. 2014. Rapid upregulation and clearance of distinct circulating microRNAs after prolonged aerobic exercise. Journal of applied physiology 116 (5), 522-531.

Barber, J. L., Zellars, K. N., Barringhaus, K. G., Bouchard, C., Spinale, F. G. & Sarzynski, M. A. 2019. The Effects of Regular Exercise on Circulating Cardiovascular-related MicroRNAs. Scientific reports 9 (1), 1-8. doi:10.1038/s41598-019-43978-x.

Bartel, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116 (2), 281-297.

Bartel, D. P. 2018. Metazoan MicroRNAs. Cell 173 (1), 20-51.

Bassett Jr, D. R. & Howley, E. T. 2000. Limiting factors for maximum oxygen uptake and determinants of endurance performance. Medicine & Science in Sports & Exercise 32 (1), 70-84.

Blumenthal, J. A., Matthews, K., Fredrikson, M., Rifai, N., Schniebolk, S., German, D., Steege, J. & Rodin, J. 1991. Effects of exercise training on cardiovascular function and plasma lipid, lipoprotein, and apolipoprotein concentrations in premenopausal and postmenopausal women. Arteriosclerosis and thrombosis: a journal of vascular biology 11 (4), 912-917.

Boon, R. A. & Vickers, K. C. 2013. Intercellular transport of microRNAs. Arteriosclerosis, Thrombosis, and Vascular Biology 33 (2), 186-192.

Bye, A., Røsjø, H., Aspenes, S. T., Condorelli, G., Omland, T. & Wisløff, U. 2013. Circulating microRNAs and aerobic fitness – the HUNT-Study. PloS one 8 (2): e57496. doi:10.1371/journal.pone.0057496.

Camont, L., Chapman, M. J. & Kontush, A. 2011. Biological activities of HDL subpopulations and their relevance to cardiovascular disease. Trends in molecular medicine 17 (10), 594-603.

Carr, M. C. 2003. The emergence of the metabolic syndrome with menopause. The Journal of Clinical Endocrinology & Metabolism 88 (6), 2404-2411.

Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., Guo, J., Zhang, Y., Chen, J. & Guo, X. 2008. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell research 18 (10), 997-1006.

Chen, X., Liang, H., Zhang, J., Zen, K. & Zhang, C. 2012. Secreted microRNAs: a new form of intercellular communication. Trends in cell biology 22 (3), 125-132.

Cho, G. J., Lee, J. H., Park, H. T., Shin, J. H., Hong, S. C., Kim, T., Hur, J. Y., Lee, K. W., Park, Y. K. & Kim, S. H. 2008. Postmenopausal status according to years since

menopause as an independent risk factor for the metabolic syndrome. Menopause 15 (3), 524-529.

Cooper, R., Mishra, G., Clennell, S., Guralnik, J. & Kuh, D. 2008. Menopausal status and physical performance in midlife: findings from a British birth cohort study. Menopause 15 (6), 1079-1085.

Cortez, M. A. & Calin, G. A. 2009. MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. Expert opinion on biological therapy 9 (6), 703-711.

Creatsas, G., Christodoulakos, G., Lambrinoudaki, I., Panoulis, C., Chondros, C. & Patramanis, P. 2003. Serum lipids and apolipoproteins in Greek postmenopausal women: association with estrogen, estrogen-progestin, tibolone and raloxifene therapy. Journal of endocrinological investigation 26 (6), 545-551.

Davis, S., Lambrinoudaki, I., Lumsden, M., Mishra, G., Pal, L., Rees, M., Santoro, N. & Simoncini, T. 2015. Menopause. Natural Reviews Disease Primers 1, 15004. doi:10.1038/nrdp.2015.4.

de Aloysio, D., Gambacciani, M., Meschia, M., Pansini, F., Modena, A. B., Bolis, P. F., Massobrio, M., Maiocchi, G., Peruzzi, E. & The Icarus Study Group. 1999. The effect of menopause on blood lipid and lipoprotein levels. Atherosclerosis 147 (1), 147-153.

de Kat, A. C., Dam, V., Onland-Moret, N. C., Eijkemans, M., Broekmans, F. & Van der Schouw, Y T. 2017. Unraveling the associations of age and menopause with cardiovascular risk factors in a large population-based study. BMC medicine 15 (1), 2. doi:10.1186/s12916-016-0762-8.

de Villiers, T. J., Hall, J. E., Pinkerton, J. V., Pérez, S. C., Rees, M., Yang, C. & Pierroz, D. D. 2016. Revised global consensus statement on menopausal hormone therapy. Maturitas 91, 153-155.

El Khoudary, S. R. 2017. HDL and the menopause. Current opinion in lipidology 28 (4), 328-336.

El Khoudary, S. R., Ceponiene, I., Samargandy, S., Stein, J. H., Li, D., Tattersall, M. C. & Budoff, M. J. 2018. HDL (High-Density Lipoprotein) Metrics and Atherosclerotic Risk in Women: Do Menopause Characteristics Matter? MESA. Arteriosclerosis, Thrombosis, and Vascular Biology 38 (9), 2236-2244.

Favari, E., Chroni, A., Tietge, U. J., Zanotti, I. & Bernini, F. 2015. Cholesterol efflux and reverse cholesterol transport. In A. von Eckardstein & D. Kardassis (eds.) High Density

Lipoproteins – From Biological Understanding to Clinical Exploitation. Cham: Springer, 181-206.

Feinberg, M. W. & Moore, K. J. 2016. MicroRNA regulation of atherosclerosis. Circulation research 118 (4), 703-720.

Fernández-Sanjurjo, M., de Gonzalo-Calvo, D., Fernández-García, B., Díez-Robles, S., Martínez-Canal, Á, Olmedillas, H., Dávalos, A. & Iglesias-Gutiérrez, E. 2018. Circulating microRNA as emerging biomarkers of exercise. Exercise and sport sciences reviews 46 (3), 160-171.

Ferreira, R., Santos, T., Amar, A., Gong, A., Chen, T. C., Tahara, S. M., Giannotta, S. L. & Hofman, F. M. 2014. Argonaute-2 promotes miR-18a entry in human brain endothelial cells. Journal of the American Heart Association 3 (3): e000968. doi:10.1161/JAHA.114.000968.

Florijn, B. W., Duijs, J. M., Levels, J. H., Dallinga-Thie, G. M., Wang, Y., Boing, A. N., Yuana, Y., Stam, W., Limpens, R. W. & Au, Y. W. 2019. Diabetic Nephropathy Alters the Distribution of Circulating Angiogenic MicroRNAs Among Extracellular Vesicles, HDL, and Ago-2. Diabetes 68 (12), 2287-2300.

