JYU DISSERTATIONS 648

Hanna-Kaarina Juppi

Associations of the Menopausal Transition with Body Composition

Examining the Influence of Hormonal Changes, Muscle RNA Signaling and Lifestyle Habits

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF SPORT AND
HEALTH SCIENCES

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ABSTRACT

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Women's hormonal aging is characterized by the cessation of the menstrual cycle during menopause. Menopause-related hormonal changes have previously been associated with increased body adiposity, decreased skeletal muscle mass and function, and decreased metabolic health. However, longitudinal data on the topic remains scarce. The aim of this study was to investigate the longitudinal associations of the menopausal transition with body composition, metabolic health indicators, and skeletal muscle tissue cellular properties and transcriptome. A further aim was to investigate whether physical activity, diet quality, and the use of exogenous hormones were associated with these same variables during mid-life. The data used in this thesis is from the Estrogenic Regulation of Muscle Apoptosis (ERMA) (n=234) and Estrogen, MicroRNAs and the Risk of Metabolic Dysfunction (EsmiRs) (n=149) studies. All the participating women (aged 47-55) were either pre- or perimenopausal at baseline, and postmenopausal at follow-up. Hormone and adipokine levels were measured from blood samples. Body composition and fat distribution were measured using dual-energy X-ray absorptiometry, quantitative computed tomography, and anthropometry. Muscle biopsies of *m. vastus lateralis* were used to determine muscle tissue properties and transcriptome. Physical activity, diet quality, and the use of exogenous hormones were examined using accelerometers and questionnaires. Menopause was associated with increased total body and waist adiposity and decreased lean and muscle mass. Systemic leptin and adiponectin levels increased, while resistin levels decreased. During the menopausal transition the expression level of 49 protein-coding genes, which take part in important cellular signaling pathways, changed. A higher physical activity level, higher diet quality, and the use of exogenous hormones were associated with less adiposity, gynoid-type fat distribution, and higher lean and muscle mass. The menopausal transition is associated with unfavorable changes in body composition, but these changes and their effects on metabolic health may be alleviated by healthier lifestyle habits. Observed changes in skeletal muscle gene expression may help to understand the mechanistic details of muscle tissue and total body health regulation during menopause.

Keywords: estrogen, physical activity, metabolic health

TIIVISTELMÄ (ABSTRACT IN FINNISH)

Juppi, Hanna-Kaarina
Vaihdevuosien siirtymävaiheen vaikutukset kehonkoostumukseen: hormonaalisten muutosten, lihaksen RNA signaloinnin ja elintapamuuttujien yhteydet.
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Naisten biologisen vanhenemisen erityispiirre ovat vaihdevuodet, jotka lähes kaikki naiset käyvät läpi keski-iässä. Vaihdevuosiin liittyvät hormonimuutokset on yhdistetty aiemmin epäsuotuisiin muutoksiin kehonkoostumuksessa ja suorituskyvyssä sekä kasvaneeseen riskiin sairastua aineenvaihduntasairauksiin. Aikaisempaa pitkittäisaineistoa vaihdevuosien siirtymävaiheesta on kuitenkin olemassa vain vähän. Tässä tutkimuksessa selvitettiin pitkittäisaineistossa vaihdevuosien siirtymävaiheen ja menopaussin läpikäymisen vaikutuksia kehon rasvoittumiseen, aineenvaihduntaterveyden indikaattoreille, sekä lihaskudoksen määrälle, transkriptomille ja lihassolujen ominaisuuksille. Lisäksi selvitettiin miten tietyt elintapatekijät olivat yhteydessä edellä mainittuihin muuttujiin. Tutkimuksessa käytettiin Estrogeeni, vaihdevuodet ja toimintakyky (ERMA, n=234)- ja Estrogeeni, mikro-RNA:t ja metabolisten toimintahäiriöiden riski (EsmiRs, n=149)-tutkimusten aineistoja. Molempien tutkimusten alussa naiset iältään 47–55 vuotta olivat joko pre- tai perimenopausaalisia ja tutkimusten lopussa postmenopausaalisia. Naisten verinäytteistä määritettiin veren hormoni- ja adipokiinitasot. Kehonkoostumusta ja rasvanjakautumista mitattiin kaksienergiaisella röntgenabsorptiometrialla, kvantitatiivisella tietokonetomografialla ja antropometrialla. Lihasbiopsioista määritettiin lihaskudoksen tarkempia ominaisuuksia ja transkriptomi. Fyysistä aktiivisuutta, ruokavalion laatua ja hormonivalmisteiden käyttöä tutkittiin kyselylomakkeilla ja aktiivisuusmittarilla. Vaihdevuosien siirtymävaiheen läpikäyminen lisäsi koko kehon ja erityisesti keskivartalon rasvamassaa, sekä vähensi lihasmassaa. Veren leptiinin ja adiponektiinin pitoisuudet nousivat ja resistiinin pitoisuus laski. Vaihdevuosien siirtymävaiheen aikana 49:n lähetti-RNA-molekyylin ilmentymistasot muuttuivat lihaksessa. Korkeampi fyysisen aktiivisuuden määrä ja ruokavalion laatu sekä sukupuolihormonivalmisteiden käyttö olivat yhteydessä matalampaan rasvamassaan, suotuisampaan rasvanjakautumiseen ja korkeampaan lihasmassaan. Vaihdevuodet muokkaavat kehonkoostumusta terveydelle epäsuotuisampaan suuntaan, mutta muutokseen ja sen vaikutuksiin aineenvaihduntaterveydelle voi vaikuttaa terveellisillä elintavoilla. Lihaskudoksessa ilmentymistasoiltaan muuttuneet geenit voivat auttaa ymmärtämään vaihdevuosien aikana havaittujen lihas- ja kehonkoostumuksen muutosten mekanismeja.

Avainsanat: estrogeeni, aineenvaihduntaterveys, fyysinen aktiivisuus

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Jyväskylä 3.5.2023
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ORIGINAL PUBLICATIONS AND AUTHOR CONTRIBUTION

This thesis is based on the following original publications, which will be referred to by their Roman numbers. The thesis also includes unpublished data.

I **Juppi, H.-K.,** Sipilä, S., Fachada, V., Hyvärinen, M., Cronin, N., Aukee, P., Karppinen, J. E., Selänne, H., Kujala, U. M., Kovanen, V., Karvinen, S., & Laakkonen, E. K. (2022). Total and regional body adiposity increases during menopause—Evidence from a follow-up study. *Aging Cell*, *21*(6), e13621. https://doi.org/10.1111/acel.13621

II Hyvärinen, M.*, **Juppi, H.-K.***, Taskinen, S., Karppinen, J. E., Karvinen, S., Tammelin, T. H., Kovanen, V., Aukee, P., Kujala, U. M., Rantalainen, T., Sipilä, S., & Laakkonen, E. K. (2021). Metabolic health, menopause, and physical activity—A 4-year follow-up study. *International Journal of Obesity*, *46*(3), 544–554. * Equal authorship. https://doi.org/10.1038/s41366-021-01022-x

III **Juppi, H.-K.**, Sipilä, S., Cronin, N. J., Karvinen, S., Karppinen, J. E., Tammelin, T. H., Aukee, P., Kovanen, V., Kujala, U. M., & Laakkonen, E. K. (2020). Role of menopausal transition and physical activity in loss of lean and muscle mass: A follow-up study in middle-aged Finnish women. *Journal of Clinical Medicine*, *9*(5),1588. https://doi.org/10.3390/jcm9051588

IV **Juppi, H.-K.,** Korhonen, T.-M., Sievänen, T., Kovanen, V., Kujala, U. M., Aukee, P., Tammelin, T., Cronin, N., Sipilä, S., Karvinen, S., & Laakkonen, E. K. Skeletal muscle mRNA transcriptome is affected by the menopausal transition. Manuscript in preparation.

I and my supervisors Eija Laakkonen, Sira Karvinen, and Sarianna Sipilä designed the research questions addressed in this thesis. I conducted all the muscle section staining procedures (*I, III*) and electrophoretic (*II*) and adipokine analyses (*I*). Manual cropping of the DXA scans *(I*) and RNA extraction (*IV*) was conducted together with laboratory staff. I was responsible for the majority of the microscopy imaging (*I, III*). I examined the images before and after image analysis for muscle section stainings (*I*) and CT scans (*I, III, IV*). In *Paper III*, I was responsible for all the muscle section image analysis. In *Paper IV*, I participated in the planning of the data filtering process and conducted the Ingenuity Pathway Analysis (IPA). I was responsible for the statistical analysis in *Papers I* and *III*, and correlation analyses in *Paper IV*. As the first author of all four publications, I drafted the manuscripts and made revisions based on comments from co-authors and reviewers. In *Paper II*, I shared the first authorship with Matti Hyvärinen and was responsible for the data on anthropometric and body composition measurements.

FIGURES

TABLES

ABBREVIATIONS

ACC-MAD	accelerometer-measured physical activity in mean amplitude deviations
ACC-MVPA	accelerometer-measured moderate-to-vigorous physical activity
ALM	appendicular lean mass
ALMI	appendicular lean mass index
BAT	brown adipose tissue
BMI	body mass index
CT	computed tomography
DE	differential expression
DHEA	dehydroepiandrosterone
DXA	dual-energy X-ray absorptiometry
E2	estradiol
EarlyMT	at baseline in the early menopausal transition
ER	estrogen receptor
FM	fat mass
FMP	final menstrual period
FSH	follicle-stimulating hormone
HT	menopausal hormone therapy
LateMT	at baseline in the late menopausal transition
LBM	total lean body mass
LBMI	total lean body mass index
LFC	$\log_2$ fold change
LH	luteinizing hormone
LM	lean mass
lncRNA	long non-coding RNA
miRNA	microRNA
mRNA	messenger RNA
ncRNA	non-coding RNA
OVX	ovariectomy
P4	progesterone
PA	physical activity
RNA	ribonucleic acid
SAT	subcutaneous adipose tissue
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHBG	sex hormone binding globulin
SR-PA	self-reported physical activity
VAT	visceral adipose tissue
WAT	white adipose tissue,
WC	waist circumference
WHR	waist-to-hip-ratio

GENE, RNA, AND PROTEIN NAME ABBREVIATIONS

ACTA1	actin
ADINR	adipogenic differentiation induced noncoding RNA
ALMS1	ALMS1 centrosome and basal body-associated protein
APC	adenomatosis polyposis coli
AR	androgen receptor
ARHGAP19	rho GTPase activating protein 19
ATP5MC2	ATP synthase membrane subunit C locus 2
BAIAP2-DT	BAR/IMD domain-containing adaptor protein 2 divergent transcript
BIRC6	baculoviral IAP repeat containing 6
C1QTNF9	C1q and TNF-related 9, CTRP9
C3	complement 3
CHPF2	chondroitin polymerizing factor 2
CKM	creatine kinase, muscle
COX1	mitochondrial cytochrome c oxidase
CS	citrate synthase
CTRP15	complement C1q TNF-related protein 15, myonectin
CYP19A1	aromatase
DES	desmin
Dum	developmental pluripotency-associated 2 upstream binding muscle lncRNA
E2F3	E2F transcription factor 3
ECM1	extracellular matrix protein 1
ELN	elastin
ENTPD1-AS1	ectonucleoside triphosphate diphosphohydrolase 1 antisense RNA 1
ESR1	estrogen receptor 1 (ERα)
ESR2	estrogen receptor 2 (ERβ)
EXTL3	exostosin-like glycosyltransferase 3
FAS	fatty acid synthase
FBXO32	F-box protein 32, MAFbx, Atrogin-1
FGF-21	fibroblast growth factor 21
FNDC5	fibronectin type III domain containing 5, irisin
FOXO1	forkhead box O1
FOXO3	forkhead box O3
FSHR	FSH receptor
GBP5	guanylate binding protein 5
GLUT4/SLC2A4	glucose transporter type 4, solute carrier family 2, member 4
GORASP1	Golgi reassembly stacking protein 1
GPD	alfa-glycerophosphate dehydrogenase
GPER1	G-protein-coupled estrogen receptor 1
GTF2F2	general transcription factor IIF subunit 2
GYG2P1	glycogenin 2 pseudogene 1

H19	H19 imprinted maternally expressed transcript
HIF-1α	hypoxia-inducible factor 1-alpha
HK1	hexokinase 1
HOTAIR	HOX transcript antisense intergenic RNA
HSD17B1 – 5	hydroxysteroid 17-beta dehydrogenases 1–5
HSD3B2	progesterone reductase
IGF1	insulin-like growth factor 1
IL-6 – 15	interleukins 6–15
IL-1ra	interleukin 1 receptor antagonist
INTU	inturned planar cell polarity protein
IQCH-AS1	IQ motif containing H antisense RNA 1
IRS-2	insulin receptor- 2
JAK2	Janus kinase 2
KANK3	KN motif and ankyrin repeat domains 3
KCNQ1OT1	potassium voltage-gated channel subfamily Q member 1 opposite transcript 1
KIAA0355/GARRE, granule associated Rac and RHOG effector 1	
LINC00667	long intergenic non-protein coding RNA 667
LINC02541	long intergenic non-protein coding RNA 2541
LINC-ROR	long intergenic non-protein coding RNA, regulator of reprogramming
LncRNA152	long non-coding RNA 152
LYPLA1	lysophospholipase 1
MAFK	MAF bZIP transcription factor K
MALAT1	metastasis-associated lung adenocarcinoma transcript 1
MAPK	mitogen-activated protein kinase 1
MAR1	muscle anabolic regulator 1
MCP1	monocyte chemoattractant protein 1
MGLL	monoglyceride lipase
MIR1-1HG	MIR1-1 host gene
MKNK1	MAPK interacting serine/threonine kinase 1
MT-CO1	mitochondrially encoded cytochrome c oxidase I
MTHFSD	methenyltetrahydrofolate synthetase domain containing
mTOR	mechanistic target of rapamycin kinase
MuRF-1/TRIM63	muscle-specific ring finger protein 1
MYD88	myeloid differentiation primary response protein MyD88
Myf5	myogenic factor 5
MYH1	myosin heavy chain 1 (IIX)
MYH2	myosin heavy chain 2 (IIA)
MYH7	myosin heavy chain 7 (I)
MyoD	myogenic differentiation 1
NAA35	N-alpha-acetyltransferase 35, NatC auxiliary subunit
NEAT1	nuclear enriched abundant transcript 1
NEB	nebulin
NLRC5	NLR family CARD domain containing 5

NORAD	non-coding RNA activated by DNA damage
Nrf2	nuclear factor erythroid 2–related factor 2
NUDT4	nudix hydrolase 4
NUTM2A-AS1	NUT family member 2A antisense RNA 1
OSER1-DT	oxidative stress-responsive serine-rich 1 divergent transcript
PCG-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PDK4	pyruvate dehydrogenase kinase 4
PFK1	phosphofructokinase 1
PGR	progesterone receptor
PI3K/Akt	phosphatidylinositol 3-kinase/Akt Serine/threonine kinase
PIDD1	p53-induced death domain protein 1
PPARα	peroxisome proliferator-activated receptor alfa
PPARγ	peroxisome proliferator-activated receptor gamma
PRKCA	protein kinase C alpha
PTPN20	protein tyrosine phosphatase non-receptor type 20
PYCR1	pyrroline-5-carboxylate reductase 1
RAM	long intergenic non-protein coding RNA 977
RBMS1	RNA binding motif single-stranded interacting protein 1
S6K1	ribosomal protein S6 kinase
SDH	succinate dehydrogenase
SESN2	sestrin 2
SGSH	N-sulfoglucosamine sulfohydrolase
SLC22A17	solute carrier family 22 member 17
SLFN11	schlafen family member 11
SNHG1 – 14	small nucleolar RNA host genes 1–14
SRD5A1	steroid 5-alpha-reductase 1
TGF-β	transforming growth factor beta
TLK2	tousled-like kinase 2
TMEM120B	transmembrane protein 120B
TMEM39B	transmembrane protein 39B
TNF-α	tumor necrosis factor alpha
TOM1L2	target of Myb1-like 2 membrane trafficking protein
TTN	titin
ULK3	Unc-51-like kinase 3
VEGF	vascular endothelial growth factor
VPS28	vacuolar protein sorting-associated protein 28 homolog
XIST	X inactive specific transcript
ZEB1	zinc finger E-box binding homeobox 1
ZNF611	zinc finger 611
ZNF710-AS1	zinc finger 710 antisense RNA 1
ZNF761	zinc finger 761
ZNF84	zinc finger 84

CONTENTS

1 INTRODUCTION

Aging is a process, that affects all of us. Based on physical performance, we reach our peak capacity around the mid-20s, and thereafter, at a tissue-specific rate, start to age (Allen & Hopkins, 2015; Huebner & Perperoglou, 2019). Biological aging is characterized by a lowering of the regeneration capacity of cells and tissues, and the consequent accumulation of cellular defects and mutations, including significant defects in important organs and leading eventually to the cessation of life. Although men and women consist mostly of the same tissues, cells, and genes, some fundamental differences exist in their respective aging processes. One of the most prominent differences is in hormonal aging specifically related to reproduction. While men experience a steady decrease in fertility and sex hormone production between their 20s and old age (Harman et al., 2001; Niederberger, 2011), women virtually come to a halt during mid-life. Cumulative aging effects in the ovaries and hypothalamus cause menopause, which ends the fertile period of women and dramatically affects their endogenous sex hormone production. Changes in the hormonal milieu include decreases in the main female sex hormone estradiol (E2), progesterone (P4), inhibin B, and anti-Müllerian hormone, and an increase in gonadotropin follicle-stimulating hormone (FSH) levels (Burger et al., 2007). These changes in the hormonal milieu have been associated with an increased risk of muscle weakness, osteoporosis, and metabolic syndrome along with decreased psychological well-being and mental health and thus highlighting the significance of menopause for public health globally (S. R. Davis et al., 2015). As women nowadays may live in the postmenopausal state for nearly one-third of their lives, this is a topic that warrants serious attention.

Body composition is one of the variables that menopause is thought to affect. Body composition, or the proportions of adipose, muscle, and bone mass, and especially the balance between adipose and muscle mass, is a significant factor in human health and functional capacity (Dulloo et al., 2010). While adipose tissue is necessary for energy storage, the protection of vital organs, insulation, and as a hormonal secretory organ, the presence of redundant adipose tissue poses several risks. Excess overall adipose tissue, or fat accumulation in a suboptimal

location such as around the intestines, inside the liver, or between muscle fibers, can, by maintaining low-grade inflammation, affect physical functioning and increase the risk for cardiovascular diseases and cancer (Chait & den Hartigh, 2020). In turn, skeletal muscle, which is one of the largest tissue types in the total of body mass, is responsible for producing motion and heat, and has an important role in energy metabolism, as an amino acid reservoir and in inter-cellular signaling (Frontera & Ochala, 2015). Therefore, low muscle mass not only affects functional capacity but also energy balance and overall health. Earlier studies indicate that during menopause muscle mass is lost and adipose tissue is gained (Marlatt et al., 2022). However, the effect of menopause on waist and visceral adiposity, a fat distribution pattern, that predisposes to an increased risk of cardiovascular disease and metabolic conditions, is less well understood (G.-C. Chen et al., 2019; M. Zhang et al., 2015). As both the accumulation of adipose tissue and decrease in muscle mass are aging- and therefore also menopause-related phenomena, they are not completely unavoidable. Nevertheless, maintaining an adequate skeletal muscle mass combined with a moderate amount of body fat is one of the most successful methods for achieving healthier aging (Baumgartner, 2006).

The mechanisms behind the menopausal changes in body composition and related decrements in metabolic health are thought to lie in hormonal changes, especially the decrease in estrogens. Estrogens, including E2, estrone (E1), and estriol (E3), are a class of steroid hormones of which E2 is the most physiologically prominent during fertile age (Gruber et al., 2002). In women, estrogen receptors have been found in several tissues, including bone, adipose tissue, and muscle (Anwar et al., 2001; Bord et al., 2001; Wiik et al., 2009). In this connection, E2 has established positive functions in adipose tissue mass and distribution regulation (Bracht et al., 2020) and skeletal muscle regeneration capacity (Collins et al., 2019; Enns & Tiidus, 2010), and it has been suggested to play a protective role against cardiovascular diseases and metabolic conditions (El Khoudary et al., 2020). In addition to E2, FSH has also recently been proposed to contribute to body composition, although the mechanisms are not yet completely understood (X. Cui et al., 2016; Veldhuis-Vlug et al., 2021).

Besides endogenous hormones, lifestyle habits, including physical activity (PA), diet quality and the use of exogenous hormones, also contribute to body composition and metabolic health in mid-life. In middle-aged women, several types of PA have been associated with greater muscle mass, lower adiposity, better cardiometabolic and mental health and higher physical functioning (Cebula et al., 2020; Elavsky & McAuley, 2007; K. M. Park et al., 2019; Sipilä et al., 2001; Ward et al., 2020). Although the specific positive effects vary across PA types, overall they are mediated through, for example, improved intra- and intercellular signaling and the release of anti-inflammatory molecules from muscle and adipose tissue (Neufer et al., 2015). Improved metabolic health, more beneficial body composition and lower inflammatory status are also well-established effects of good diet quality (Galland, 2010; Mozaffarian et al., 2011). A diet including a high amount of vegetables and fruits, minimally processed

foods, soft fats, more fish than red meat and low amounts of dairy products with moderate energy content has been shown to associate with lower incidence of cardiovascular disease, certain cancers and inflammatory diseases in several cohorts including menopausal women (Tosti et al., 2018). Overall, the PA and diet quality guidelines for the adult population, aimed at achieving significant health benefits and reducing health risks, can also be applied to menopausal women (The Nordic Council of Ministers, 2014; World Health Organization, 2003, 2020).

In addition to exercise and diet, another important lifestyle choice for women is the use of exogenous sex hormones, mainly E2 and P4 and their derivates. Menopausal hormone therapy (HT), which aims to alleviate bothersome symptoms related to the changing hormone levels that occur in midlife, has also been associated with lower adiposity (Papadakis et al., 2018; Yüksel et al., 2007), decreased risk for metabolic conditions (Salpeter et al., 2006) and higher bone density (L. Zhu et al., 2016) in postmenopausal women. The beneficial results of HT on muscle mass and strength have become more controversial following the neutral results obtained from recent meta-analyses (Javed et al., 2019; Xu et al., 2020). While HT seems to have beneficial effects on cognition and cardiovascular disease, some of these effects may be related to the therapeutic window, i.e., the timing of the therapy (Vigneswaran & Hamoda, 2022).

Nonetheless, the changes in body composition, skeletal muscle properties and metabolic health together with lifestyle factors in women undergoing menopause have not been adequately studied in a longitudinal design. Although several cross-sectional studies have been conducted in which premenopausal women have been compared to postmenopausal women in these parameters (e.g., Kanaley et al., 2001; Karvinen et al., 2019; Sternfeld et al., 2005; Svendsen et al., 1995), longitudinal studies following the same women throughout menopause are less abundant (e.g., Abdulnour et al., 2012; Greendale et al., 2019; Marlatt et al., 2020; Simkin-Silverman et al., 2003). Studies aimed at capturing the immediate menopausal hormonal change-induced changes over the other aging-related changes in a close timeframe around the final menstrual period are even fewer (C. G. Lee et al., 2009). Moreover, no previous longitudinal studies have been conducted on the effects of hormonal changes on skeletal muscle fiber properties and RNA signaling.

Therefore, the aim of this thesis, using two longitudinal datasets of middle-aged women going through menopause, was to study the factors and associations affecting women's health during and after mid-life. The specific aims were to investigate how the menopausal transition associates with changes in body composition and, in particular, skeletal muscle properties, such as muscle fiber type and RNA transcriptome. A further aim was to study whether PA, diet quality and the use of exogenous hormones have additional associations with these same parameters.

2 LITERATURE REVIEW

2.1 Reproductive aging in women

2.1.1 Fertile age

During the reproductive years following puberty, female sex hormones (gonadal/ovarian hormones) and gonadotropins fluctuate periodically during the menstrual cycle, which on average lasts for 28 days (Taylor et al., 2019). At the beginning of the follicular phase, starting from menses, the systemic levels of the four main regulatory hormones E2, P4, FSH and the luteinizing hormone (LH) are low. Gradually the systemic levels of pituitary secreted FSH increase, which leads to the maturation of usually one dominant follicle. During maturation, the follicle begins to secrete E2, P4 and inhibin B. This forms a negative feedback loop with FSH release to control follicle growth. When the E2 level reaches a threshold level, the hypothalamus reacts and releases gonadotropin-releasing hormone (GnRH) to induce a surge of LH from the anterior pituitary gland. This LH peak further induces release of the oocyte from the follicle in ovulation. The remaining follicle shell, the corpus luteum, begins to secrete both P4 and E2 in preparation for potential implantation simultaneously thickening the endometrium. If fertilization does not occur, the corpus luteum degenerates, hormone production ends and menstrual bleeding starts as the endometrium is no longer maintained (Taylor et al., 2019).

2.1.2 Menopausal transition and menopause

Menopause marks the end of the female fertile period. The cause of menopause are aging-related changes that mainly occur in the ovaries, although some aging modifications can also be seen in the pituitary gland and hypothalamus (Hall, 2015). The key event behind the age-related loss of ovarian function is the

follicular depletion. At birth, the ovaries contain several hundreds of thousands of resting follicles, whereas around mid-life this number has decreased to hundreds or even less (Gougeon, 1996; Richardson et al., 1987). Serum anti-Müllerian hormone has been proposed as a good marker of ovarian reserve (van Rooij et al., 2005). Typically, a few years before the final menstrual period (FMP) or menopause, the first signs of ovarian failure begin to show, as the menstrual cycle becomes irregular and systemic sex hormone and FSH levels start to fluctuate more due to endogenous ovarian hyperstimulation (Taylor et al., 2019). This is the start of perimenopause and the menopausal transition (Harlow et al., 2012), and it is also the time when women may start to experience menopausal symptoms, such as hot flashes or sleep disturbance. The menopausal transition can be divided into early and late stages, that are differentiated by cycle length (consecutive menstrual cycle lengths extended by seven or more days/amenorrhea of more than 60 days) and blood FSH levels (slightly elevated in the early phase and frequently more than 25 IU/L in the late phase) (Harlow et al., 2012). Along with ovarian follicular depletion, the systemic E2, P4 and inhibin B levels also decrease, thereby allowing more FSH to be released from the pituitary. Loss of tight hormonal regulation manifests in anovulatory cycles and, finally, FMP and the end of the menopausal transition. After 12 months lack of menstrual bleeding, a woman is retrospectively considered to be postmenopausal and hence the perimenopausal period has also ended (Harlow et al., 2012). (Due to terminological variation, the term perimenopausal in the present data for this thesis refers only to women who are still in the menopausal transition). In postmenopause, E2 and P4 levels soon permanently stabilize at low levels and FSH and LH at high levels until older age (Hall, 2015). The stabilization period for E2 and FSH lasts about two years (Harlow et al., 2012). Although the most distinct decrease in sex hormone levels occurs in E2, women also experience a decrease in testosterone, androstenedione, sulfated dehydroepiandrosterone (DHEA-S) and E1 (C. Kim et al., 2017; Rannevik et al., 1995). After menopause, sex hormones are produced mainly by converting adrenal gland-derived DHEA (Figure 1) in peripheral tissues (Labrie et al., 2017).

Overall, the duration, timing and symptomology related to the menopausal transition and menopause are highly individual. In developed countries, menopause occurs typically between ages 48–52 (S. R. Davis et al., 2015) and in developing countries, such as those in African countries and Latin America, a few years earlier (Gold, 2011). Several factors are known to affect natural menopausal age. For example smoking, nulliparity and low socioeconomic status are associated with earlier menopause, while parity and the use of oral contraceptives have been associated with a later menopausal age (S. R. Davis et al., 2015; Gold, 2011). The effect of BMI on menopausal age has also been widely studied, and a meta-analysis of international studies indicated a positive association between menopausal age and BMI (D. Zhu et al., 2018). The mechanisms behind this are not clear, but it seems, for example, that adipose tissue-derived leptin may contribute to this by augmenting female fertility (M. Mitchell et al., 2005). Moreover, as much as 44–85% of the variance in natural

menopausal age is estimated to be heritable (Hall, 2015). Apart from natural menopause, women can experience secondary menopause due to surgical removal of ovaries or radiation- or chemotherapy.

2.1.3 Specific actions of estrogens, progestagens and gonadotropins

Estrogens. Estrogens are a group of steroid hormones comprising of E2, E1 and E3. Estrogens are produced from cholesterol via steroidogenesis mainly in the ovaries but also in smaller amounts in the adrenal gland, skeletal muscle, adipose tissue, skin and liver (Figure 1) (Gruber et al., 2002; Taylor et al., 2019). The last step in steroidogenesis is aromatization, where E2 is converted from testosterone and E1 from androstenedione. E3 is further converted from E2 and E1 mainly in the liver (Gruber et al., 2002). During fertile age, E2 is the most abundant estrogen, with high bioactivity in several tissues, whereas after menopause E1 becomes the main estrogen (Table 1). E3 becomes biologically active only during pregnancy (J. Cui et al., 2013; Kuijper et al., 2013). In blood, estrogens are reversibly bonded to sex hormone binding-globulin (SHBG) and albumin (Taylor et al., 2019).

In tissues, estrogens act through estrogen receptors (ERs), of which the genomic actions of ERα and ERβ are the most well established. After ligand binding and receptor dimerization, ERα- and ERβ-ligand-complexes move to the nucleus and act as transcription factors in estrogen-responsive elements (EREs, 5'-GGTCAnnnTGACC-3') of the DNA to alter gene expression. In addition, ligand-receptor-complexes modulate the activity of other transcription factors resulting in enhanced transcription (Klinge, 2001). Genes such as *ESR2*, *C3*, *PGR* and many others are known to include EREs (Ikeda et al., 2015; Klinge, 2001). ERα is more widely expressed in gonadal tissues (ovaries, uterus, testes and breast), while ERβ is more abundant in non-gonadal tissues, such as brain, lung and bladder (J. Cui et al., 2013). In addition to genomic actions, expression of ERs has also been found in the mitochondria, where they regulate mitochondrial gene expression (Klinge, 2017). Of the three estrogens, E2 has the greatest binding potential to ERα and -β and E3 has the lowest (Kuiper et al., 1997).

In addition to nuclear receptors, estrogens have been confirmed to act through the G-protein-coupled estrogen receptor (GPER) (Ding et al., 2019) and ER-X (Toran-Allerand et al., 2002). GPER and ER-X are localized in the cell membrane and exert their actions through activating a cytoplasmic signaling cascade by interacting with other cellular receptors and protein kinases (J. Cui et al., 2013). It has been proposed that non-nuclear ERs may contribute to the fine-tuning of the beneficial effects of estrogens in metabolic health (Gourdy et al., 2018). Estrogen signaling through nuclear interaction takes from minutes to hours to alter protein synthesis, while non-nuclear signaling induces immediate responses, such as vasodilatation of the coronary arteries (Gruber et al., 2002). In addition to natural estrogens, synthetic ligands can also bind to ERs. These include derivates of natural estrogens (e.g., ethinylestradiol and conjugated equine estradiol), synthetic inhibitors (e.g., fulvestrant) and selective ER modulators (SERMs, e.g., tamoxifen and raloxifene), which may function either as ER inhibitors or activators depending on the tissue (McDonnell, 1999).

The fact that ERs have been found in several tissues indicates that estrogens have an important role in several cell types. Besides reproduction and important roles in adipose and muscle tissue (discussed in later sections), estrogens promote the expression of secondary sex characteristics (Stingl, 2011), have neuroprotective effects (Garcia-Segura et al., 1999), improve vascular function (Sherwood et al., 2007), and help to maintain bone density (L. Zhu et al., 2016).

Progestagens. Progestagens are early intermediate products in steroidogenesis and include pregnenolone, P4, 17a-hydroxypregnenolone and 17a-hydroxyprogesterone, of which P4 is the main end product in gonadal steroidogenesis (Miller & Auchus, 2011) (Table 1). While the primary site of P4 synthesis is the ovaries, the adrenal gland also contributes to serum levels (Taylor et al., 2019). In blood, P4 binds to cortisol-binding globulin and albumin (Taraborrelli, 2015) and in the tissues acts mainly through progesterone receptors (PRs) PR-A, and -B, which, like ERα and ERβ, function as transcription factors targeting the hormone response element (HRE) of the DNA (Grimm et al., 2016). However, P4 also has a non-genomic mechanism of action through membrane-bound G-protein receptor (Taraborrelli, 2015; Taylor et al., 2019). PRs are expressed primarily in the female reproductive and central nervous system (Grimm et al., 2016), but are also found in bone, striated muscle and adipose tissue (Boivin et al., 1994; Gras et al., 2007; O'Brien et al., 1998). The primary target of progestagens is the endometrium, in order to maintain pregnancy, but major actions in the brain and nervous system, including sexual desire (Boozalis et al., 2016), neuroprotection (Soltani et al., 2017), addictive behavior (Tosun et al., 2019) and myelin production (Hussain et al., 2011) have also been reported. Estrogens are known to promote PR expression (Jacobsen & Horwitz, 2012). Like in estrogens, synthetic progestagens, sometimes referred as progestogens or progestins, such as dihydrogesterone, norethisterone, medroxyprogesterone acetate, or levonorgestrel can also bind to PRs (Hapgood et al., 2018; Vigneswaran & Hamoda, 2022).