Friedman, R. C., Farh, K. K., Burge, C. B. & Bartel, D. P. 2009. Most mammalian mRNAs are conserved targets of microRNAs. Genome research 19 (1), 92-105.

Godsland, I. F. 2001. Effects of postmenopausal hormone replacement therapy on lipid, lipoprotein, and apolipoprotein (a) concentrations: analysis of studies published from 1974–2000. Fertility and sterility 75 (5), 898-915.

Gohlke-Bärwolf, C. 2000. Coronary artery disease – is menopause a risk factor? Basic research in cardiology 95 (1), I77-I83.

Gold, E. B. 2011. The timing of the age at which natural menopause occurs. Obstetrics and Gynecology Clinics 38 (3), 425-440.

Greendale, G. A., Sowers, M., Han, W., Huang, M., Finkelstein, J. S., Crandall, C. J., Lee, J. S. & Karlamangla, A. S. 2012. Bone mineral density loss in relation to the final menstrual period in a multiethnic cohort: results from the Study of Women's Health Across the Nation (SWAN). Journal of bone and mineral research 27 (1), 111-118.

Gregersen, I., Høibraaten, E., Holven, K. B., Løvdahl, L., Ueland, T., Mowinckel, M., Dahl, T. B., Aukrust, P., Halvorsen, B. & Sandset, P. M. 2019. Effect of hormone replacement

therapy on atherogenic lipid profile in postmenopausal women. Thrombosis research 184, 1-7.

Haddock, B. L., Hopp, H. P., Mason, J. J., Blix, G. & Blair, S. N. 1998. Cardiorespiratory fitness and cardiovascular disease risk factors in postmenopausal women. Medicine & Science in Sports & Exercise 30 (6), 893-898.

Harlow, S. D., Gass, M., Hall, J. E., Lobo, R., Maki, P., Rebar, R. W., Sherman, S., Sluss, P., de Villiers, T & STRAW + 10 Collaborative Group. 2012. Executive summary of the Stages of Reproductive Aging Workshop + 10: Addressing the unfinished agenda of staging reproductive aging. The Journal of Clinical Endocrinology & Metabolism, 97 (4), 1159-1168.

Hodis, H. N., Mack, W. J., Henderson, V. W., Shoupe, D., Budoff, M. J., Hwang-Levine, J., Li, Y., Feng, M., Dustin, L. & Kono, N. 2016. Vascular effects of early versus late postmenopausal treatment with estradiol. New England Journal of Medicine 374 (13), 1221-1231.

Howard, B. V., Kuller, L., Langer, R., Manson, J. E., Allen, C., Assaf, A., Cochrane, B. B., Larson, J. C., Lasser, N. & Rainford, M. 2005. Risk of cardiovascular disease by hysterectomy status, with and without oophorectomy: The Women's Health Initiative Observational Study. Circulation 111 (12), 1462-1470.

Imboden, M. T., Harber, M. P., Whaley, M. H., Finch, W. H., Bishop, D. L. & Kaminsky, L. A. 2018. Cardiorespiratory fitness and mortality in healthy men and women. Journal of the American College of Cardiology 72 (19), 2283-2292.

Jensen, J., Nilas, L. & Christiansen, C. 1990. Influence of menopause on serum lipids and lipoproteins. Maturitas 12 (4), 321-331.

Kajani, S., Curley, S. & McGillicuddy, F. C. 2018. Unravelling HDL – looking beyond the cholesterol surface to the quality within. International journal of molecular sciences 19 (7), 1971. doi:10.3390/ijms19071971.

Kangas, R., Pöllänen, E., Rippo, M. R., Lanzarini, C., Prattichizzo, F., Niskala, P., Jylhävä, J., Sipilä, S., Kaprio, J. & Procopio, A. D. 2014. Circulating miR-21, miR-146a and Fas ligand respond to postmenopausal estrogen-based hormone replacement therapy–a study with monozygotic twin pairs. Mechanisms of ageing and development 143-144, 1-8.

Kangas, R., Törmäkangas, T., Fey, V., Pursiheimo, J., Miinalainen, I., Alen, M., Kaprio, J., Sipilä, S., Säämänen, A., Kovanen, V. & Laakkonen, E. K. 2017. Aging and serum exomiR content in women-effects of estrogenic hormone replacement therapy. Scientific reports 7, 42702. doi:10.1038/srep42702.

Karlamangla, A. S., Lachman, M. E., Han, W., Huang, M. & Greendale, G. A. 2017. Evidence for cognitive aging in midlife women: study of women's health across the nation. PLoS One 12 (1): e0169008. doi:10.1371/journal.pone.0169008.

Karvinen, S. M., Jergenson, M. J., Hyvärinen, M. V., Aukee, P., Tammelin, T. H., Sipilä, S., Kovanen, V. M., Kujala, U. M. & Laakkonen, E. K. 2019. Menopausal status and physical activity are independently associated with cardiovascular risk factors of healthy middle-aged women: cross-sectional and longitudinal evidence. Frontiers in endocrinology 10, 589. doi:10.3389/fendo.2019.00589.

Kodama, S., Saito, K., Tanaka, S., Maki, M., Yachi, Y., Asumi, M., Sugawara, A., Totsuka, K., Shimano, H. & Ohashi, Y. 2009. Cardiorespiratory fitness as a quantitative predictor of all-cause mortality and cardiovascular events in healthy men and women: a meta-analysis. Jama 301 (19), 2024-2035.

Kodama, S., Tanaka, S., Saito, K., Shu, M., Sone, Y., Onitake, F., Suzuki, E., Shimano, H., Yamamoto, S. & Kondo, K. 2007. Effect of aerobic exercise training on serum levels of high-density lipoprotein cholesterol: A meta-analysis. American Medical Association 167 (10), 999-1008.

Kontush, A. & Chapman, M. J. 2006. Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. Pharmacological reviews 58 (3), 342-374.

Kontush, A., Lhomme, M. & Chapman, M. J. 2013. Unraveling the complexities of the HDL lipidome. Journal of lipid research 54 (11), 2950-2963.

Kontush, A., Lindahl, M., Lhomme, M., Calabresi, L., Chapman, M.J., & Davidson, W.S. 2015. Structure of HDL: Particle Subclasses and Molecular Components. In A. von Eckardstein & D. Kardassis (eds.) High Density Lipoproteins – From Biological Understanding to Clinical Exploitation. Cham: Springer, 3-51.

Kotecha, P. T., Godsland, I. F., Crook, D. & Stevenson, J. C. 2020. Effects of tibolone or continuous combined oestradiol and norethisterone acetate on lipids, high-density lipoprotein subfractions and apolipoproteins in postmenopausal women in a two-year,

randomized, double-blind, placebo-controlled trial. Clinical endocrinology 92 (4), 303-311.