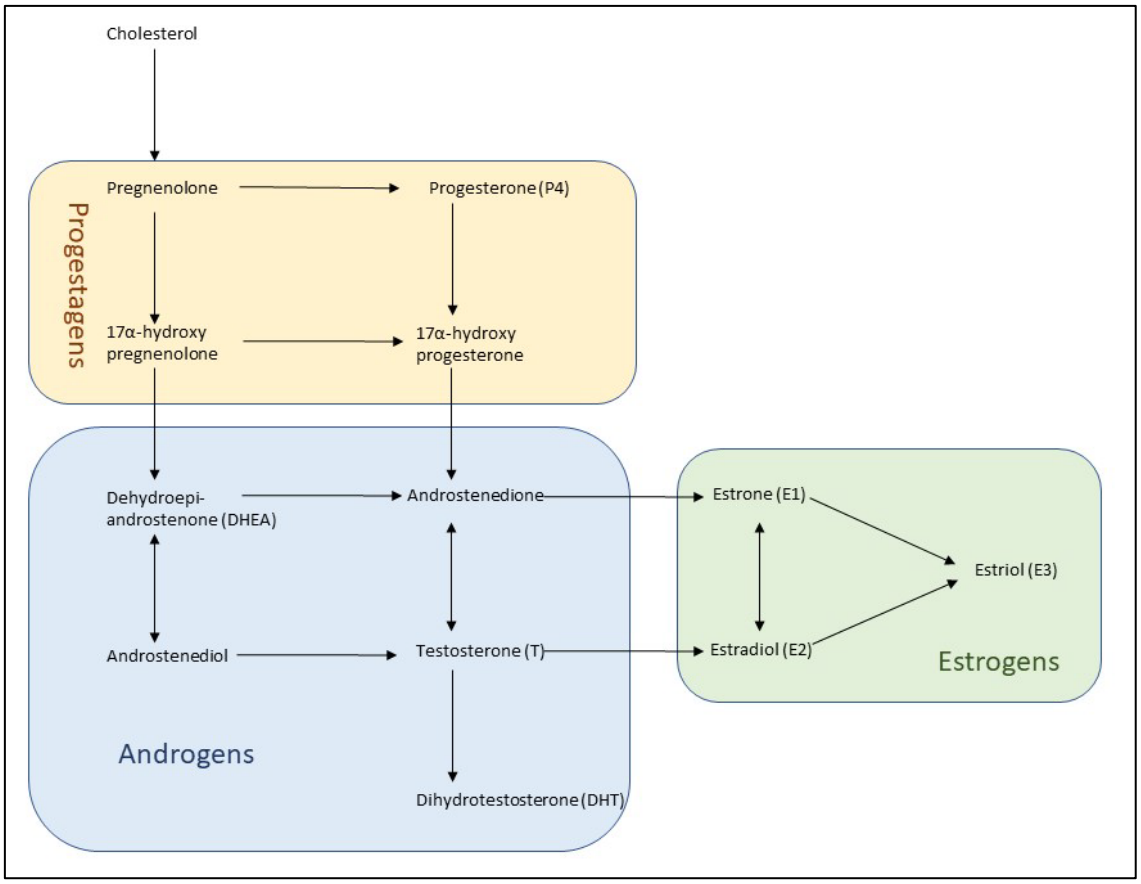

FIGURE 1 Overview of the steroidogenic pathways for synthesis of progestagens, androgens and estrogens. (Adapted from Miller & Auchus, 2011; Taylor et al., 2019).

Gonadotropins. FSH and LH belong to the group of gonadotropins, which control the sex steroid synthesis. FSH is released from the pituitary gland after hypothalamic release of GnRH and in women has a key role in stimulating the growth of pre-ovulatory follicle. FSH stimulates ovarian aromatase to increase E2 production, which in turn downregulates FSH production (Erickson & Hsueh, 1978). FSH also sensitizes the maturing follicle to LH receptors and prepares it for ovulation (Vegetti & Alagna, 2006). FSH receptors, FSHRs, are G-protein-coupled receptors located in gonadal granulosa cells and adipose tissue (X. Liu et al., 2015; Simoni et al., 1997). Attachment of the ligand initiates a signaling cascade leading to target protein phosphorylation by protein kinase A. Nuclear phosphorylation and the attachment of transcription factors to FSH-responsive elements leads to transcription of the target genes (Simoni et al., 1997). Like FSH, LH is also released from the anterior pituitary, but only after mid-cycle high levels of E2. The LH surge initiates ovulation and the development of the corpus luteum. In females, LH receptors are found in the ovaries, uterus and, for example, brain (reviewed in Dufau, 1998). After menopause, the inhibitory effect of E2 is lost due not only to significantly decreased levels, but also due to an attenuated gonadotropin response (Shaw et al., 2010), and hence the levels of FSH and LH remain high (Table 1).

TABLE 1 Sex hormone and gonadotropin levels in blood during female life.

Hormone	Prepubertal	Fertile age*	Postmeno-pausal	Method
Estradiol (nmol/L)				
[1]	<0.09	0.06–1.29	<0.04	LC-MS/MS
[2]	0.04	0.08–2.38	<0.10	LC-MS/MS
Estrone (nmol/L)				
[1]	0.04–0.12	0.06–0.74	0.03–0.15	LC-MS/MS
[2]	-	0.14–0.91	0.13–0.36	RIA
Estriol (nmol/L)				
[1]	<0.27	<0.27	<0.27	ELISA
Progesterone (nmol/L)				
[1]	<1.1	2.8–76.3	<0.60	ECLIA
[2]	-	0.2–75.9	<0.4	ECLIA
Follicle-stimulating hormone (IU/L)				
[1]	0.3–5.8	1.4–23.2	16–157	ECLIA
[2]	<1	1.7–21.5	26–135	ECLIA
Luteinizing hormone (IU/L)				
[1]	<3.1	0.7–118	5.3–65.4	ECLIA
[2]	<0.3	1–96	7.7–59	ECLIA

ECLIA, electrochemiluminescent immunoassay; ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, Liquid chromatography with tandem mass spectrometry; RIA, radioimmunoassay; * Includes all menstrual cycle phases.
[1] Reference values from a USA/International reference laboratory (Mayo Clinic, 2022), [2] Reference values from a Finnish laboratory center (Fimlab, 2022).

2.2 Body composition

Body mass consists predominantly of the three main tissue types: bone, muscle, and adipose tissue. Measurements of body composition often focus on estimating the proportions of the two largest tissue types, adipose and skeletal muscle tissue, due to their important function in health and metabolism. While body composition is highly affected by lifestyle habits, such as the level of PA and caloric intake, genetics are also known to play a part in this (Livingstone et al., 2021). Sex chromosomes and hormones also play a role in determining body composition, especially after puberty, as women tend to have higher overall and gluteofemoral adiposity, while men typically have more total and upper body muscle mass, but also higher central adiposity (Elbers et al., 1999; Heid et al., 2010; Wells et al., 2007). During aging, however, these differences in shape diminish (Kirchengast, 2010; Wells et al., 2007). Given the topic of the thesis, the following sections will only describe the characteristics of adipose and skeletal muscle tissue.

2.2.1 Adipose tissue and adipokines

Adipose tissue function. Adipose tissue can be divided into subtypes based on function or location. The two main subtypes of adipose tissue based on function are white and brown, although recently beige and pink subtypes have also been recognized (Cypess, 2022). The main function of white adipose tissue (WAT) is to store energy and insulate, but it also has an important role in metabolism and hormonal supply (Kershaw & Flier, 2004). In fat cells, adipocytes, energy is stored in lipid droplets in the form of neutral lipids. Lipogenesis is controlled at the nutritional level by circulating fatty acids and glucose, and in hormonal levels by insulin (activating), growth hormone and, for example, leptin (inhibiting) (Kersten, 2001). At the transcriptional level, nuclear transcription factor PPARγ has an important role in lipogenesis induction. PPARγ is activated by free fatty acids and increases the transcription of, for example, *IRS-2* (U. Smith et al., 2001), *GLUT4* (Z. Wu et al., 1998) and fatty acid transporter genes (Motojima et al., 1998), and inhibits the expression of inflammatory cytokines, such as TNF-α (Jiang et al., 1998). Adipocyte lipolysis, which produces fatty acids and glycerol for bloodstream mobilization, is controlled by intracellular enzymes called lipases, of which the most well known are hormone-sensitive lipase, triacylglycerol hydrolase, adipose triglyceride lipase and monoglyceride lipase (Duncan et al., 2007). Another lipase, lipoprotein lipase (LPL), which resides in the adipose tissue capillaries and is responsible for breakdown of chylomicrons and very-low density lipoprotein particles into free fatty acids, also contributes to lipogenesis (Eckel, 1989). Of the other adipose tissue subtypes, brown adipose tissue is probably the most well established. The main role of brown adipose tissue is to produce heat through "wasting" of the electrochemical gradient in the mitochondria and to take part in systemic triglyceride clearance and the regulation of total body metabolism in mammals (Chait & den Hartigh, 2020; Wibmer et al., 2021). Besides adipocytes, adipose tissue is also composed of other cell types, including fibroblasts, nerve cells, immune cells and vascular cells (Cypess, 2022).

Adipose tissue depots. Subcutaneous WAT is the largest adipose tissue depot in lean healthy individuals and the main location of long-term energy storage during fasting and endurance exercise (Chait & den Hartigh, 2020). When the storage capacity of subcutaneous depot is exceeded, fat begins to accumulate in ectopic regions. Ectopic WAT can be found in various regions, including muscle, liver, heart, blood vessels and pancreas (Chait & den Hartigh, 2020), and several of them have been associated with increased risk for cardiovascular disease and metabolic conditions (K. A. Britton & Fox, 2011). One significant ectopic depot is the visceral adipose tissue (VAT) surrounding the intestines. VAT is metabolically highly active and responsible for the fast supply of systemic free fatty acids (Richelsen et al., 1991; Tchernof & Després, 2013). The reasons for this include higher sensitivity to catecholamine-induced lipolysis and lower sensitivity to insulin-induced inhibition of lipolysis compared to WAT (Meek et al., 1999; Richelsen et al., 1991). VAT is also a highly vascular and innervated

tissue, that contains a large number of inflammatory and immune cells in addition to adipocytes (Ibrahim, 2010). In a variety of BMI values, the adipocytes in VAT are, on average, smaller than the subcutaneous adipocytes. However, in obesity, the number of large adipocytes is increased especially in VAT (Reynisdottir et al., 1997; Verboven et al., 2018).

During aging or due to disuse, ectopic fat may also begin to accumulate in skeletal muscle. Intermuscular adiposity includes lipid accumulation under the muscle fascia both inside muscle cells (intramyocellular) and around muscle fibers (intramuscular/intermuscular) (Addison et al., 2014). In muscle, excess lipid accumulation has been associated with total body adiposity (Goodpaster, Theriault, et al., 2000; Ryan & Nicklas, 1999), functional decrements (Straight et al., 2019), and, in non-athletes, also with lowered insulin sensitivity and impaired metabolic health (Dube et al., 2011; Goodpaster et al., 2001) due to lipotoxicity. In muscle, an increase in triglyceride concentration interferes with insulin-stimulated PI3K activation and, further, with glucose transporter localization and glucose uptake (Roden & Shulman, 2019).

In women, estrogens specifically impact adipose tissue location and accumulation. The distribution of female-type adipose tissue (gynoid obesity) is influenced by estrogenic actions that suppress adipocyte lipolysis and increase preadipocyte proliferation in SAT (Anderson et al., 2001; Gavin et al., 2013; Pedersen et al., 2004), and increase lipolysis and decrease the expression of lipogenic genes in VAT (Al-Qahtani et al., 2017; Bryzgalova et al., 2008; Pedersen et al., 2004). Moreover, the high level of LPL activity in women's gynoidal adipocytes further contributes to regional adipose tissue accumulation (Pouliot et al., 1991). Among the factors contributing to the tissue specific responses of estrogens are ERs. In females, different adipose tissue depots express ERs at varying densities (Ahmed et al., 2022; Blouin et al., 2009), and recent evidence suggests that the ratio of ERα and -β in adipose tissue might be important in obesity (Shin et al., 2007). ERα activation has been proposed to promote anti-obesity effects (K. E. Davis et al., 2013), whereas signaling through ERβ has been linked to impairment of metabolic health (Foryst-Ludwig et al., 2008). In women, aging and postmenopausal status have been associated with decreased ERα in subcutaneous tissue, thus potentially contributing to the enhanced signaling through ERβ (Ahmed et al., 2022; Y.-M. Park et al., 2017).

Adipokines. The metabolic actions of adipose tissue includes the release of several hormones, cytokines and growth factors. The secretion pattern of these molecules differs across adipose tissue depots, leading to variation in systemic levels depending on body shape (Chait & den Hartigh, 2020). Collectively, all the secreted molecules originating from adipose tissue can be referred as adipokines (Ouchi et al., 2011). Adipokines are known to have roles in, e.g., regulating appetite and fertility, affecting insulin sensitivity, and promoting an either inflammatory or anti-inflammatory environment (Fasshauer & Blüher, 2015; M. Mitchell et al., 2005). To date, several adipokines have been identified, including adiponectin, omentin, resistin, leptin, IL-6, TNF-α and FGF-21 (Chait & den Hartigh, 2020; Ouchi et al., 2011). Among the most studied of these are

adiponectin, leptin and resistin. As they were also investigated in this dissertation research, the following paragraphs will focus on their functions.

Adiponectin is mainly produced by adipocytes and plays a role in insulin-sensitizing and increased fatty acid oxidation in several target tissues, such as skeletal muscle (through PPARα), pancreas, liver and immune cells. Hence, adiponectin is often considered to be an anti-inflammatory adipokine (Chait & den Hartigh, 2020). Moreover, adiponectin is also known to increase adipocyte hyperplasia, the less metabolically harmful type of obesity (Asterholm & Scherer, 2010). Adiponectin expression is inversely related to total adiposity and BMI, but women overall tend to have higher adiponectin levels than men (Kern et al., 2003). Women may have higher adiponectin levels because subcutaneous adipose tissue, and especially gluteal fat is a greater source of adiponectin compared to VAT (F. M. Fisher et al., 2002; Lihn et al., 2004; Samaras et al., 2010). In fact, increases in VAT have been associated with decreases in adiponectin levels (Ryo et al., 2004). The mechanism through which central obesity decreases adiponectin expression is thought to comprise adipocyte dysfunction, an increase in proinflammatory cytokines and the inhibitory role of insulin (Möhlig et al., 2002; Ouchi et al., 2011). In postmenopausal healthy women, adiponectin has also been positively associated with lean mass (Banh et al., 2019).

Leptin, which is also predominantly secreted from adipocytes, is a hormone responsible for signaling full energy reservoirs, enhancing fatty acid oxidation, and controlling appetite and fertility at the central nervous level (Mantzoros et al., 2011). Leptin levels correlate positively with total fat mass (FM) (Rosenbaum et al., 1996; J. Zhang et al., 2015) and leptin is more abundantly expressed in subcutaneous adipose tissue than in VAT (Samaras et al., 2010). E2 levels also associate positively with leptin levels (Chan et al., 2002; Konukoglu et al., 2000). Presumably, it is for these reasons that women usually have higher leptin levels than men (Rosenbaum et al., 1996; Yannakoulia et al., 2003). Unlike adiponectin, leptin has been negatively associated with muscle mass in postmenopausal women (Banh et al., 2019).

Resistin is one of the more recently found adipokines secreted by the adipose tissue macrophages (Patel et al., 2003). Resistin has a role in preventing insulin actions and enforcing an inflammatory environment, and is thus an inflammatory adipokine (Benomar et al., 2013; Reilly et al., 2005). The mechanisms suggested for this are the increment in lipolysis from adipocytes and actions in preventing adiponectin secretion (N. Chen et al., 2014). Resistin levels have been associated with increasing adiposity, insulin resistance, and determinants of metabolic syndrome (Norata et al., 2007; Su et al., 2019). However, the precise role of resistin in metabolic health remains under investigation (Chait & den Hartigh, 2020).