Kovanen, V., Aukee, P., Kokko, K., Finni, T., Tarkka, I. M., Tammelin, T., Kujala, U. M., Sipilä, S. & Laakkonen, E. K. 2018. Design and protocol of Estrogenic Regulation of Muscle Apoptosis (ERMA) study with 47 to 55-year-old women's cohort: novel results show menopause-related differences in blood count. Menopause 25 (9), 1020-1032.

Kozomara, A., Birgaoanu, M. & Griffiths-Jones, S. 2019. miRBase: from microRNA sequences to function. Nucleic acids research 47 (D1), D155-D162. doi:10.1093/nar/gky1141.

Krause, B. J., Carrasco-Wong, I., Dominguez, A., Arnaiz, P., Farias, M., Barja, S., Mardones, F. & Casanello, P. 2015. Micro-RNAs Let7e and 126 in plasma as markers of metabolic dysfunction in 10 to 12 years old children. PLoS One 10 (6): e0128140. doi:10.1371/journal.pone.0128140.

Kuai, R., Li, D., Chen, Y. E., Moon, J. J. & Schwendeman, A. 2016. High-density lipoproteins: nature's multifunctional nanoparticles. ACS nano 10 (3), 3015-3041.

Kuh, D., Langenberg, C., Hardy, R., Kok, H., Cooper, R., Butterworth, S. & Wadsworth, M. 2005. Cardiovascular risk at age 53 years in relation to the menopause transition and use of hormone replacement therapy: a prospective British birth cohort study. BJOG: An International Journal of Obstetrics & Gynaecology 112 (4), 476-485.

Kujala, U. M., Kaprio, J., Sarna, S. & Koskenvuo, M. 1998. Relationship of leisure-time physical activity and mortality: the Finnish twin cohort. Jama 279 (6), 440-444.

Laffont, B. & Rayner, K. J. 2017. MicroRNAs in the pathobiology and therapy of atherosclerosis. Canadian Journal of Cardiology 33 (3), 313-324.

Li, P., Wei, J., Li, X., Cheng, Y., Chen, W., Cui, Y., Simoncini, T., Gu, Z., Yang, J. & Fu, X. 2017. 17 β-Estradiol Enhances Vascular Endothelial Ets-1/miR-126-3p Expression: The Possible Mechanism for Attenuation of Atherosclerosis. The Journal of Clinical Endocrinology & Metabolism 102 (2), 594-603.

Lin, X., Zhang, X., Guo, J., Roberts, C. K., McKenzie, S., Wu, W., Liu, S. & Song, Y. 2015. Effects of exercise training on cardiorespiratory fitness and biomarkers of cardiometabolic health: a systematic review and meta-analysis of randomized controlled trials. Journal of the American Heart Association 4 (7): e002014. doi:10.1161/JAHA.115.002014.

Loh, F. H., Chen, L. H., Yu, S. L. & Jorgensen, L. N. 2002. The efficacy of two dosages of a continuous combined hormone replacement regimen. Maturitas 41 (2), 123-131.

Lopez, Y. O. N., Garufi, G. & Seyhan, A. A. 2017. Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes. Molecular biosystems 13 (1), 106-121.

Lynch, N. A., Ryan, A. S., Berman, D. M., Sorkin, J. D. & Nicklas, B. J. 2002. Comparison of VO2max and disease risk factors between perimenopausal and postmenopausal women. Menopause 9 (6), 456-462.

Mandrup, C. M., Egelund, J., Nyberg, M., Slingsby, M. H. L., Andersen, C. B., Løgstrup, S., Bangsbo, J., Suetta, C., Stallknecht, B. & Hellsten, Y. 2017. Effects of high-intensity training on cardiovascular risk factors in premenopausal and postmenopausal women. American Journal of Obstetrics and Gynecology 216 (4), 384. doi: 10.1016/j.ajog.2016.12.017.

Manson, J. E., Hsia, J., Johnson, K. C., Rossouw, J. E., Assaf, A. R., Lasser, N. L., Trevisan, M., Black, H. R., Heckbert, S. R., Detrano, R. & Strickland, O. L. 2003. Estrogen plus Progestin and the Risk of Coronary Heart Disease. New England Journal of Medicine 349 (6), 523-534.

Matthews, K. A., Crawford, S. L., Chae, C. U., Everson-Rose, S. A., Sowers, M. F., Sternfeld, B. & Sutton-Tyrrell, K. 2009. Are changes in cardiovascular disease risk factors in midlife women due to chronological aging or to the menopausal transition? Journal of the American College of Cardiology 54 (25), 2366-2373.

Mendell, J. T. & Olson, E. N. 2012. MicroRNAs in stress signaling and human disease. Cell 148 (6), 1172-1187.

Mendis, S., Puska, P., Norrving, B. & World Health Organization. 2011. Global atlas on cardiovascular disease prevention and control. Geneva: World Health Organization. Cited 31.1.2020. https://apps.who.int/iris/handle/10665/44701.

Mercuro, G., Saiu, F., Deidda, M., Mercuro, S., Vitale, C. & Rosano, G. M. 2006. Impairment of physical exercise capacity in healthy postmenopausal women. American Heart Journal 151 (4), 923-927.

Mikkola, T. 2019. Vaihdevuodet. In J. Tapanainen, O. Heikinheino & K. Mäkikallio (eds.) Naistentaudit ja synnytykset. 6th revised edition. Helsinki: Duodecim, 175-183.

Mikkola, T. S., Anthony, M. S., Clarkson, T. B. & Clair, R. W. S. 2002. Serum cholesterol efflux potential in postmenopausal monkeys treated with tibolone or conjugated estrogens. Metabolism – Clinical and Experimental 51 (4), 523-530.

Moldovan, L., Batte, K. E., Trgovcich, J., Wisler, J., Marsh, C. B. & Piper, M. 2014. Methodological challenges in utilizing mi RNA s as circulating biomarkers. Journal of Cellular and Molecular Medicine 18 (3), 371-390.

Mori, M. A., Ludwig, R. G., Garcia-Martin, R., Brandão, B. B. & Kahn, C. R. 2019. Extracellular miRNAs: from biomarkers to mediators of physiology and disease. Cell metabolism 30 (4), 656-673.

Munk-Jensen, N., Ulrich, L. G., Obel, E. B., Nielsen, S. P., Edwards, D. & Meinertz, H. 1994. Continuous combined and sequential estradiol and norethindrone acetate treatment of postmenopausal women: effect on plasma lipoproteins in a two-year placebo-controlled trial. American Journal of Obstetrics and Gynecology 171 (1), 132-138.

Nabulsi, A. A., Folsom, A. R., White, A., Patsch, W., Heiss, G., Wu, K. K. & Szklo, M. 1993. Association of hormone-replacement therapy with various cardiovascular risk factors in postmenopausal women. New England Journal of Medicine 328 (15), 1069-1075.