In addition to secreting adipokines, adipose tissue is also a source of estrogens through steroidogenesis from precursor hormones (Figure 1). This characteristic becomes evident after menopause specifically in E1 production (Blouin et al., 2009; Szymczak et al., 1998) and also translates to serum levels (Oh et al., 2017; Szymczak et al., 1998). SAT is the main E1 producer, while VAT may

contribute to increased E2 levels especially in waist obesity (Hetemäki et al., 2017). Studies have also reported a positive correlation between body fatness and BMI and E2 levels in both pre- and postmenopausal women (Oh et al., 2017; Tin Tin et al., 2020). However, the same direct correlation has not been repeated in all populations (Colleluori et al., 2018).

Obesity and metabolic health. When systemic energy intake exceeds energy consumption, adipose tissue expands, causing obesity. Adipocytes can either grow in size (hypertrophy) or become more abundant (hyperplasia). These alternatives have different metabolic consequences (Cypess, 2022). Hypertrophic adipose tissue is considered to be metabolically more harmful, as it induces an inflammatory response in the tissue by recruiting immune cells and releasing pro-inflammatory cytokines, such as IL-6 and IL-8 (Skurk et al., 2007). One of the causes for this is suggested to be adipose tissue hypoxia (Y. S. Lee et al., 2014). Large adipocytes have also been found to release more leptin, but not adiponectin (Skurk et al., 2007). Hyperplasic adipose tissue, in turn, releases fewer pro-inflammatory cytokines, but more adiponectin, while also remaining more insulin sensitive (reviewed in Stenkula & Erlanson-Albertsson, 2018).

Recent studies have revealed the importance of investigating obesity in greater detail, as phenotypes such as metabolically healthy and metabolically abnormal obesity have been identified (Blüher, 2020; Peppa et al., 2013). Metabolically healthy obese individuals have excess FM, but are typically characterized by a smaller trunk-to-leg FM ratio, less fluctuation in body weight and lower risk factor levels for developing the metabolic conditions traditionally associated with obesity (Peppa et al., 2013). The factors contributing to the metabolically healthy obese phenotype have been suggested to be less sedentary behavior and beneficial dietary choices, leading to a higher cardiorespiratory-fitness level and skeletal muscle insulin sensitivity (Camhi et al., 2015; Jones et al., 2021). On the other hand, the metabolically obese non-obese phenotype may be expressed even in individuals with relatively normal BMI, but who, due to elevated blood pressure, central obesity, and physical inactivity are at increased risk for obesity related conditions (Ruderman et al., 1998).

Different adipose tissue depots contribute to metabolic health in different ways. Gluteofemoral SAT has been associated with better metabolic health and insulin sensitivity (reviewed in Manolopoulos et al., 2010), while excess adipose tissue in the abdominal area has been associated with increased risk for cardiometabolic incidences and type II diabetes in both men and women in several populations (G.-C. Chen et al., 2019; Fox et al., 2007; H. Kwon et al., 2017; M. Zhang et al., 2015). The ratio between abdominal and gynoid or total FM seems to be especially important (Okosun et al., 2015; Peppa et al., 2013; Wiklund et al., 2008). The favourable metabolic effects of gynoid SAT are proposed to be linked to anti-inflammatory adipokine release, although it may also directly buffer against abdominal adipose tissue accumulation (Hernandez et al., 2011). In women, gluteofemoral and gynoid fat have been found to positively associate with bone mass density after menopause (Aedo et al., 2020; Namwongprom et

al., 2019), while excess total and trunk fat are associated with increased breast cancer risk also after adjusting for BMI (Arthur et al., 2020).

As stated earlier, obesity is one of the major contributors to metabolic health. Metabolic health or the risk for cardiometabolic conditions can be assessed using the metabolic syndrome criteria pertaining to measurements of the blood lipid and glucose profiles, waist adiposity and blood pressure (Grundy et al., 2004), or, for example, using the plasma adipokine profile (Mauriège et al., 2020). Worsening metabolic health leads to metabolic syndrome, which is a collection of inflammatory conditions and increases the risk for type II diabetes and cardiovascular disease (P. L. Huang, 2009). For example, a woman with the co-occurrence of at least three of the risk factors (waist circumference of 88 cm or more, blood pressure of ≥130/≥85 mmHg, triglycerides of ≥1.7mmol/l, high-density cholesterol levels below 1.29 mmol/l or fasting glucose ≥5.6 mmol/l) is considered to have metabolic syndrome (Grundy et al., 2004). Increased waist adiposity, due to the release of inflammatory cytokines and free fatty acids from VAT, seems to be a particularly important contributory factor (Wajchenberg, 2000). The systemic adipokine profile has also been proposed to be useful in estimating metabolic health. For example, in postmenopausal women, a higher ratio between blood adiponectin and leptin levels has been suggested as a valid marker for better metabolic health irrespective of the presence of obesity (Mauriège et al., 2020). No reference values for adiponectin or leptin levels for middle-aged women exist, but earlier studies suggest that an adiponectin-leptin ratio over one can be considered normal, while a ratio below 0.5 indicates a severe risk for cardiometabolic events (Frühbeck et al., 2018). Metabolic risk factors can be effectively managed by reducing obesity, especially in the waist area, increased PA and dietary modifications (Grundy et al., 2004).

2.2.2 Skeletal muscle tissue

In humans, skeletal muscle accounts for approximately 40% of total body weight. Moreover, alongside its role in movement and balance, it participates in total body energy metabolism and heat production and functions as an amino acid reserve. Muscle mass is dependent not only on the number of muscle cells (myofibers), but also on the balance of muscle protein synthesis and rate of degradation, which are responsive to hormonal and nutritional status, exercise and, for example, injuries (Sartori et al., 2021). After birth, human myofibers do not divide, and hence the expansion of muscle mass is achieved through hypertrophy and the inclusion of new myofibers and myonuclei from muscle stem cells. Muscle stem cells, or satellite cells, reside between the sarcolemma (muscle cell membrane) and basal lamina (lowest layer of the endomysium), and are responsible for the regeneration of muscle tissue (Mauro, 1961; Snijders et al., 2015). After stimulus, quiescent satellite cells are activated and begin to proliferate. Depending on the need for regeneration, proliferated satellite cells may either differentiate into myoblasts, myotubes and further form a new myofiber or fuse as new myonuclei to an existing myofiber (Petrella et al., 2008; Webster et al., 2016). A fraction of the proliferated satellite cell population returns

to quiescence. In aged muscle, the muscle satellite cell pool has been found to be reduced (Shefer et al., 2006) and the regeneration commitment impaired, due to the lack of proliferation of satellite cells and their differentiation into non-myogenic lineage (Asakura et al., 2001; Conboy et al., 2003).

Individual myofibers are multinucleated cells that are surrounded by sarcolemma and composed of numerous myofibrils (Figure 2). The main components of myofibrils are the contractive proteins myosin in the thick filament, actin, troponin and tropomyosin in the thin filament, and titin as the elastic filament. After receiving the signal to contract, the thin and thick filaments of each contractile unit, i.e., sarcomere, slide over each other using adenosine triphosphate (ATP) as energy and causing the sarcomeres and eventually the whole myofiber to shorten (Frontera & Ochala, 2015). Each myofiber is also enveloped by a thin layer of connective tissue called the endomysium. A group of myofibers form larger units, muscle bundles, which are further surrounded by the perimysium, while on the whole muscle level, the individual muscles are surrounded by the epimysium. At the limb or region level, muscle groups are surrounded by a thicker connective tissue layer called the fascia, which separates the muscle tissue from subcutaneous adipose tissue. The role of these different connective tissue layers is to guide muscle fiber development, accommodate the tissue with nerves and capillaries in the tissue and to transmit contracting forces (Purslow, 2020).

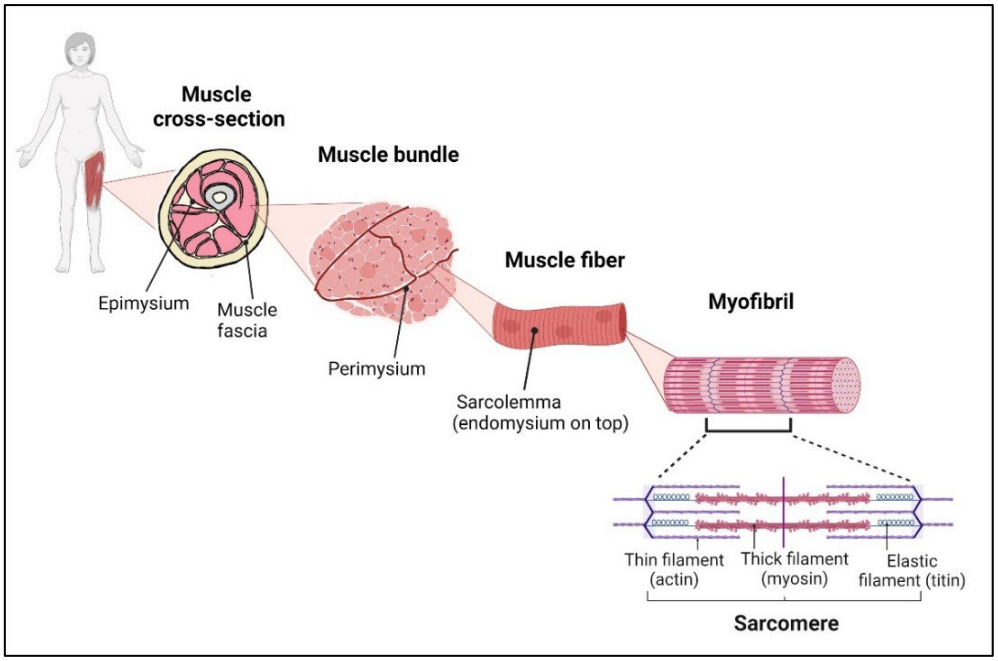

FIGURE 2 Basic structure and contracting unit of the muscles. Figure created with Biorender.com.

Muscle fibers can be classified based on their myosin heavy chain (MHC) composition, ATPase activity or biochemical properties. Sarcomeric myosins comprise of two MHC molecules, each with a globular head, neck region with

two myosin light chains attached, and a tail, which intertwine with each other to form a helical coil. The head component includes both actin- and ATP-binding sites (Schiaffino & Reggiani, 2011). In adult human muscle, according to the composition of the MHC isoform, three types of fibers can be differentiated: I, IIA and IIX. Type I fibers are mostly composed of MHCI and can maintain movement for long periods owing to their efficient oxidative metabolism, for which high vascularity supplies oxygen. Because of the low ATPase activity related to MHCI, type I fibers are also called slow fibers. Of all three fiber types, slow fibers have the largest cross-sectional area (Gouzi et al., 2013) and typically contain the largest amount of lipid droplets (J. He et al., 2001). Type IIA fibers, mainly composed of MHCIIA, can contract more rapidly due to more active ATPase, but as they are simultaneously more glycolytic compared to type I fibers, they are unable to maintain contraction for extended periods. Lastly, type IIX fibers are the fastest and most glycolytic fibers, but also rarest in human muscle tissue, accounting for approximately only 10% of the fiber population (Vikne et al., 2020). Subgrouping by MHC is based on the expression of the main isoform, since skeletal muscles fibers are rarely "pure", but rather mixtures of more than one myosin heavy chain (Pette & Staron, 2000).

In addition, muscle fiber type composition, and overall metabolism, can be estimated from the fibers oxidative and glycolytic properties. In oxidative metabolism, energy is produced through oxidative phosphorylation from carbohydrates (muscle glycogen and extracellular glucose) and lipids. Oxidative metabolism requires oxygen, and hence highly vascular and mitochondria-rich type I fibers typically have high oxidative capacity (Schiaffino & Reggiani, 2011). Aerobic energy production yields ATP efficiently and therefore renders fibers more resistant to fatigue and suitable, for example, for maintaining posture. Oxidative fibers are also rich in lipid droplets, as lipids provide a stable source of energy, especially for prolonged exercise bouts (Gemmink et al., 2017). Studies investigating the properties of muscle fiber in relation to the whole-body metabolism have found, that lower oxidative metabolism and high muscle fiber lipid content are associated with obesity and lowered insulin sensitivity (G. Fisher et al., 2017; J. He et al., 2001; Sucharita et al., 2019; Tanner et al., 2002). However, the overall oxidative capacity of a muscle can be increased with PA (J. He et al., 2004), although in athletic muscle, increased oxidative capacity is accompanied with increased lipid content, a phenomenon known as the "athletes' paradox" (Goodpaster et al., 2001). The other main route to energy production is through glycolysis. Glycolytic fibers (IIA and IIX) primarily produce energy, independent of oxygen, from phosphocreatine and muscle glycogen, which supplies the cell with less ATPs per cycle than oxidative metabolism (Westerblad et al., 2010). Although energy is produced rapidly, the fibers experience fatigue relatively soon. Increased glycolytic capacity in muscles has been associated with type II diabetes and decreased muscle density (Oberbach et al., 2006; J. A. Simoneau et al., 1995; J.-A. Simoneau & Kelley, 1997). Conversely, exercise, especially resistance exercise, has been shown to increase the total muscle glycolysis rate (Nitzsche et al., 2020). Additionally, the increase in the number of

fast fibers has been associated with a significant decline in FM in mice (Izumiya et al., 2008).

At the tissue level, whole human skeletal muscles are mixtures of different fiber types. For example, *m. vastus lateralis* comprises approximately equal proportions of type I and II (Gouzi et al., 2013; Lexell et al., 1986), while some other muscles tend to contain slightly more of the oxidative type and others slightly more of the glycolytic type (Elder et al., 1982). The composition of fiber types in a muscle is thought to be determined mainly by genetics, but several studies propose that aging, physical activity and inactivity, and hormones might also affect fiber composition (reviewed in Haizlip et al., 2015; Pette & Staron, 2001). Exercise, especially of the endurance type, and aging seem to increase the proportion of slower, more oxidative fibers (W.-S. Lee et al., 2006; Moreillon et al., 2019), while inactivity shifts the balance toward the faster forms (D'Antona et al., 2003). Sex has also been proposed to affect fiber distribution, as women have been observed to have more type I fibers compared to men (Staron et al., 2000; Welle et al., 2008). In addition to fiber type, also the single fiber cross-sectional area is affected by the same factors. Resistance exercise, young age and male sex have been previously associated with larger fiber size, especially of type II fibers (Andersen & Aagaard, 2000; Barnouin et al., 2017; Lexell et al., 1988; Lexell & Taylor, 1991; Martel et al., 2006). Moreover, sex also seems to affect satellite cell pool, as compared to men, women have more satellite cells around their type I fibers, but a lower total number of satellite cells (Horwath, Moberg, et al., 2021).

2.2.3 Measuring body composition

Depending on the required accuracy, body composition measurements can be conducted using several methods. One option is to use multicomponent models, which utilize several assumptions and theoretical equations (Heymsfield et al., 2015). In the simplest version, a 2-component model (2C), body mass is divided into FM and fat-free mass (FFM). The assessment can be done with bioelectrical impedance analysis (BIA) or densitometric analyses, such as hydrostatic underwater weighting (S. Y. Lee & Gallagher, 2008). As the 2C-model analysis includes build assumptions about body water content and FFM density, it is not ideal for all populations, such as children or non-healthy participants (S. Y. Lee & Gallagher, 2008). The more detailed model is the three-component model (3C), where body mass is divided into FM, water, and fat-free dry mass (including proteins and minerals). 3C-measurement requires information on total body mass, body water (e.g., BIA) and body volume (densitometry), and therefore allows a more accurate separation of the muscle hydration fraction (Wells & Fewtrell, 2006). Dual-energy X-ray absorptiometry (DXA) measurement is also sometimes considered as a 3C model, as it divides total mass into fat, lean and bone mass (Kuriyan, 2018; Toomey et al., 2015). The four-component model (4C), which is considered to be the gold standard for body composition measurements (Gallagher et al., 2013), divides body mass into FM, water, mineral content and residuals (including protein, glycogen and soft tissue mineral) (Fosbøl & Zerahn, 2015). A 4C-measurement can be obtained by combining data from, for example,

densitometry (FM), BIA or isotope dilution (water) and DXA (mineral). The residual mass is then calculated by subtracting other compartments from total body mass (Toomey et al., 2015). However, a recent study suggests that, in healthy adults, results comparable to those obtained with the 4C model could also be obtained by simply using just DXA in combination with BIA (Ng et al., 2018). In addition to the theoretical compartment models, body composition can be assessed using imaging technologies, such as DXA, quantitative computed tomography (qCT) and magnetic resonance imaging (MRI), and predictive and anthropometric measures, such as skin-fold measurement and waist and hip circumference. In the following sections focus is confined to the body composition measurement methods used in this thesis.