Nelson, H. D. 2008. Menopause. The Lancet 9614 (371), 760-770.

Niculescu, L. S., Simionescu, N., Sanda, G. M., Carnuta, M. G., Stancu, C. S., Popescu, A. C., Popescu, M. R., Vlad, A., Dimulescu, D. R. & Simionescu, M. 2015. MiR-486 and miR-92a identified in circulating HDL discriminate between stable and vulnerable coronary artery disease patients. PloS one 10 (10): e0140958. doi:10.1371/journal.pone.0140958.

Olivieri, F., Rippo, M. R., Procopio, A. D. & Fazioli, F. 2013. Circulating inflamma-miRs in aging and age-related diseases. Frontiers in genetics 4, 121. doi:10.3389/fgene.2013.00121.

Pansini, F., Bonaccorsi, G., Calisesi, M., Campobasso, C., Franze, G. P., Gilli, G., Locorotondo, G. & Mollica, G. 1993. Influence of spontaneous and surgical menopause on atherogenic metabolic risk. Maturitas 17 (3), 181-190.

Pereira-da-Silva, T., Cruz, M. C., Carrusca, C., Ferreira, R. C., Napoleão, P. & Carmo, M. M. 2018. Circulating microRNA profiles in different arterial territories of stable atherosclerotic disease: a systematic review. American journal of cardiovascular disease 8 (1), 1-13.

Pérez-Cremades, D., Mompeón, A., Vidal-Gómez, X., Hermenegildo, C. & Novella, S. 2018. Role of miRNA in the regulatory mechanisms of estrogens in cardiovascular ageing. Oxidative medicine and cellular longevity 2018, 6082387. doi:10.1155/2018/6082387.

Peters, H. W., Westendorp, I., Hak, A. E., Grobbee, D. E., Stehouwer, C., Hofman, A. & Witteman, J. 1999. Menopausal status and risk factors for cardiovascular disease. Journal of internal medicine 246 (6), 521-528.

Prud'homme, G. J., Glinka, Y., Lichner, Z. & Yousef, G. M. 2016. Neuropilin-1 is a receptor for extracellular miRNA and AGO2/miRNA complexes and mediates the internalization of miRNAs that modulate cell function. Oncotarget 7 (42), 68057-68071.

Pu, D., Tan, R., Yu, Q. & Wu, J. 2017. Metabolic syndrome in menopause and associated factors: A meta-analysis. Climacteric 20 (6), 583-591.

Rader, D. J. & Hovingh, G. K. 2014. HDL and cardiovascular disease. The Lancet 384 (9943), 618-625.

Redgrave, T. G., Roberts, D. & West, C. E. 1975. Separation of plasma lipoproteins by density-gradient ultracentrifugation. Analytical Biochemistry 65 (1-2), 42-49.

Romero-Corral, A., Somers, V. K., Sierra-Johnson, J., Korenfeld, Y., Boarin, S., Korinek, J., Jensen, M. D., Parati G. & Lopez-Jimenez, F. 2010. Normal weight obesity: A risk factor for cardiometabolic dysregulation and cardiovascular mortality. European Heart Journal 31 (6), 737–746

Rosano, G., Vitale, C., Marazzi, G. & Volterrani, M. 2007. Menopause and cardiovascular disease: the evidence. Climacteric 10 (sup1), 19-24.

Ross, R., Blair, S. N., Arena, R., Church, T. S., Després, J., Franklin, B. A., Haskell, W. L., Kaminsky, L. A., Levine, B. D. & Lavie, C. J. 2016. Importance of assessing cardiorespiratory fitness in clinical practice: a case for fitness as a clinical vital sign: a scientific statement from the American Heart Association. Circulation 134 (24), e653-e699. doi:10.1161/CIR.0000000000000461.

Roth, G. A., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., Abbasi, N., Abbastabar, H., Abd-Allah, F., Abdela, J. & Abdelalim, A. 2018. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet 392 (10159), 1736-1788.

Russo, A., Bartolini, D., Mensà, E., Torquato, P., Albertini, M. C., Olivieri, F., Testa, R., Rossi, S., Piroddi, M. & Cruciani, G. 2018. Physical Activity Modulates the Overexpression of the Inflammatory miR-146a-5p in Obese Patients. IUBMB life 70 (10), 1012-1022.

Salpeter, S. R., Walsh, J. M., Greyber, E. & Salpeter, E. E. 2006b. Brief report: coronary heart disease events associated with hormone therapy in younger and older women. Journal of general internal medicine 21 (4), 363. doi:10.1111/j.1525-1497.2006.00389.x.

Salpeter, S. R., Walsh, J. M., Ormiston, T. M., Greyber, E., Buckley, N. S. & Salpeter, E. E. 2006a. Meta-analysis: effect of hormone-replacement therapy on components of the metabolic syndrome in postmenopausal women. Diabetes, Obesity and Metabolism 8 (5), 538-554.

Sapp, R. M., Shill, D. D., Roth, S. M. & Hagberg, J. M. 2017. Circulating microRNAs in acute and chronic exercise: more than mere biomarkers. Journal of applied physiology 122 (3), 702-717.

Sapp, R. M., Shill, D. D., Dash, C., Hicks, J. C., Adams-Campbell, L. L. & Hagberg, J. M. 2019. Circulating microRNAs and endothelial cell migration rate are associated with metabolic syndrome and fitness level in postmenopausal African American women. Physiological reports 7 (14): e14173. doi:10.14814/phy2.14173.

Silva, G. J., Bye, A., el Azzouzi, H. & Wisløff, U. 2017. MicroRNAs as important regulators of exercise adaptation. Progress in cardiovascular diseases 60 (1), 130-151.

Simonsen, J. B. 2017. What are we looking at? Extracellular vesicles, lipoproteins, or both? Circulation research 121 (8), 920-922. Cited 29.11.2019. circres_circres-2017-311767_supp1.pdf.

Soules, M. R., Sherman, S., Parrott, E., Rebar, R., Santoro, N., Utian, W. & Woods, N. 2001. Executive summary: stages of reproductive aging workshop (STRAW). Climacteric 4 (4), 267-272.

Tabet, F., Vickers, K. C., Torres, L. F. C., Wiese, C. B., Shoucri, B. M., Lambert, G., Catherinet, C., Prado-Lourenco, L., Levin, M. G. & Thacker, S. 2014. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. Nature communications 5 (1). doi:10.1038/ncomms4292.

Tangney, C. C., Mosca, L. J., Otvos, J. D. & Rosenson, R. S. 2001. Oral 17β-estradiol and medroxyprogesterone acetate therapy in postmenopausal women increases HDL particle size. Atherosclerosis 155 (2), 425-430.

Taskinen, M., Puolakka, J., Pyorala, T., Luotola, H., Bjorn, M., Kaariainen, J., Lahdenpera, S. & Ehnholm, C. 1996. Hormone replacement therapy lowers plasma lp (a) concentrations: Comparison of cyclic transdermal and continuous estrogen-progestin regimens. Arteriosclerosis, Thrombosis, and Vascular Biology 16 (10), 1215-1221.

Teede, H. J., Liang, Y., Kotsopoulos, D., Zoungas, S., Craven, R. & McGrath, B. P. 2001. A placebo-controlled trial of long-term oral combined continuous hormone replacement therapy in postmenopausal women: Effects on arterial compliance and endothelial function. Clinical Endocrinology 55 (5), 673-682.

The Emerging Risk Factors Collaboration. 2009. Major lipids, apolipoproteins, and risk of vascular disease. Jama 302 (18), 1993-2000.

Tikkanen, M. J., Nikkilä, E. A., Kuusi, T. & Sipinen, S. 1982. High density lipoprotein-2 and hepatic lipase: reciprocal changes produced by estrogen and norgestrel. The Journal of Clinical Endocrinology & Metabolism 54 (6), 1113-1117.

Turchinovich, A., Samatov, T. R., Tonevitsky, A. G. & Burwinkel, B. 2013. Circulating miRNAs: cell–cell communication function? Frontiers in genetics 4, 119. doi:10.3389/fgene.2013.00119.

Uhari, M. & Nieminen, P. 2012. Epidemiologia ja biostatistiikka. 2nd revised edition. Helsinki: Duodecim.

Uhlemann, M., Möbius-Winkler, S., Fikenzer, S., Adam, J., Redlich, M., Möhlenkamp, S., Hilberg, T., Schuler, G. C. & Adams, V. 2014. Circulating microRNA-126 increases after different forms of endurance exercise in healthy adults. European journal of preventive cardiology 21 (4), 484-491.

Urbich, C., Kuehbacher, A. & Dimmeler, S. 2008. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. Cardiovascular Research 79 (4), 581-588.

Vadlamudi, S., MacLean, P., Israel, R. G., Marks, R. H., Hickey, M., Otvos, J. & Barakat, H. 1998. Effects of oral combined hormone replacement therapy on plasma lipids and lipoproteins. Metabolism 47 (10), 1222-1226.

Van Craenenbroeck, A. H., Van Craenenbroeck, E. M., Van Ackeren, K., Hoymans, V. Y., Verpooten, G. A., Vrints, C. J. & Couttenye, M. M. 2016. Impaired vascular function contributes to exercise intolerance in chronic kidney disease. Nephrology Dialysis Transplantation 31 (12), 2064-2072.

Vickers, K. C., Palmisano, B. T., Shoucri, B. M., Shamburek, R. D. & Remaley, A. T. 2011. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nature cell biology 13 (4), 423.

Vickers, K. C. & Remaley, A. T. 2012. Lipid-based carriers of microRNAs and intercellular communication. Current opinion in lipidology 23 (2), 91-97.

Vickers, K. C. & Remaley, A. T. 2014. HDL and cholesterol: life after the divorce? Journal of lipid research 55 (1), 4-12.

von Eckardstein, A., Huang, Y. & Assmann, G. 1994. Physiological role and clinical relevance of high-density lipoprotein subclasses. Current opinion in lipidology 5 (6), 404-416.

von Eckardstein, A., Nofer, J. & Assmann, G. 2001a. High density lipoproteins and arteriosclerosis: role of cholesterol efflux and reverse cholesterol transport. Arteriosclerosis, Thrombosis, and Vascular Biology 21 (1), 13-27.

von Eckardstein, A., Schmiddem, K., Hövels, A., Gülbahçe, E., Schuler-Lüttmann, S., Elbers, J., Helmond, F., Bennink, H. J. C. & Assmann, G. 2001b. Lowering of HDL cholesterol in post-menopausal women by tibolone is not associated with changes in cholesterol efflux capacity or paraoxonase activity. Atherosclerosis 159 (2), 433-439.

Wagner, J., Riwanto, M., Besler, C., Knau, A., Fichtlscherer, S., Röxe, T., Zeiher, A. M., Landmesser, U. & Dimmeler, S. 2013. Characterization of levels and cellular transfer of circulating lipoprotein-bound microRNAs. Arteriosclerosis, Thrombosis, and Vascular Biology 33 (6), 1392-1400.

Walsh, B. W., Schiff, I., Rosner, B., Greenberg, L., Ravnikar, V. & Sacks, F. M. 1991. Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. New England Journal of Medicine 325 (17), 1196-1204.

Wardle, S. L., Bailey, M. E., Kilikevicius, A., Malkova, D., Wilson, R. H., Venckunas, T. & Moran, C. N. 2015. Plasma microRNA levels differ between endurance and strength athletes. PloS One 10 (4): e0122107. doi:10.1371/journal.pone.0122107.

Williams, C. J., Williams, M. G., Eynon, N., Ashton, K. J., Little, J. P., Wisloff, U. & Coombes, J. S. 2017. Genes to predict VO_{2max} trainability: a systematic review. BMC genomics 18 (8), 831. doi:10.1186/s12864-017-4192-6.

World Health Organization (WHO). 1996. Research on the Menopause in the 1990s: report of a WHO scientific group. WHO Technical Report Series 866. Cited 8.10.2019. https://apps.who.int/iris/bitstream/handle/10665/41841/WHO_TRS_866.pdf.

Woodard, G. A., Brooks, M. M., Barinas-Mitchell, E., Mackey, R. H., Matthews, K. A. & Sutton-Tyrrell, K. 2011. Lipids, menopause and early atherosclerosis in SWAN Heart Women: menopausal transition and lipids. Menopause 18 (4), 376–384.

Writing Group for the Women's Health Initiative Investigators. 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the women's health initiative randomized controlled trial. Jama 288 (3), 321-333.

Ylikorkala, O., Lim, P. & Caubel, P. 2000. Effects on serum lipid profiles of continuous 17 β-Estradiol, intermittent norgestimate regimens versus continuous combined 17 β-Estradiol/norethisterone acetate hormone replacement therapy. Clinical therapeutics 22 (5), 622-636.

Yuana, Y., Levels, J., Grootemaat, A., Sturk, A. & Nieuwland, R. 2014. Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation. Journal of extracellular vesicles 3 (1), 23262. doi:10.3402/jev.v3.23262.

Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A., Pais, P. & Varigos, J. 2004. Investigators IS. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. The Lancet 364 (9438), 937-952.