Dual energy X-ray absorptiometry. Although the 4C-model is considered as the reference method for in-vivo assessment, it cannot separate differences in regional lean and fat masses. For this reason as well as easier accessibility and high reproducibility (Cordero-MacIntyre et al., 2002; Lohman et al., 2009), DXA has gained popularity in measuring body composition and is often regarded as the gold standard for muscle and bone mass measurement (Buckinx et al., 2018; Roux & Briot, 2017). DXA uses X-rays of two wavelengths that are projected onto body and detected from the opposite site. The attenuation in energy is then used to calculate bone mineral content and soft tissue density (Figure 3). The amount of lean mass and FM is further estimated from soft tissue using predetermined equations (Bazzocchi et al., 2016). The terminology used in reporting DXA results should be borne in mind here: *fat mass (FM)* includes only fat mass, *fat-free mass (FFM)* consists of non-adipose soft tissue and bone mass, and *lean body mass (LM/LBM)* consists of non-adipose soft tissue mass including muscle, skin, organs and connective tissues (Buckinx et al., 2018). However, the DXA lean mass measurement is frequently used as a surrogate variable for muscle mass and its correlation with especially appendicular muscle mass is high (J. Kim et al., 2002; Shih et al., 2000). In fact, it has been estimated that appendicular lean mass (ALM) forms 75% of total muscle mass and thus could be used as the most reliable proxy for total muscle mass (Buckinx et al., 2018). Although DXA uses ionizing radiation, the single dose is below or equivalent to background levels allowing for relatively safe measurements in longitudinal studies (Bazzocchi et al., 2016). The disadvantages of DXA, apart from its two-dimensional image and limited resolution, include its inability to separate, for example, leg intermuscular fat from the leg subcutaneous adipose tissue (Beaudart et al., 2016), which have distinct metabolic consequences. Moreover, SAT and VAT cannot be separated using DXA, although calculational algorithms have been developed for the purposes of estimation (Kelly et al., 2010). While normal variation in hydration status has no or only a minor effect on body composition measurements with DXA, in obese participants, tissue thickness could affect the estimation of body fat percentage (Bazzocchi et al., 2016; LaForgia et al., 2009).

Several studies have compared the correlations between muscle and adipose tissue quantities in DXA and then further compared these to MRI or the 4C-model as the gold standards. For adiposity at the total body level, DXA has

been shown to correlate well with other methods in adults. A small underestimation of the percentage of body fat obtained by DXA (2–5%), but otherwise high overall correlations (r ~ 0.9) of DXA measurements with those found using 4C-models have been reported (Bergsma–Kadijk et al., 1996; Gallagher et al., 2000; Prior et al., 1997; van der Ploeg et al., 2003). In the case of MRI, DXA estimates of total FM and VAT estimation correlated strongly (r ≥ 0.9) with those obtained using MRI in middle-aged men and women (Borga et al., 2018; Maskarinec et al., 2022; Mohammad et al., 2017). For lean mass, MRI-measured total body skeletal muscle mass correlated highly (r = 0.94) with DXA-derived LBM in postmenopausal older women (Z. Chen et al., 2007). Additionally, MRI and DXA scans of thigh muscularity have also been reported to correlate strongly (r^2 ~ 0.8–0.9) in both young and old populations (Cameron et al., 2020; Z. Chen et al., 2007; Maden-Wilkinson et al., 2013), although some studies have also reported a decrease in the accuracy of DXA when measuring longitudinal changes (Delmonico et al., 2008; Tavoian et al., 2019).

Quantitative computed tomography. Besides 2D imaging performed with DXA, especially regional body composition can also be measured with sectional imaging methods such as qCT. qCT utilizes information from X-ray attenuation on a 360° platform, creating slice images and, based on density measures, enabling high-definition separation of bone, muscle, adipose tissue and intestines (Figure 3) (Fosbøl & Zerahn, 2015). Section densities are calculated in Hounsfield units (HU) using calibrations phantoms, where water is assigned a HU value of 0 and air a HU value of ~ -1000. For adipose tissue, the HU value is below zero (-190 to -30) and for muscle it is between 30–100 (Fosbøl & Zerahn, 2015). Cross-sectional areas can be calculated from the images for each tissue type. In addition to MRI, CT, owing to its excellent accuracy and reproducibility, is considered as the reference standard for the diagnosis of, for example, sarcopenia (Y.-H. Lee et al., 2017; Mitsiopoulos et al., 1998). Although highly accurate, CT is not easily usable with large populations, owing to its delivery of a moderate radiation dose, limitations on patient size and the high cost of the equipment (Beaudart et al., 2016).

For CT, high correlations with MRI adipose tissue measurements have been reported in the chest (r ~ 0.90) (Faron et al., 2020), waist (r ~ 0.8-0.9) (Zaffina et al., 2022) and leg areas (r ~ 0.8) (Niklasson et al., 2022). Additionally, CT-measured thigh muscle density and intermuscular fat has correlated positively with BMI- and DXA-measured total body adiposity in adults and in older female populations (Goodpaster, Kelley, et al., 2000; Straight et al., 2019). High correlations between skeletal muscle CT and MRI (r ~ 0.8–0.9) scans have also been reported in the adult population (Faron et al., 2020; Niklasson et al., 2022; Zaffina et al., 2022). Thus, based on the literature, both DXA and CT produce measures of fat and lean mass that correlate highly with those produced using the gold standard methods of MRI and the 4C-model.

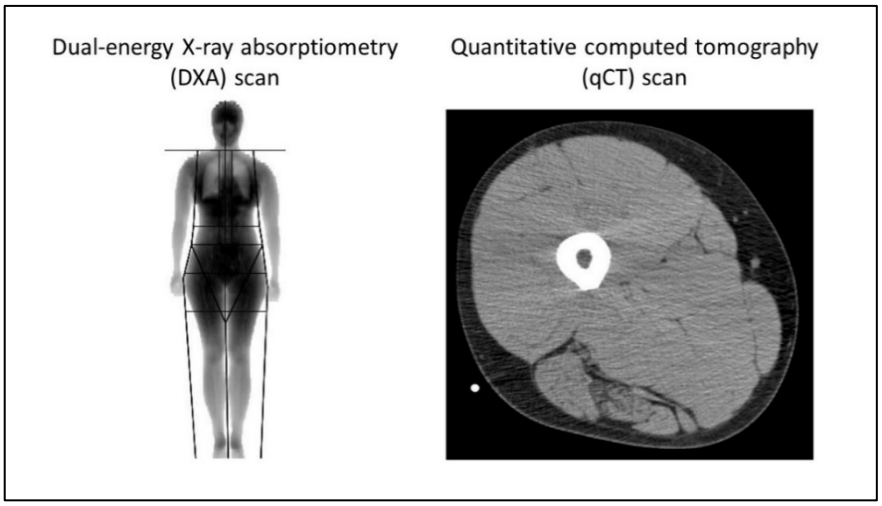

FIGURE 3 Representative images of total body DXA and mid-thigh qCT scans used in the analysis of body composition.

Anthropometric measures of adiposity. In addition to laboratory measurements, adiposity and body shape can be estimated using anthropometric measurements. Measures such as waist and hip circumference (WC and HC), and waist-to-hip-ratio (WHR) are easily, economically and safely measured at home or in a nurse's office and thus possess practical properties for large-scale population studies, but also for personal follow-up.

WC is commonly used as a surrogate measure of abdominal adiposity. The anatomical landmarks guiding WC measurement vary to some extent between the studies, as WC can be measured either at the 1) approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest (recommended), 2) top of the iliac crest, 3) level of the umbilicus or navel, or 4) at the point of the minimal waist (World Health Organization, 2011). In several populations, a WC of 88 centimeters or over in women has been widely associated with an increased risk for metabolic syndrome and cardiovascular diseases (World Health Organization, 2011), as it has been associated positively with total FM in addition to abdominal SAT and VAT ($r \sim 0.7–0.9$), although the correlation varies across populations (Camhi et al., 2011; Fox et al., 2007). The measurement site also affects the correlation coefficient, as two recent studies have found highest correlations with the "minimal waist" measurement site (Pinho et al., 2018; Seimon et al., 2018). In a longitudinal study of more than 700 middle-aged women, a 10 cm increase in WC was associated with a doubled risk of type II diabetes (Snijder et al., 2003). The limitation of WC measurement is, that it does not separate abdominal SAT from VAT.

Measurement of the WHR (WC divided by HC) is used to describe the fat accumulation pattern in regards to the mid-region and gluteal areas. As with WC, HC can also be measured from slightly differing locations. World Health Organization expert panel lists only "the widest portion of the buttocks" (World Health Organization, 2011), but studies report measurements also from the "widest level over the greater trochanters" (Snijder et al., 2003) and "maximum

excursion of the buttocks" (Greendale et al., 2021). A WHR of ≥ 0.85 has been associated with a higher risk of unhealthy metabolic conditions in women (World Health Organization, 2011). A cross-sectional study of mostly middle-aged participants similarly found a WHR cut-off limit of 0.9 in men and 0.88 in women to predict metabolic syndrome (Bener et al., 2013). In a longitudinal study, an increase in WHR of 0.07 units was associated with a 2.15 odds ratio of developing type II diabetes in middle-aged women (Snijder et al., 2003). In addition to metabolic consequences, central obesity measured with WC and WHR has been associated with increased breast cancer risk in both pre- and postmenopausal women (Houghton et al., 2021).

2.3 Skeletal muscle RNA signaling and body composition

2.3.1 Protein-coding and non-coding RNAs

In mammalian cells, DNA encodes for all the proteins needed for cellular growth, metabolism, development and controlled death. In addition to serving as an instruction for protein-coding genes, DNA also functions as a template for non-protein-coding RNAs (ncRNAs), which regulate DNA transcription and protein function.

Messenger RNA (mRNA) is the RNA species needed to produce proteins. mRNA, like all the other RNA species, is produced from template DNA during transcription. Here, the target DNA sequence is copied into single-stranded mRNA by RNA polymerase. The resulting pre-mRNA is released, spliced or otherwise modified, and transferred to the cytoplasm as mature mRNA. In the cytoplasm it attaches to ribosomes and can be translated into a functional protein or part of one. Several protein coding genes can produce multiple slightly variable mRNA transcripts and the mRNAs can also be modified before translation. Thus from the around 20 000 human protein-coding genes currently known (Salzberg, 2018), a considerable number of different mRNA transcripts and thereby different protein variants can be produced for the needs of different cell types.

In addition to the protein-coding RNA molecules, a variety of ncRNAs act as regulating factors in gene expression at different levels. Based on their length, ncRNAs can be divided into small and long ncRNAs. One class of widely studied small RNAs are microRNAs (miRNAs), which are ~22 nucleotides long RNA molecules coded from the non-protein coding regions of the genome. miRNAs are produced from miRNA genes and go through specific pri- and pre-miRNA processing before reaching their single-stranded mature state (O'Brien et al., 2018). Some miRNAs, such as muscle myomiRNAs and brain specific miRNAs, are known to be expressed in a highly tissue-specific manner (Lagos-Quintana et al., 2002; McCarthy, 2011). Several miRNAs are also observed in body fluids and suggested to have a role in the inter-tissue signaling (Boon & Vickers, 2013). Currently, ~ 2 600 recognized mature miRNAs have been found in humans

(miRBase v22.1)(Kozomara et al., 2019). miRNAs are most known for their function in downregulating gene expression. Interaction between target mRNAs and miRNAs occurs through miRNA binding first into a RNA-induced silencing complex (RISC), a multiprotein complex including, for example, nucleases. After miRNA loading, the complex attaches to target mRNA mainly through 3' UTR (untranslated region) binding and, based on the level of miRNA-mRNA complementary, mRNA translation is either repressed or the target mRNA is cleaved (Bartel, 2004). However, miRNAs have also been observed to increase gene expression through binding to a gene promoter or mRNA 5' UTR (Dharap et al., 2013; Ørom et al., 2008).

Another large class of ncRNAs are the long non-coding RNAs (lncRNAs). These are defined as RNA molecules of more than 200 nucleotides in length which do not have protein-coding potential (Fernandes et al., 2019). lncRNAs are estimated to be encoded by ~15 000 genes (Salzberg, 2018), either from the intronic area or partially overlapping with the protein-coding exones. lncRNAs share many similar properties with mRNAs, as they also consist of introns and exones, are spliced, and may contain a polyA-tail. lncRNAs may localize either in the nucleus or cytoplasm and can both activate or repress target gene transcription or regulate protein translation post-transcriptionally (Statello et al., 2021). Regulation mechanisms include the opening and formation of heterochromatin, recruiting and decoying DNA regulation proteins, splicing regulation, miRNA binding and mRNA stabilization and degradation. Overall, although there is debate on the amount of functional lncRNAs, there is no question that lncRNAs are crucial regulators of the transcriptome.

The RNA composition of a sample is comprehensively determined using RNA sequencing. In short, extracted RNA molecules are first fragmented, reverse-transcribed to complementary DNA molecules, tagged with identifiers and amplified with polymerase chain reaction. The samples are further loaded into a sequencing system, single-stranded DNA fragments are attached to an immobile media as a template and amplified once again. After cluster formation, using repetitive cycles of adding free nucleotides and detecting the attachment signal, the sequences of the original sample molecules are determined (Illumina, 2022; Rizzo & Buck, 2012). Using bioinformatics, the raw sequencing reads are further aligned and compared to the reference genome. Due to the previously added identifiers, the number of RNA molecules or fragments from each gene can be counted per sample and this information used for, e.g., differential expression (DE) analysis (Pereira et al., 2020).

2.3.2 RNA signaling and contribution to body composition and metabolic health

As proteins are the basis of all cellular functions, mRNA synthesis and its regulation are also key determinants of total body metabolic health and body composition. Due to the extensive nature of the field, this and the following sections will mainly focus on describing the current knowledge on muscle tissue, although blood and adipose tissue are to some extent also covered.

For skeletal muscle tissue, myogenic transcription factors (e.g., MyoD, Myf5) and structural proteins (e.g., actin, myosin, troponin, titin), among others, are crucial for function and homeostasis. Muscle mass with respect to atrophy and hypertrophy is regulated by, for example, the PI3K/Akt-pathway, which involves the participation of mTOR and S6K1 for increased protein synthesis and the involvement of FoxO, MuRF1 and Atrogin-1 for decreased protein synthesis (Sandri, 2008). Muscle energy metabolism is regulated by gene products involved in, for example, glycolysis (e.g., HK1 and PFK1), fat oxidation (e.g., CS and PPARα) and oxidative phosphorylation (SDH and COX). The expression level changes in energy metabolism related genes have been linked to obesity and type II diabetes (Debard et al., 2004; Gerhart-Hines et al., 2007). For example, the expression of oxidative phosphorylation regulating genes has been found to decrease in insulin resistance (Mootha et al., 2003).

In humans, an increasing number of miRNAs and lncRNAs have been associated with the regulation of muscle tissue properties, body composition and metabolic health. miR-222 has been shown in vitro to regulate muscle fiber type conversion and the biogenesis of mitochondria (Gan et al., 2020). In addition, insulin has been shown to regulate the expression of several miRNAs, such as miR-1, -95 and -133a in human skeletal muscle, thus possibly also contributing to metabolic health (Granjon et al., 2009). Skeletal muscle tissue is known to express myomiRNAs: miR-1, miR-133a/b, miR-206, miR-208a/b, miR-486 and miR-499, which regulate muscle proliferation, differentiation and regeneration (Horak et al., 2016). While for lncRNAs, similar muscle-specific species have not been found, the lncRNAs H19, NEAT1, RAM and Dum have been observed to associate with the regeneration of muscle tissue (Dey et al., 2014; Martone et al., 2020), and the lncRNAs Chronos, MALAT1 and MAR1 have been previously linked with possible roles in skeletal muscle atrophy (Neppl et al., 2017; Ruan et al., 2022; Z.-K. Zhang et al., 2018). Little is known about the effects of skeletal muscle lncRNAs on total body metabolism. In rats, several hundred lncRNA transcripts were differentially expressed between diabetic and control phenotypes (W. Zhang et al., 2020), whereas in mice and human cells, only muscle H19 has been found to be downregulated in insulin- resistant individuals (Gao et al., 2014). H19 interacts with miRNA let-7 (Kallen et al., 2013), which further represses the insulin signaling pathway, including insulin receptor (H. Zhu et al., 2011). In adipose tissue, lncRNAs such as H19 and ADINR have been associated with increases in adipogenic differentiation (Y. Huang et al., 2016; Xiao et al., 2015). Circulating miR-374a-5p has also been shown to associate with the metabolically healthy rather than metabolically abnormal obesity phenotype in women (Doumatey et al., 2018) and miR-24 and -29b to associate with higher T2D incidence (X. Wang et al., 2014). In lncRNAs, the increased expression of circulating RP11-20G13.3 and the decreased expression of GYG2P1, lncRNA-p21015 and -p5549 have previously been associated with increased adiposity (Y. Liu et al., 2018; Sun et al., 2016).

2.4 Lifestyle habits and body composition

Lifestyle habits are known to contribute considerably to body composition. Of all the possible lifestyle habits, PA, diet quality and the use of exogenous hormones were investigated in this thesis, and hence the following sections will focus on exploring these.

2.4.1 Physical activity

PA can be defined as "any bodily movements produced by skeletal muscles that result in energy expenditure", whereas *exercise* is a more structured form of PA engaged in to promote health or fitness (Caspersen et al., 1985). PA is a widely known contributor to the overall health, as it reduces the risk for cardiovascular diseases, obesity, hypertension, certain cancers, and type II diabetes, while improving musculoskeletal health, mental health, cognitive function and sleep (Warburton et al., 2006). Acutely, PA increases, for example, energy and oxygen consumption, heart rate and blood flow (Thyfault & Bergouignan, 2020). The positive long-term effects of PA on health are mediated by systemic anti-inflammatory response, as muscle contraction increases the levels of IL-6, -10, -1ra and soluble receptors of TNF, and inhibits the secretion of TNF-α from adipose tissue (A. M. W. Petersen & Pedersen, 2005). IL-6 can be considered also as an proinflammatory cytokine, although after acute exercise response, the elevation of IL-6 increases lipolysis and fat oxidation and drives the secretion of anti-inflammatory cytokines, such as IL-10 and IL-1ra (E. W. Petersen et al., 2005; Steensberg et al., 2003). Other myokines released by muscle in response to exercise include IL-8 (angiogenesis), IL-15 (anabolic factor), FNDC5/irisin (browning of AT) and myostatin (insulin sensitivity) (reviewed in Leal et al., 2018). In addition, PA has been shown to increase mitochondrial biogenesis (Wright et al., 2007), insulin sensitivity (Houmard et al., 2004) and, for example, muscle hypertrophy (Schoenfeld, 2010). In adipose tissue, PA increases lipolysis and mitochondrial function, and reduces inflammatory macrophages (Y.-M. Park et al., 2014). The WHO's PA recommendations for adults (aged 18–64 years) are >150 minutes of moderate or 75 minutes of vigorous PA and resistance exercise twice a week. In addition, as much light exercise as possible and avoiding sedentary time is also recommended (World Health Organization, 2020).

PA can be separated into four dimensions: mode (activity type), frequency (how often), duration (how long) and intensity (rate of energy expenditure) (Strath et al., 2013). Energy consumption during PA can be estimated using the doubly labelled water method, indirect calorimetry or, for example, by heart rate monitoring, but due to their limitations of cost, including labor, and sensitivity, self-reported questionnaires or diaries, and devices such as accelerometers are preferred in large-scale measurements (Strath et al., 2013). Questionnaires with varying numbers of questions aiming to capture either leisure or all-time PA are inexpensive and easily distributed, but may not be robust enough to reflect the

amount of light activity and energy expenditure (Sylvia et al., 2014). Some of the widely used PA questionnaires include, for example, the International Physical Activity Questionnaire (Craig et al., 2003), and the Saltin and Grimby instrument and its derivates (Saltin & Grimby, 1968). While accelerometers do not rely on participant memory and can capture large amounts of data, they can be costly and depending on their placement on the body, may not capture all types of PA, such as gym exercise, to a similar extent (Sylvia et al., 2014). Accelerometers record PA using, for example, one- or triaxial acceleration data binned in "counts", where one count can be simplified as a movement with acceleration, that exceeds the filter limit (Neishabouri et al., 2022). Thus, using several different assessment methods might be beneficial for more accurate estimation of PA level, as the results from questionnaires and accelerometers never fully correlate (Hagstromer et al., 2010; Hyvärinen et al., 2019).

Some of the most often used measures of PA include metabolic equivalents of a task (METs) and the amount of moderate-to-vigorous PA (MVPA). One MET represents the energy expenditure required for sitting of a 70 kg person and is equivalent of 3.5 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$. Thus an activity, which requires oxygen consumption of 10.5 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$, such as normal pace walking, is equivalent to three METs (Ainsworth et al., 2011). The daily or weekly amount of PA in METs can be estimated as intensity, duration and frequency (intensity (METs) * duration (mins/h) * frequency (times per day/week)). MVPA by definition is an activity of consuming between 3 and 6 METs, that is, an activity in which the heart rate increases but only to a level that enables one to perform it for a longer time (World Health Organization, 2020). Like METs, daily or weekly MVPA in minutes or hours can also be calculated (duration * frequency). Recently, the measurement of mean amplitude deviations (MAD) has also become popular in studies of PA. MADs are produced by accelerometers, but instead of binning PA in counts, it captures activity volume from the whole intensity range without filtering (Vähä-Ypyä et al., 2015).

2.4.2 Diet quality

Diet and caloric intake are well known contributors to both body composition and metabolic health. They contribute to health through nutrients, calories, fiber, vitamins and antioxidants. A healthy diet consists of a suitable amount of energy, adequate amounts of protein, carbohydrates and soft fats, and limited amounts of hard fats, free sugars and salt (World Health Organization, 2003). According to the Nordic Nutrition Recommendations for adults, in a health-promoting diet fats should form 25–40 percentages of the total energy intake (E%), protein 10–20E% and carbohydrates 45–60E%. Such a diet should predominantly be derived from plant-based, high-fiber and low energy-density protein and carbohydrate sources. More specifically, saturated fats and added sugar should not exceed more than 10E% (The Nordic Council of Ministers, 2014). A diet rich in fats, and especially saturated fats, increases the risk for cardiovascular and metabolic conditions and is associated with obesity (Bray & Popkin, 1998; de Souza et al., 2015). While higher protein intake has been associated with higher LM and

quadriceps strength in adults (Sahni et al., 2015), very high protein diets (>1.5g protein/kg/day), especially those containing animal protein, may be harmful for kidney function in the long-term, particularly for individuals with predisposing conditions (reviewed in Ko et al., 2020). Diets with a very high carbohydrate concentration (>60E%), such as the typical Asian diet, have recently also been associated with increased metabolic risk (Bikman et al., 2022; Y. J. Lee et al., 2018). Some of the cellular mechanisms underlying how different macronutrients and diet affect the body and health are relatively well understood. For example, high fat-diet and overnutrition overall leads to accumulation of lipid molecules in the cells, further to inhibition of insulin receptor signaling (insulin resistance), adipose tissue hypoxia and inflammation, and the release of inflammatory cytokines (Roden & Shulman, 2019). A low protein diet may lead to a reduced rate of protein turnover due to deficiency of essential amino acids and an attenuated anabolic signal, and may, according to the 'protein leverage' hypothesis, even predispose to the overconsumption of calories (Atherton & Smith, 2012; Hursel et al., 2015; Simpson & Raubenheimer, 2005).

Diet quality can be measured with food frequency questionnaires, food diaries and, for example, interviews. Several different indices have been created, including the Healthy Eating Index-2015 (Krebs-Smith et al., 2018), the various Mediterranean diet indices (Aoun et al., 2019) and the Dietary Approaches to Stop Hypertension (DASH) diet (Mellen et al., 2008) to name a few. The reason for the creation of these different scores are differences in food cultures between countries as well as constantly emerging new innovations for popular foods, such as different plant-based "milk" and protein products. Depending on the index, associations of blood lipid values with vitamins, as well as with BMI, cardiovascular disease and cancer risk have been found (Wirt & Collins, 2009).

2.4.3 Exogenous hormone use

During fertile age, hormonal preparations for contraception or treating medical conditions are widely used by women. Oral, injection, transdermal or intravaginal/uterine preparations for these purposes include either progestogen, or estrogen combined with progestogen as their effective agent. Progestogen is the main contraceptive agent as it prevents ovulation by inhibiting LH surge, alters the vaginal mucus composition to prevent sperm from moving into the uterus and induces uterine endometrium atrophy. The estrogen component inhibits the secretion of FSH and thus the maturation of the dominant follicle (L. E. Britton et al., 2020). In midlife, similar or even identical preparations can be used in menopausal HT. Here the purpose is no longer to prevent pregnancy, but to alleviate menopausal symptoms, such as sleeping difficulties, irregular bleeding or vasomotor symptoms caused mainly by low estrogen levels (S. R. Davis & Baber, 2022). Thus, menopausal HT can consist of either estrogen alone or, in women with a uterus, estrogen combined with progestogen to reduce endometrial hyperplasia (Vigneswaran & Hamoda, 2022). In addition, testosterone, DHEA and tibolone, which possess estro-, andro- and progestogenic properties can used for specific symptoms such as sexual

dysfunction (S. R. Davis & Baber, 2022). The different HT treatments include oral, transdermal, subcutaneous, and intrauterine preparations, in addition to local intravaginal therapy. Both synthetic and bioidentical hormones are used. In Finland, approximately 12% of the women ≥ 45 years in age use exogenous sex hormone preparations (Finnish Medicines Agency & Social Insurance Institution, 2021). The effects of HT on the body composition of menopausal women will be discussed further in chapter 2.6.3.

2.5 Changes in body composition during aging and menopause

2.5.1 Body composition and aging

During aging, muscle mass is lost and adipose tissue is gained in both men and women (Gába & Přidalová, 2014; Guo et al., 1999). Longitudinal increases in FM vary between 0.1 kg to 1.4 kg per year, while the decrease in muscle mass varies between zero and three percent per decade, corresponding to approximately 0.1 kg per year (Guo et al., 1999; Hughes et al., 2002; Jackson et al., 2012; Kyle et al., 2006). Age and sex both contribute to these changes. Aging-related muscle loss (sarcopenia), characterized by decreased muscle strength and lowered ALM, has a significant impact on health as it is associated with lowered physical functioning, cognitive impairment, metabolic health and increased risk for cardiac disease (Cruz-Jentoft et al., 2019; S. H. Kim et al., 2021). Age-related muscle atrophy is characterized by a decrease in fiber size, especially in type II fibers, but also by a reduction in the total number of fibers (Larsson et al., 1978; Lexell et al., 1988; Lexell & Taylor, 1991). Muscle quality also affects physical functioning and health. During aging, muscle tissue is partially replaced by non-contractile connective and adipose tissue (Delmonico et al., 2009). Lower muscle density, decreased muscle cross-sectional area and increased fat infiltration inside muscle fascia have been associated with an increased fall risk, lower gait speed, and decreased muscle power and postural balance (Edmunds et al., 2018; Straight et al., 2019; Vitale et al., 2021), thus contributing strongly to overall wellbeing.

2.5.2 Change in adiposity and adipokines during the menopausal transition

Compared to aging, the impact of hormonal aging and menopause on changes on body composition and metabolic health in women is less well understood. The main mechanism behind menopausal tissue changes may be the dramatic decrease in systemic E2, as E2 has been shown to have several beneficial effects on both fat and muscle tissues at the cellular level. In adipose tissue, E2 is known to increase preadipocyte proliferation (Anderson et al., 2001), regulate adipose tissue browning, lipolysis and lipogenesis (Al-Qahtani et al., 2017; Pedersen et al., 2004) and affect glucose uptake (Ahmed et al., 2022). Estrogen deficiency induces adipose tissue hypertrophy, immune cell invasion and

fibrosis (Abildgaard et al., 2021). E2 also affects the regulatory system in the hypothalamus, which controls hunger and increases energy expenditure, thereby possibly preventing excess lipid accumulation (reviewed in Bracht et al., 2020). Decrease in overall energy expenditure due to hormonal changes has been widely suggested to be a key factor for increased adiposity in postmenopause (Lovejoy et al., 2008), but recently the role of age and decreased PA have also been introduced (Duval et al., 2013; Karppinen et al., 2023). As previously mentioned, PR and FSHR are also expressed in adipose tissue. In adipocytes, P4 increases lipogenesis (Lacasa et al., 2001), mitochondrial biogenesis (Rodríguez-Cuenca et al., 2007) and adipocyte hypertrophy, decreases IL-6 production, and affects the adipokine expression (Pektaş et al., 2015; Stelmanska et al., 2012). In adipocytes, FSH has been observed to increase lipid droplet accumulation and to decrease adiponectin and increase leptin release (H. Cui et al., 2012; X. Liu et al., 2015). In animal models, blocking the effects of FSH with an antibody resulted in lower adiposity and increased energy expenditure after OVX (X. Han et al., 2020; P. Liu et al., 2017). In humans serum FSH levels have been found to associate positively with body adiposity especially before 60 years of age (Gavaler & Rosenblum, 2003; M. Sowers et al., 2007; Veldhuis-Vlug et al., 2021).

The associations between menopause and body composition have been investigated in both cross-sectional and longitudinal studies such as Melbourne Women's Midlife Health Project (MWMHP, 1991–2000), Study of Women's Health Across the Nation (SWAN, 1994->), Montreal-Ottawa New Emerging Team group study (MONET, 2004–2009), Healthy Transitions (1997–2002), and Women's Healthy Lifestyle Project (WHLP, 1992–1999). Several studies investigating the effects of HT or ovarian suppression on body composition have also been conducted (e.g., Dam et al., 2021; Gavin et al., 2020; Ronkainen et al., 2009; Sipilä et al., 2001). Studies have varied in the menopausal status assignment, measurement interval and methodology used in body composition measurements, somewhat complicating the comparisons between them. In addition, studies such as SWAN include participants from different ethnic backgrounds, whereas participant ethnicity has been more limited in other studies. A selection of the longitudinal results most relevant for this thesis are presented in Table 2, while the results of the cross-sectional studies are discussed only in the text. Here, the focus is on studies, in which women underwent natural menopause. Studies on HT use are discussed in more detail in chapter 2.6.3

Most of the cross-sectional studies comparing the adiposity between pre- and postmenopausal women have reported increases in adiposity, but only in some of the variables. For example, in their multi-ethnic study, Sternfeld et al. (2005) observed increases in total fat percentage only in the Chinese group, but no change in total FM in any of the other ethnic groups. In a study of French middle-aged women, postmenopausal status was associated with higher trunk and leg adiposity (Panotopoulos et al., 1996), whereas another study in age-matched Japanese women found no differences in appendicular, trunk or total FM between pre- and postmenopause (Douchi et al., 2007). Although significant increases have been reported in total and abdominal FM in groups with larger

age ranges (Douchi et al., 2007; Svendsen et al., 1995), two studies of age-matched middle-aged women with differing menopausal statuses only found trends (Gould et al., 2022; Svendsen et al., 1995). Increases in VAT adiposity between post- and premenopausal women has been reported in two cross-sectional studies (Abildgaard et al., 2013; Kanaley et al., 2001).

In the majority of the longitudinal menopausal studies an increase in total body FM and fat percentage at the group mean level has been reported. For example, in a large multiethnic study of more than 1 000 participants FM increased by 0.45 kilograms and body fat percentage with an relative increase of 1% per year during the menopausal transition (Greendale et al., 2019). In another cohort of mostly Caucasian women, similar increases were observed two years prior to FMP, although the group did not find statistically significant differences when comparing the timepoints to the FMP year (year 0) (Abdulnour et al., 2012). Several studies focused on the abdominal area have reported menopause-related increases in both subcutaneous and intra-abdominal (including VAT) adiposity (Franklin et al., 2009; C. G. Lee et al., 2009; Lovejoy et al., 2008; Marlatt et al., 2020), while one study concluded that the changes were not related to menopause, but to a decline in the level of PA (Kanaley et al., 2001).