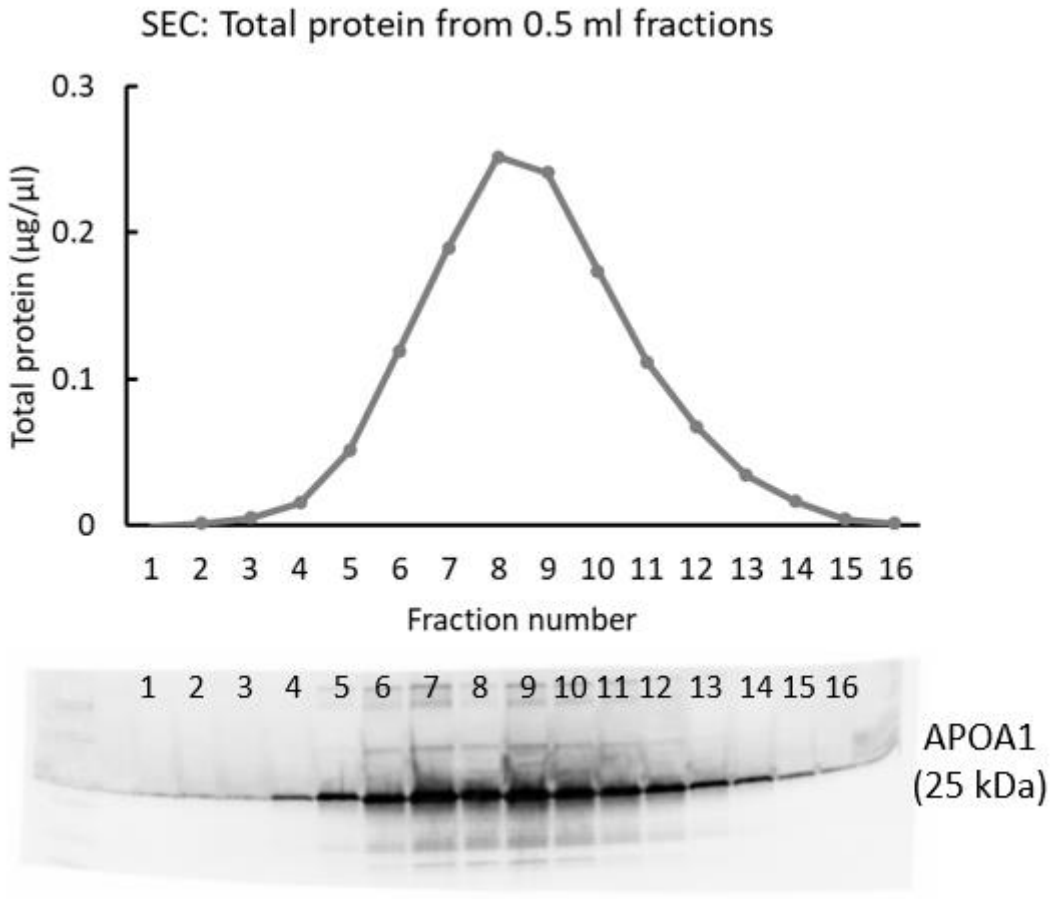
Zampetaki, A., Kiechl, S., Drozdov, I., Willeit, P., Mayr, U., Prokopi, M., Mayr, A., Weger, S., Oberhollenzer, F. & Bonora, E. 2010. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circulation research 107 (6), 810-817.

Zhao, C., Sun, X. & Li, L. 2019. Biogenesis and function of extracellular miRNAs. ExRNA 1 (1), 38. doi:10.1186/s41544-019-0039-4.

Zhou, S., Jin, J., Wang, J., Zhang, Z., Freedman. J. H., Cheng, Y. & Cai, L. 2018. miRNAS in cardiovascular diseases: potential biomarkers, therapeutic targets and challenges. Acta Pharmacologica Sinica 39, 1073–1084. doi:10.1038/aps.2018.30.

APPENDICES

APPENDIX 1. Western blot detection of apoA-I in SEC HDL-fractions.



APPENDIX 2. Qiagen miRNeasy protocol (manual).

Quick-Start Protocol March 2016

miRNeasy Serum/Plasma Kit

The miRNeasy Serum/Plasma Kit (cat. no. 217184) is shipped at ambient temperature. Store the RNeasy® MinElute® spin columns immediately at 2–8°C. Store the miScript® Primer Assay at –30 to –15°C. QIAzol® Lysis Reagent can be stored at room temperature (15–25°C) or at 2–8°C. Store the remaining components dry at room temperature. All kit components are stable for at least 9 months under these conditions if not otherwise stated on label.

Further information

- *miRNeasy Serum/Plasma Handbook*: **www.qiagen.com/HB-1002**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- This protocol is for purifying total RNA, including small RNAs, from small volumes (up to 200 µl) of serum, plasma or other body fluids.
- If necessary, redissolve any precipitate in Buffer RWT by warming.
- Except for phase separation (step 7), all steps should be performed at room temperature (15–25°C). Work quickly.
- Add ethanol (96–100%) to Buffer RWT and Buffer RPE concentrates before use (see bottle label for volume).
- Before starting with step 1 for the first time, read the recommendations for preparing serum or plasma in the *miRNeasy Serum/Plasma Handbook*.
- The miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610) must be purchased separately. For recommendations on how to prepare a working solution, see the *miRNeasy Serum/Plasma Handbook*.

1. Prepare serum or plasma, or thaw frozen samples.
2. Add 5 volumes QIAzol Lysis Reagent to the sample (e.g., for 200 µl sample, add 1 ml QIAzol Lysis Reagent). Mix by vortexing or pipetting up and down.

3. Incubate the homogenate at room temperature (15–25°C) for 5 min.
4. Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (at 1.6 x 10^8 copies/µl).
5. Add chloroform of an equal volume to the starting sample and cap tube securely (e.g., for 200 µl sample, add 200 µl chloroform). Shake vigorously for 15 s.
6. Incubate at room temperature for 2–3 min.
7. Centrifuge for 15 min at 12,000 x *g* at 4°C.
8. Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transferring any interphase. Add 1.5 volumes of 100% ethanol (e.g., for 600 µl aqueous phase, add 900 µl ethanol). Mix thoroughly by pipetting.
9. Pipet up to 700 µl sample, including any precipitate, into an RNeasy MinElute spin column in a 2 ml collection tube. Close the lid and centrifuge at ≥8000 x *g* for 15 s at room temperature. Discard the flow-through.
10. Repeat step 9 using the remainder of the sample.
11. Add 700 µl Buffer RWT to the RNeasy MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
12. Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
13. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid, and centrifuge for 2 min at ≥8000 x *g*. Discard the flow-through and the collection tube.
14. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column and centrifuge at full speed for 5 min to dry the membrane. Discard the flow-through and the collection tube.
15. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Scan QR code for handbook.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. Trademarks: QIAGEN®, Sample to Insight®, QIAzol®, MinElute®, miScript®, RNeasy® (QIAGEN Group). 1101202 03/2016 HB-0982-002 © 2016 QIAGEN, all rights reserved.