Waist adiposity and body shape around menopause have also been investigated using WC and WHR. In cross-sectional studies, WC and WHR have not been reported to differ between pre- and postmenopausal women (P. Gupta et al., 2008; Sternfeld et al., 2005), while several, but not all (Abdulnour et al., 2012; Franklin et al., 2009), longitudinal studies have reported an increase in these parameters in postmenopausal women (Greendale et al., 2021; Guthrie et al., 1999; Janssen et al., 2008; Marlatt et al., 2020; M. Sowers et al., 2007). In a recent meta-analysis of longitudinal studies, the average annual increase in WC was 0.51 cm and in body fat percentage 0.41% during midlife (Ambikairajah et al., 2019).

Besides natural menopause, the effects of artificial menopause have also been investigated. In a study of premenopausal women (aged ~46 years), who went through ovarian suppression with GnRH-agonist for 24 weeks, a significant increase in FM and a decrease in leg FFM was observed (Gavin et al., 2020). In addition, surgical menopause (oophorectomy) has also been shown to increase FM in a several age groups (Karia et al., 2021).

Adipokines as indicators of adipose tissue and metabolic health have been rarely studied in menopausal women. For adiponectin, cross-sectional studies have reported both an increase (Gavrila et al., 2003; Tamakoshi et al., 2007) and no differences between middle-aged pre- and postmenopausal women (P. Gupta et al., 2008; Matsui et al., 2012). However, in longitudinal studies, adiponectin levels have been found both to increase during the transition from peri- to postmenopause (C. G. Lee et al., 2009; M. R. Sowers et al., 2008), but to correlate also negatively with intra-abdominal fat (C. G. Lee et al., 2009). Interestingly, the Michigan Bone Health and Metabolism Study reported a drop in adiponectin levels in perimenopause (M. R. Sowers et al., 2008). Given that menopause and related E2 deficiency have been associated with decreased metabolic health and increased risk for cardiovascular disease (El Khoudary et al., 2020) and that

adiponectin has been suggested to be an indicator of metabolic health, the previously reported increases in adiponectin levels seems counterintuitive.

As with adiponectin, the associations of leptin with menopause and the menopause-related hormonal environment are mixed. In middle-aged women, several cross-sectional and longitudinal studies, with or without body fat adjustment, report no associations between menopausal status and leptin levels (P. Gupta et al., 2008; Hong et al., 2007; Kanaley et al., 2001; C. G. Lee et al., 2009), although in separate studies both E2 and FSH have been proposed to associate positively with leptin levels (Di Carlo et al., 2002; Fungfuang et al., 2013; Geber et al., 2012; X. Liu et al., 2015; M. R. Sowers et al., 2008). Due to leptin's well-established role in fertility (reviewed in Mitchell et al., 2005), it seems that interaction between leptin and sex hormones is rather bi- than one directional. However, although E2 and FSH seem to play a role in leptin signaling, it is widely suggested that adipose tissue mass and its subcutaneous location is the main contributor to leptin levels in several populations (Ahtiainen et al., 2012; Rissanen et al., 1999; Sherk et al., 2011).

Resistin has been found to associate with menopausal status in only one study (M. R. Sowers et al., 2008), while others have found no association (Chalvatzas et al., 2009; Hong et al., 2007). In the study by Sowers et al. (2008) resistin levels were found to decrease between pre- and postmenopause also when the models were adjusted for WC. The results from in vitro and animal models are also conflicting as some suggest up- and some downregulation of resistin by E2 and P4 (Caja & Puerta, 2007; Y.-H. Chen et al., 2006). Nevertheless, like leptin, resistin may also be a part of the signaling loop related to sex hormones (Messini et al., 2019).

TABLE 2 Review table of the most important longitudinal studies and their results relevant to this thesis. The participant column describes the main baseline characteristics and follow-up time.

Study	Participants + follow-up time	Adiposity	WC/ WHR	Adipokines	Lean and muscle mass
MWMHP (Guthrie et al., 1999)	Age 46–57, pre- and perimenopausal, Australian-born. 5-year follow-up. N = 112	Skinfold ↑	WC and WHR ↑	-	-
SWAN (M. Sowers et al., 2007)	Age 42–52, pre- or early perimenopausal, African-American and Caucasian. 6-year follow-up, annual measurements. N = 130	Total FM ↑	WC ↑	-	Total muscle mass ↓
HT (Lovejoy et al., 2008)	Age 48>, pre- or perimenopausal, Caucasian and African-American. 4-year follow-up with annual measurements. N = 51	Total FM, SAT and VAT ↑	-	-	Total LBM ↔
(Franklin et al., 2009)	Age 49, premenopausal, Caucasian, 8-year follow-up. N = 8	Total abdominal fat, SAT and VAT↑, total FM and fat% ↔	WC ↔	-	Total LBM ↔
SMWHS (C. G. Lee et al., 2009)	Age 45–55, premenopausal, mainly Caucasian, individualized follow-up (~ 4.1 ± 1.4 years). N = 69	SAT, intra-abdominal fat and trunk fat% ↑, total fat% ↔	-	Adiponectin ↑, leptin ↔	-
MONET (Abdulnour et al., 2012)	Age 47–55, premenopausal, Caucasian, 5 -year follow-up, annual measurements. N = 61	Total FM and fat%, trunk FM, VAT and total abdominal fat area ↑	WC ↔	-	Total FFM↔
SWAN (Greendale et al., 2019)	Age 42–52, premenopausal, White, Black, Hispanic, Chinese or Japanese, 16-year follow-up with annual measurements. N = 1246	Total FM and fat%↑	-	-	Total LBM and lean%↓

continues

TABLE 2 continues

Study	Participants + follow-up time	Adiposity	WC/WHR	Adipokines	Lean and muscle mass
HT (Marlatt et al., 2020)	Age ≥ 43, premenopausal, White and Black, 7-year follow-up with annual measurements. N = 94	Total FM and fat%, SAT and VAT ↑	WC↑	-	Total FFM↔
SWAN (Greendale et al., 2021)	Age 42–52, premenopausal, White, Black or Japanese, 17-year follow-up with annual measurements. N = 390	VAT, android FM, gynoid FM ↑	WC and HC↑	-	-
Oxford Biobank (Dehghan et al., 2021)	Age 44–48, premenopausal, White. Follow-up 3–7 years after baseline. N = 97	Total, abdominal and android FM, VAT, leg FM ↑	-	-	Total LBM and leg lean mass ↓

N=number of participants who became postmenopausal during the study. FFM, fat-free mass (lean mass + bone); FM, fat mass; HC, hip circumference; HT, Healthy Transitions; LBM, lean body mass; MONET, Montreal-Ottawa New Emerging Team group study; MWMHP, Melbourne Women's Midlife Health Project; SAT, subcutaneous abdominal fat; SMWHS, Seattle Midlife Women's Health Study; SWAN, Study of Women's Health Across the Nation; VAT, visceral fat; WC, waist circumference; WHR, waist-to-hip ratio. ↑ increased in postmenopause, ↓ decreased in postmenopause, ↔ no change between pre- and postmenopause.

2.5.3 Changes in lean and muscle mass and skeletal muscle tissue during the menopausal transition

Skeletal muscle is known to express ERs and PR (Ekenros et al., 2017; Pöllänen et al., 2007; Wiik et al., 2009), and thus can be regulated by the change in sex hormones during menopause. In skeletal muscle, E2 has been found to improve mitochondrial function (Torres et al., 2018), protect from apoptosis (La Colla et al., 2017; Vasconsuelo et al., 2008), and contribute to stem cell number and function (Baltgalvis et al., 2010; Collins et al., 2019; Enns & Tiidus, 2008; Larson et al., 2020), all effects contributing to muscle tissue energy expenditure, mass and repair. In addition, estrogen deficiency has been observed to decrease myofiber size (McCormick et al., 2004), induce a shift to faster fiber type (Kitajima & Ono, 2016), and cause dysfunction of myosin function and contractability (Moran et al., 2007). In myofibers, P4 has been found to increase mitochondrial hydrogen peroxide release and lower glucose oxidation, possibly contributing to also to insulin resistance (Gras et al., 2007; Kane et al., 2011). In vivo, progestogen supplement after OVX did not affect muscle protein content or force (Cabelka et al., 2019), whereas in humans, high progestogen supplementation increased the protein synthesis rate (Hansen et al., 2011; G. I. Smith et al., 2014). In muscle, although the expression of FSHR has not been reported, FSH has also been found to increase lipid biosynthesis and to promote the accumulation of intramuscular

fat (X. Cui et al., 2016). Serum FSH levels have also been negatively associated with LBM (Gourlay et al., 2012; Veldhuis-Vlug et al., 2021).

While most studies report a menopause-related increase in body adiposity, the results for the simultaneous decrease in lean and muscle mass are more controversial. In cross-sectional studies, total LBM has been reported to be ~5–10% lower in middle-aged postmenopausal than pre- or perimenopausal women (Abildgaard et al., 2013; Panotopoulos et al., 1996; Y.-M. Park et al., 2020; Sternfeld et al., 2005; Svendsen et al., 1995). The results of studies investigating regional changes in lean and muscle mass indicate decreases between 3 and 9% in the appendicular and leg region when transitioning from pre- to postmenopause (Panotopoulos et al., 1996; Y.-M. Park et al., 2020; Sipilä et al., 2020). In longitudinal studies spanning several years around menopause, an association between lean and muscle mass and menopause has, surprisingly, been less frequently reported (Dehghan et al., 2021; Greendale et al., 2019; M. Sowers et al., 2007). In these studies, which have observed a change, the decrease in LBM has been ~1% over four to six years. Most of these studies have investigated LBM, while only one has investigated possible regional differences, although the significance of, in particular, leg muscles to physical functioning is vital. This study reported a significant decrease in leg LM of ~1% during a 5-year follow-up (Dehghan et al., 2021). Similarly in a 24-week ovarian suppression study of middle-aged premenopausal women, leg FFM was observed to decrease (Gavin et al., 2020). In addition to muscle mass, muscle quality as density was reported to decrease during menopause in one cross-sectional study (Pöllänen et al., 2011), while longitudinal studies are completely lacking.

At a deeper level, little is known about the effects of menopause on myofibers in human skeletal muscle tissue. On the morphological level, no differences were observed in capillary density, fiber cross-sectional area, fiber distribution or calcium-induced contractability between pre- and postmenopausal women (Pérez-Gómez et al., 2021).

2.5.4 Menopause and RNA signaling in muscle and adipose tissue

In human skeletal muscle cells, E2 has been found to regulate the expression of energy metabolism and cell cycle-related genes (E. K. Laakkonen, Soliymani, et al., 2017) and in rodents, antioxidative gene, fat oxidation and mitochondrial biogenesis markers (Baltgalvis et al., 2010; Barbosa et al., 2016; Campbell et al., 2003). In human muscle, systemic E2 levels associate with the mRNA expression of anabolic *AR* and catabolic *Atrogin-1* (Pöllänen et al., 2007). Between pre- and postmenopausal women, the mRNA levels of *MyoD*, follistatin and *FOXO3* are increased in postmenopausal women, and P4 treatment, but not E2, further increases *MyoD* expression (G. I. Smith et al., 2014). In a study of middle-aged pre-, peri- and postmenopausal women, no differences were found in gene expression pertaining to muscle fat oxidation properties (e.g., *PPARa*, *PCG-1a* and *PDH4*) (Abildgaard et al., 2013). At the protein level, postmenopausal E2 levels have been shown to associate with signaling pathways related to, for example, cell death (E. K. Laakkonen, Soliymani, et al., 2017). An acute E2

treatment was also associated with a decrease in protein breakdown markers in early postmenopausal women, while in late postmenopausal women the effect was the opposite (Y.-M. Park et al., 2019). In mice, low systemic E2 levels have been associated with increased *ESR1* mRNA levels in muscle (Baltgalvis et al., 2010), but the same has not been observed in humans (Pöllänen et al., 2007). In the case of progestagens, P4 supplement after OVX reverses *PPARa* and *PDK4* mRNA levels in rodent skeletal muscle (Campbell et al., 2003).

In human adipose tissue, postmenopausal status has been associated with lower expression of *ER*s and *FAS*, and, with depot-specific differences, increased *PPARγ*, *HIF1a*, *IL-6*, *IL-18*, *MCP 1* and *VEGF-A* (Abildgaard et al., 2021; Ahmed et al., 2022; Y.-M. Park et al., 2017). E2 treatment has been observed to increase the expression of α-adrenergic receptor, *ESR2* and to reduce the expression of genes related to fatty acid synthesis and *PPARγ* in human SAT (Ahmed et al., 2022; Lundholm et al., 2008; Pedersen et al., 2004). In turn, FSH has been observed to increase the expression of lipogenic genes (e.g., *FAS*, *LPL* and *PPARγ*) in adipocytes (X. Liu et al., 2015).

Certain miRNAs and lncRNAs have also been found responsive to menopause-related hormone levels. Among lncRNAs, there are to date rather few findings linking menopause to lncRNA expression. Most have focused on postmenopausal osteoporosis, although some results on healthy individuals are also available. In the only human study found, systemic SNHG1 levels were found to be downregulated in postmenopause when compared to premenopausal participants in a cross-sectional set-up, but when the postmenopausal women were followed for six years, no further decrease was observed (S. Huang et al., 2019). In mice, the effect of OVX was found to be the opposite to systemic SNHG1 (X. Yu et al., 2021). SNHG1 is a well-known cell proliferation inducer in many cancers and contributes to bone homeostasis (Thin et al., 2019; X. Yu et al., 2021). In mouse muscle, 12 weeks of OVX was associated with upregulation of eight and downregulation of five lncRNAs, which were found to regulate mRNAs associated with, for example, RNA transport, p53 signaling pathway and fatty acid biosynthesis (Chai et al., 2019). Estrogen has been found to induce the upregulation of HOTAIR, LncRNA152 and H19, and downregulation of MALAT1 and LINC-ROR, although most of these data derive from cancer tissues outside muscle or adipose tissue (reviewed in Sedano et al., 2020). Similarly, while P4 has been found to regulate NEAT1 in endometrial cancer cells (X. Huang et al., 2019), no data is currently available on skeletal muscle or adipose tissue. In the case of FSH, most of the recent literature has investigated the effects of lncRNAs on gonadotropin secretion rather than the effects of gonadotropins on lncRNA expression. Yet, in humans and in vitro, there is emerging evidence, that FSH associates with gonadal lncRNA expression (Hu et al., 2021; Li et al., 2021). Whether this also applies to other tissues expressing FSH receptors is not yet understood.

In the case of miRNAs, in humans, systemic E2 levels have been found to associate with miR-142-3p, -146a, -182 and -223 in muscle (Kangas et al., 2014; Olivieri et al., 2014), with miR-19a-3p in adipose tissue (Kangas et al., 2018) and

with e.g., miR-21, -27b-3p, -30a-5p and -146a in serum (Kangas et al., 2014, 2017). Several of these miRNAs have been linked to regulation of insulin, FOXO and TGF-β signaling pathways and substrate use (Butz et al., 2012; Chemello et al., 2019; Olivieri et al., 2014). In animals, associations of miR-27a-5p, -122-5p, -133a-3p, -199a-3p and -483-3p measured from muscle have been found with systemic E2 levels (Karvinen et al., 2021; Martignani et al., 2019). These miRNAs have been shown to, e.g., target the apoptotic pathway, and to regulate proliferation and metabolism (Chemello et al., 2019; J.-F. Chen et al., 2006; Karvinen et al., 2021). In human tissues other than serum, muscle or adipose tissue, P4 has been associated with the expression of, for example, miR-20, -21, -26a, -29, -453-3p and let-7i (reviewed in Cochrane et al., 2012). Recently, more miRNAs have been reported to participate in P4 signaling pathways (Nothnick, 2022), but no such information has been reported for skeletal muscle or adipose tissue. In addition, the effects of FSH on miRNA expression in muscle or adipose tissue remain unknown.

2.6 Lifestyle habits as contributors to body composition during menopause

2.6.1 Physical activity and menopause

The associations between PA, body composition, skeletal muscle tissue and metabolic health during menopause have been explored in only in a handful of studies. Even fewer results based on longitudinal data have been reported. In animals, spontaneous PA decreases after ovariectomy (Cabelka et al., 2019; Gorzek et al., 2007). Results on PA changes in humans during the menopausal transition vary across studies. For example, a study of 51 women reported a two-fold decrease in PA around the FMP (Lovejoy et al., 2008), while another study reported a small increase at around the same time (Do et al., 2000). In three other studies, no change was observed (Franklin et al., 2009; Gould et al., 2022; Guérin et al., 2019). The possible factors impacting midlife PA level have been concluded to include severeness and type of menopausal symptoms, possible muscle and joint pain, time pressures at work and possible care of grandchildren (Grindler & Santoro, 2015; McArthur et al., 2014).