APPENDIX 3. Qiagen miScript protocol (manual).

Quick-Start Protocol March 2016

miScript® II RT Kit

The miScript II RT Kit (cat. nos. 218160, 218161, also provided as part of the miScript PCR Starter Kit, cat. no. 218193) should be stored at –30 to –15°C upon arrival.

Further information

- *miScript PCR System Handbook*: **www.qiagen.com/HB-0235**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Total RNA, containing miRNA, should be used as the starting material. It is not necessary to enrich for small RNA. We recommend miRNeasy Kits or PAXgene® miRNA Kits for purification of total RNA including miRNA. For further information, visit **www.qiagen.com/miRNA**.
- The miScript II RT Kit includes 2 buffers: **5x miScript HiSpec Buffer** and **5x miScript HiFlex Buffer**. Only one of these buffers should be used in each reverse-transcription reaction. The correct buffer to use depends on the subsequent PCR application:
  Use 5x miScript HiSpec Buffer to prepare cDNA for subsequent mature miRNA profiling using miScript miRNA PCR Arrays or quantification of mature miRNAs only using miScript Primer Assays (see Table 1). miScript HiSpec Buffer should also be used to prepare cDNA for subsequent preamplification using the miScript PreAMP PCR Kit.
  Use 5x miScript HiFlex Buffer to prepare cDNA for subsequent real-time PCR applications that may involve quantification of mature miRNAs in parallel with precursor miRNAs, mRNAs and/or other noncoding RNAs using miScript Primer Assays, miScript Precursor Assays and/or QuantiTect® Primer Assays (see Table 1).
- **IMPORTANT: Only 5x miScript HiSpec Buffer should be used to prepare cDNA for real-time PCR with miScript miRNA PCR Arrays.**
- **IMPORTANT: Only 5x miScript HiSpec Buffer should be used to prepare cDNA for preamplification with the miScript PreAMP PCR Kit.**

- **IMPORTANT**: Do not use 5x miScript HiFlex Buffer with the miScript PreAMP PCR Kit or miScript miRNA PCR Arrays.
- This protocol is for use with up to 2 µg RNA if 5x miScript HiSpec Buffer is used, or for up to 1 µg RNA if 5x miScript HiFlex Buffer is used. If using higher RNA amounts, scale up the reaction linearly. Recommended amounts of template RNA to use in this protocol depend on the downstream PCR application and are shown in Table 1. If RNA sample is limiting (10 ng–100 ng), and the sample is intended for use with miScript miRNA PCR Arrays, we highly recommend preparing cDNA with 5x miScript HiSpec Buffer and preamplifying with the miScript PreAMP PCR Kit.

Table 1. Recommended RNA starting amounts and buffers

PCR application	Assay/array	Buffer	Recommended RNA input*
Preamplification	Any miScript Array	5x miScript HiSpec Buffer	10–100 ng per RNA sample
Pathway profiling of mature miRNA	Pathway-Focused miScript miRNA PCR Arrays	5x miScript HiSpec Buffer	125–250 ng per RNA sample†
Pathway profiling of mature miRNA (high content)	miScript miRNA HC PCR Arrays	5x miScript HiSpec Buffer	250–500 ng for one 384-well plate†
Whole miRNome profiling of mature miRNA	miRNome miScript miRNA PCR Arrays	5x miScript HiSpec Buffer	250–500 ng per 384-well plate or per 4 x 96-well plates/Rotor-Discs (the number of plates provided in a miRNome miScript miRNA PCR Array varies depending on the species of interest)†
Mature miRNA quantification only	miScript Primer Assays	5x miScript HiSpec Buffer	Depends on abundance and number of target miRNAs to be quantified; from 10 ng up to a maximum of 2 µg
Parallel real-time PCR quantification of mature miRNAs, precursor miRNAs, mRNAs and/or other noncoding RNAs	miScript Primer Assays, miScript Precursor Assays and/or QuantiTect Primer Assays	5x miScript HiFlex Buffer	Depends on abundance and number of target miRNAs to be quantified; up to a maximum of 1 µg
Precursor miRNA detection	miScript Precursor Assays	5x miScript HiFlex Buffer	Depends on abundance and number of target miRNAs to be quantified; up to a maximum of 0.5 µg

* If the RNA sample is not limiting, use the upper limit of the recommended range. For more information on sample input, refer to the *miScript PCR System Handbook* or *miScript miRNA PCR Array Handbook*.

† These recommended RNA starting amounts result in 0.5–1 ng cDNA per array well.

1. Thaw template RNA on ice. Thaw 10x miScript Nucleics Mix and either 5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer at room temperature (15–25°C). Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes and then store on ice.
2. Prepare the reverse-transcription master mix on ice according to Table 2. Mix and then store on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA.

   IMPORTANT: **If cDNA will be used for preamplification with the miScript PreAMP PCR Kit, use a final reaction volume of 10 µl**. For next steps, refer to the *miScript PreAMP PCR Kit Quick-Start Protocol* and the *miScript PreAMP Handbook*.

   Note: miScript Reverse Transcriptase Mix should be removed from the –20°C freezer just before preparation of the master mix and placed on ice. It should be returned to the freezer immediately after use.

Table 2. Reverse-transcription reaction components

Component	Volume/reaction	Volume/reaction for later preamplification
5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer*	4 µl	2 µl
10x miScript Nucleics Mix	2 µl	1 µl
RNase-free water	Variable	Variable
miScript Reverse Transcriptase Mix	2 µl	1 µl
Template RNA (added in step 3)	Variable (see Table 1)	Variable (see Table 1)
Total volume	**20 µl**	**10 µl**

* The correct buffer to use depends on the subsequent PCR application, see "Notes before starting" and Table 1.

3. Add template RNA to each tube containing reverse-transcription master mix. Mix gently, briefly centrifuge and then place on ice.
4. Incubate for 60 min at 37°C.
5. Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and place on ice. To proceed immediately, dilute the reactions as described in Table 3, mix gently, briefly centrifuge and continue with real-time PCR or preamplification. Alternatively, to store prior to real-time PCR or preamplification, transfer undiluted to a –20°C freezer.

Table 3. cDNA dilution prior to PCR or preamplification

PCR application	Assay/array	Reaction dilution
Preamplification	Any miScript Array	Add 40 µl RNase-free water to the 10 µl reverse-transcription reaction
Pathway profiling	Pathway-Focused miScript miRNA PCR Arrays	Add 200 µl RNase-free water to each 20 µl reverse-transcription reaction
Pathway profiling (high content)	miScript miRNA HC PCR Arrays	Add 90 µl RNase-free water to the 20 µl reverse-transcription reaction
Whole miRNome profiling	miRNome miScript miRNA PCR Arrays	Dilution depends on the number of plates/Rotor-Discs: For 1 x 384-well plate or 4 x 96-well plates/Rotor-Discs: add 90 µl RNase-free water to the 20 µl reverse-transcription reaction For 2 x 384-well plate or 8 x 96-well plates/Rotor-Discs: add 200 µl RNase-free water to the 20 µl reverse-transcription reaction For 3 x 384-well plate or 12 x 96-well plates/Rotor-Discs: add 310 µl RNase-free water to the 20 µl reverse-transcription reaction For 4 x 384-well plate or 16 x 96-well plates/Rotor-Discs: add 420 µl RNase-free water to the 20 µl reverse-transcription reaction
Mature miRNA quantification	miScript Primer Assays	Depends on abundance of miRNAs of interest; ensure 50 pg–3 ng cDNA per PCR by adding at least 200 µl RNase-free water, or more if necessary, to the 20 µl reverse-transcription reaction
Parallel quantification of mature miRNAs, precursor miRNAs, mRNAs, other noncoding RNAs	miScript Primer Assays, miScript Precursor Assays and/or QuantiTect Primer Assays	Depends on abundance of RNAs of interest; for parallel detection of mature miRNA with either precursor miRNA and/or mRNA, ensure 10–20 ng cDNA per PCR; for parallel detection of mature miRNA and other noncoding RNAs, ensure 50 pg–3 ng cDNA per PCR
Precursor miRNA detection	miScript Precursor Assays	Depends on abundance of precursor miRNA of interest; ensure 10–20 ng cDNA per PCR

Scan QR code for handbook.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. Trademarks: QIAGEN®, Sample to Insight®, miScript®, QuantiTect®, Rotor-Disc® (QIAGEN Group); PAXgene® (PreAnalytiX GmbH). 1101233 03/2016 HB-1192-002 © 2016 QIAGEN, all rights reserved.

APPENDIX 4. Questions for assessing self-reported leisure time physical activity (EsmiRs).

VASTAUSOHJEET

Lomakkeen täyttäminen on helppoa. Lukekaa ensin koko kysymys ja vastatkaa sitten merkitsemällä rasti sopivimman vaihtoehdon mukaiseen ruutuun tai kirjoittamalla vastauksenne sille varatulle viivalle (_____). Virheen sattuessa älkää käyttäkö pyyhekumia, vaan täyttäkää väärin merkitsemänne ruutu kokonaan ja rastittakaa oikea vaihtoehto.

Esimerkiksi: ■ virhe ☒ oikea

Liikunta

46. Montako kertaa kuukaudessa harrastatte nykyään vapaa-ajan liikuntaa?

- ☐ harvemmin kuin kerran kuukaudessa
- ☐ 1-2 kertaa kuukaudessa
- ☐ 3-5 kertaa kuukaudessa
- ☐ 6-10 kertaa kuukaudessa
- ☐ 11-19 kertaa kuukaudessa
- ☐ yli 20 kertaa kuukaudessa

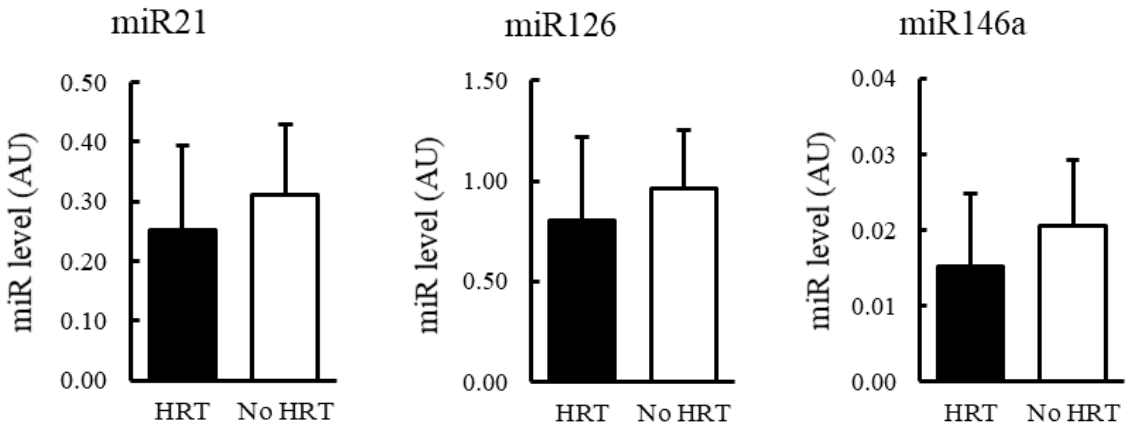
47. Harrastamanne vapaa-ajan liikunta on yleensä rasittavuudeltaan suunnilleen yhtä raskasta kuin

- ☐ kävely
- ☐ kävelyn ja kevyen juoksun vuorottelu
- ☐ kevyt juoksu (hölkkä)
- ☐ reipas juoksu

48. Kuinka kauan keskimäärin yksi vapaa-ajan liikuntakerta kestää?

- ☐ alle 15 min
- ☐ 15 min – alle puoli tuntia
- ☐ puoli tuntia – alle tunnin
- ☐ tunti – alle kaksi tuntia
- ☐ yli kaksi tuntia

APPENDIX 5. miR-21, -126 and -146a content of EVs in HRT using and non-using women.



SUPPLEMENTARY FIGURE 1. The relative expression level of miR-21, -126 and -146a in HRT using and non-using women in arbitrary units (AU). Values are presented as mean. SD bars describe SD. HRT = postmenopausal women using hormone replacement therapy (n = 5), No HRT = postmenopausal women not using hormone replacement therapy (n = 5).

APPENDIX 6. NGS read counts of miRNA content of HDL of a premenopausal woman.

Prevalence	miRNA	READs
1	let-7a-5p	4 910
2	miR-16-5p	4 068
3	let-7f-5p	3 209
4	let-7b-5p	3 163
5	miR-142-3p	2 956
6	miR-223-3p	2 880
7	miR-423-5p	1 997
8	miR-26b-5p	1 374
9	miR-199a-3p	1 369
10	let-7i-5p	1 299
11	miR-26a-5p	1 237
12	miR-486-5p	1 035
13	**miR-21-5p**	**1 021**
14	**miR-126-3p**	**981**
15	**miR-146a-5p**	**925**
16	miR-328-3p	869
17	miR-191-5p	859
18	miR-4433b-5p	797
19	miR-6728-5p	744
20	miR-3613-3p	712