The effectiveness of PA on improving body composition, skeletal muscle tissue properties and metabolic health during the menopausal transition has mostly been studied in cross-sectional designs or in postmenopausal women only. While both the cross-sectional and few longitudinal studies existing have reported an association between higher PA level and leaner body composition (Dugan et al., 2010; Kanaley et al., 2001; Sternfeld et al., 2004, 2005), PA alone has not been able to completely prevent weight gain and adipose tissue accumulation during the menopausal transition (Abdulnour et al., 2012; Brown et al., 2005; Wing et al., 1991). In a large study of postmenopausal women with a six-year follow-up, higher PA level (+1200 MET-mins/week ~ 1h walk (3 MET) per day) was only associated with a lower increase in FM and BMI in women aged 50 to

59, whereas in the older age groups FM decreased irrespective of the amount of PA (Sims et al., 2013). In the same study, the researchers found no association with the change in LBM and PA. In another study of Brazilian postmenopausal women (aged 45–65 years), frequent resistance exercise ≥ 3 times per week was found to be beneficial for lower adiposity, but not, surprisingly, for LM variables (Magalhães et al., 2022). PA has, however, been found to improve blood lipid levels and reduce blood pressure and IL-6 levels in middle-aged women (Gudmundsdottir et al., 2013; Pérez-López et al., 2022; L. Wu et al., 2014). Intervention studies have found that weekly aerobic exercise and high intensity training following the MVPA recommendations are beneficial for fat loss, especially in total and abdominal fat, and improve cardiovascular function in both pre- and postmenopausal women (Cebula et al., 2020; Dupuit, Maillard, et al., 2020; Dupuit, Rance, et al., 2020; Friedenreich et al., 2010). Despite this, it seems that, overall energy expenditure rather than intensity may be more determining to the amount of fat loss (La New & Borer, 2022). For gaining muscle mass and improving the adipokine profile in mid-age, resistance exercise has been found especially effective (Dupuit, Rance, et al., 2020; Son et al., 2020; Ward et al., 2020). Thus both aerobic and resistance exercise are effective in improving the body composition and metabolic health of women, including those in midlife, although they may not be able to completely counteract the changes caused by hormonal aging and also naturally individual differences in responses occur (Orsatti et al., 2022). Moreover, results from animal studies suggest that PA is a much stronger contributor to metabolic health than HT (S. B. Silva et al., 2022).

2.6.2 Diet quality and menopause

The associations between diet quality and menopause are sparsely studied. Cross-sectional studies report both no change (Gould et al., 2022), and increased diet quality after the menopausal transition (García-Arenzana et al., 2012; Massé et al., 2004). Longitudinal results on the changes in total diet quality during the menopausal transition have not been published, although some groups have reported results for individual nutrient groups or in relation to metabolic health or physical functioning (Guthrie et al., 2000; Tomey et al., 2008; D. Wang et al., 2020).

The dietary needs for optimal health in menopause-aged women have recently been reviewed (T. R. Silva et al., 2021). While the conclusions drawn do not greatly differ from the WHO recommendations, they highlight certain aspects. For aging women, protein intake is especially crucial for muscle mass. In observational studies, daily intakes between 1.2 and 1.6 grams of protein/kg of body weight were found to associate with a lower risk of frailty, higher skeletal muscle mass index and better physical function (T. R. Silva et al., 2021). However, in a randomized controlled weight loss study, no additional benefit for the maintenance of FFM was observed with a high protein diet (1.5g/kg weight) compared to recommendations (0.8g/kg weight) (Englert et al., 2021). Besides protein intake, the Mediterranean diet, consisting of whole-grain cereals, pulses, soft vegetable oils and nuts, fruits, fish and lesser amounts of red meat and

sweetened beverages has been found to be beneficial for BMI, WC and total and waist adiposity in menopausal women (Flor-Alemany et al., 2020). The Mediterranean diet includes plenty of dietary antioxidants such as beta-carotene, magnesium and vitamins C, D and E, which may protect cells from oxidative stress and improve energy metabolism (T. R. Silva et al., 2021). The Mediterranean diet with modest caloric reduction and even without exercise has been shown to beneficial for decreasing adiposity and metabolic health indicators around menopause (Lombardo et al., 2020). Studies investigating separate food groups have also found that fruit, rich chocolate and high calcium intake and coffee consumption are beneficial for lean and bone mass and lower adiposity in middle-aged women (Bae, 2020; Bredariol et al., 2020; Garcia-Yu et al., 2021; Yonekura et al., 2020). Overall, a low-energy, low-fat diet with fiber-rich carbohydrates and adequate amounts of protein, calcium, vitamin D and antioxidants seem to offer middle-aged women the greatest health benefits (T. R. Silva et al., 2021). In addition, a 12-month intervention study in which the study group received both diet and PA guidance showed clear benefits for body composition and cardiovascular risk factors during menopause (L. Wu et al., 2014).

2.6.3 Menopausal hormone therapy

In observational studies, the long term use of estrogen- or tibolone-based HT has been associated with a lower body fat percentage and WC (Ahtiainen et al., 2012; Perrone et al., 1999), whereas intervention studies of at least 12 months in duration have found HT to reduce, but not to completely prevent, adipose tissue accumulation on both the total body and thigh level compared to controls (S. R. Davis et al., 2000; Kristensen et al., 1999; Sipilä et al., 2001). In an earlier meta-analysis, HT use was found to be associated with reduced WC and abdominal fat percentage (Salpeter et al., 2006). In a more recent meta-analysis, subgroup analysis revealed that although HT use was associated with a higher body fat percentage, it was also associated with a lower trunk fat percentage, possibly indicating a protective role of HT, especially for the harmful waist adiposity (Ambikairajah et al., 2019). Thus, it seems that while estrogen-containing HT use does not completely prevent FM gain, it attenuates it, especially in the waist region. These observations suggest that, in addition to ovarian hormone loss, increasing adiposity is also related to aging changes and lifestyle habits, such as diet and the level of PA. Studies investigating the use of progestogen-only preparations during the menopausal transition are scarce, although the few that exist indicate that, depending on the administration method, progestogen use may also have an effect on body adiposity. In perimenopausal women, 12 months of cyclical progestogen use was associated with a decrease in body FM, whereas continuous progestogen use was seen to increase FM and WC compared to controls (Cagnacci et al., 2006; Napolitano et al., 2016). Therefore, as estrogen and progestogen alone may affect body composition on opposite ways, the choice of specific HT preparation may have a critical effect on changes in the user's adiposity.

For muscle mass, the effects of exogenous hormone use are also conflicting. Long term HT use has been associated with higher relative thigh muscle area (Ronkainen et al., 2009) and in a randomized placebo-controlled study, the use of estrogen-containing HT for 12 months was associated with an increase in quadriceps muscle area in postmenopausal women (Sipilä et al., 2001). Another study comparing E2 and E2 + T found, that in postmenopausal women only E2 + T treatment for two years was beneficial for increasing FFM (S. R. Davis et al., 2000). Two large meta-analyses have been conducted on the effects of HT on muscle mass. The earlier analysis found HT use to increase LBM, while the more recent one, by Javed et al. (2019), found estrogen-containing HT to slightly decrease the amount of lost LBM compared to non-users, but the association failed to reach significance (Javed et al., 2019; Salpeter et al., 2006). Thus, while estrogen alone might not have especially strong effects on muscle mass during menopause, its anabolic effects seem to be highlighted when combined with PA (Dam et al., 2020; Sipilä et al., 2001). In addition, although a recent meta-analysis reported that HT does not further improve muscle strength when compared to non-HT users in controlled trials (Xu et al., 2020), an earlier larger meta-analysis (Greising et al., 2009) and studies outside these meta-analyses have reported a connection between HT use and improved strength and functional capacity (Dam et al., 2020; Ronkainen et al., 2009). In turn, the use of estrogen-based HT has been found to associate with higher muscle attenuation (Taaffe et al., 2005), maximum voluntary contraction per unit (Onambele-Pearson et al., 2021) and involuntary force-generation (Finni et al., 2011). One study found that tibolone, but not estrogen, was associated with muscle attenuation (Ronkainen et al., 2009). On the cellular level, in a study of postmenopausal twins, while HT use was not associated with fiber size or composition, it was associated with more beneficial contractability properties and myonuclei organization (Qaisar et al., 2013; Widrick et al., 2003).

In addition to body composition, cardiovascular health and systemic metabolic health indicators are also affected by HT use. The use of both estrogen-only and combined HT use has previously been associated with lower adiponectin levels (Im et al., 2006; Kunnari et al., 2008). HT use has also been associated with lower leptin levels, but only before adjusting for FM (Gower et al., 2000; Kristensen et al., 1999). In a study of HT-discordant twins, no difference was observed in adiponectin and leptin levels between the sisters, even before adjusting for FM (Ahtiainen et al., 2012). Interestingly, in another study, six months of HT use was reported to increase leptin levels, although the results were not adjusted for FM (Konukoglu et al., 2000). For resistin, in turn, no effect of HT use has been reported (Kunnari et al., 2008). Postmenopausal HT has been shown to be associated with lower levels of total and non-HDL cholesterol, higher HDL levels, better blood glucose management and lower risk for type II diabetes (Crespo et al., 2002; Pentti et al., 2009; Salpeter et al., 2006). Overall, the initiation of HT, after menopause, especially in the early stage, decreases the mortality risk of cardiovascular diseases (Savolainen-Peltonen et al., 2016).

Although HT seems to possess several beneficial health outcomes it also presents risks. For example, increased breast cancer risk was associated with all HT use in the Million Women Study (Beral & Million Women Study Collaborators, 2003). The risk-increasing agent has since been pinpointed to synthetic progestogens (Vinogradova et al., 2020). In fact, a recent follow-up study from Women's Health Initiative, with nearly 30 000 women found that conjugated estrogen use was associated with a lower incidence of breast cancer and related mortality (Chlebowski et al., 2020). Similar results on overall HT use have also been observed in other cohorts (Mikkola et al., 2016). Furthermore, it seems that the route of administration affects the observed responses and risks. For example, transdermal preparations are associated with a lower risk for thrombosis, whereas oral preparations have been associated with a higher risk for thromboembolic events and increases in systemic HDL and triglyceride levels. The use of synthetic progestogens and >10 years of HT use in women over 60 has also been associated with increased risks (S. R. Davis & Baber, 2022; Vigneswaran & Hamoda, 2022).

3 AIMS OF THE STUDY

The aim of this study was to determine the associations between the menopausal transition and changes in body adiposity, metabolic health parameters, lean and muscle mass, skeletal muscle cellular properties and gene expression. In addition, the associations of physical activity, diet quality and exogenous hormone use with the abovementioned parameters were evaluated. The associations were investigated by using longitudinal data from a Finnish cohort of middle-aged women.

The specific aims of this thesis were:

1. To investigate the changes in body adiposity measures, systemic adipokines and lean and muscle mass during the menopausal transition (*Papers I, II* and *III*)

   Hypothesis: Body adiposity will increase, particularly in the android region. Waist circumference and waist-to-hip-ratio will increase. Serum adiponectin will decrease and leptin and resistin levels will increase. Lean and muscle mass will decrease during the menopausal transition.

2. To investigate structural changes and changes in RNA expression in skeletal muscle fibers during the menopausal transition (*Papers I, III* and *IV*)

   Hypothesis: During menopausal transition, muscle fiber cross-sectional area will decrease, and more lipid droplets will be accumulated inside the fibers. Oxidative and glycolytic enzyme activities will decrease. Changes in skeletal muscle RNA expression will be associated with the hypothesized increase in body adiposity and decrease in lean and muscle mass.

3. To investigate the associations between lifestyle habits (physical activity, diet quality and exogenous hormone use) and body adiposity, lean and muscle mass and RNA expression during the menopausal transition (*Papers I-IV*)

   Hypothesis: Higher physical activity, healthier diet quality and exogenous hormone use will be associated with lower adiposity and higher lean and muscle mass. Waist circumference and waist-to-hip-ratio will be negatively associated with higher physical activity. Serum leptin and resistin levels will be negatively and adiponectin levels positively associated with physical activity. Physical activity will be associated with muscle RNA transcriptome.

4 METHODS

4.1 Study design and participants

This doctoral thesis includes four sub-studies (*Papers I-IV*), resulting in four original publications (listed on page 8). Data for all four substudies were drawn from the ERMA (Estrogenic Regulation of Muscle Apoptosis) and EsmiRs (Estrogen, MicroRNAs and the Risk of Metabolic Dysfunction)-studies (Figure 4) (E. Laakkonen et al., 2022). In 2015, a total of 6 878 women aged 47 to 55 years and resident in the Jyväskylä area were invited by letter to take part in the ERMA-baseline assessments. The women were reached using contact information from the Population Register Center (82% of the whole cohort). In total 3 229 women responded and 3 064 women returned the prequestionnaires. An invitation to the first laboratory visit for menopausal assignment was sent to 1 627 women. Exclusion criteria for the first measurements included conditions or medications affecting ovarian function, muscle function, obesity or systemic inflammation status, and thus potentially also the molecular mechanisms of interest, such as use of estrogen-containing medication during the previous three months, pregnancy or lactation, self-reported BMI > 35 kg/m^2 and musculoskeletal conditions affecting everyday physical functioning (Kovanen et al., 2018). Altogether, the menopausal statuses of 1 393 women were assigned for baseline measurements using the modified STRAW +10 guidelines (Harlow et al., 2012). Based on their FSH levels and self-reported menstrual data for the preceding six to twelve months, 389 women were categorized as premenopausal, 323 as early perimenopausal, 242 as late perimenopausal and 530 as postmenopausal. After menopausal status assignment, participants with conditions potentially affecting their daily mental or physical function or systemic hormone or inflammatory status, such as insulin-treated diabetes, cortisone-medicated conditions, cancer diagnosed less than five years previously or severe gastrointestinal disease or mental illness, were excluded. After exclusions, a laboratory visit with

physiological measurements and a thorough medical examination was conducted for 1 158 participants. Based on the exclusion criteria, to ensure safe participation in the physiological measurements and willingness to participate in all the measurements, another 235 participants were excluded, and nine participants withdrew, yielding a baseline sample of N = 914. Of them, 234 were premenopausal, 183 were early perimenopausal, 198 were late perimenopausal and 299 were postmenopausal.

Altogether 381 participants in the perimenopausal group entered the ERMA longitudinal study, during which the women's menopausal transition was followed individually with a laboratory visit every three to six months. Three premenopausal women, whose menopausal status had originally been assigned as perimenopausal, but later recategorized as premenopausal, were also included. During each laboratory visit, serum FSH levels and menstrual diary markings were checked. After two consecutively risen FSH values and approximately six months without menstrual bleeding, the woman was deemed early postmenopausal and was invited to the final follow-up measurements. Women who started using HT during the follow-up, were invited to the final follow-up measurements six months after hormone use had started. Six months was considered a sufficient time for HT to manifest its potential effects on body composition and skeletal muscle properties. During the follow-up, three participants were excluded due to inconsistent HT use, 29 discontinued, 69 did not reach postmenopause, 48 had unclear menopausal status at study end and one person died. Altogether 234 participants transitioned to early postmenopause before the end of 2018 when the data collection was ended. This study is from now on referred as the short-term follow-up (Figure 4).

EsmiRs longitudinal study was a 4-year follow-up study from the ERMA baseline. Data for EsmiRs was collected between 2018 and 2020. Invitations to participate in the EsmiRs study were sent to 811 women who had consented to be contacted with new research participation invitations at the ERMA baseline. Altogether 494 participants returned the 4-year follow-up questionnaires. Of these, 46 were excluded due to being more than seven years since menopause, two due to insulin-treated diabetes, and eight due to cancer or severe cardiac dysfunction. A further 41 participants were not willing to continue to physiological measurements or did not consent, and 99 participants could not be measured owing to COVID-19 restrictions. Thus, a final of 298 women visited the laboratory for a repeat of all the ERMA baseline physiological measurements. This study is henceforth referred as the long-term follow-up (Figure 4).

Participant inclusions for *Papers I–IV* of this thesis are presented in Figure 4. *Paper I* included participants from both the short-term (n = 230) and those from the long-term follow-up, who transitioned from pre- or perimenopause to postmenopause (n = 148). From the total short-term population (n = 234), four participants were afterwards excluded due to reported cancer. Of the short-term follow-up participants, 62 were re-measured in the long-term follow-up measurements (n = 60 for body composition). Thus, for these women three measurement points around menopause (peri-, early postmenopause and later

postmenopause) were available. *Paper II* comprised participants from the long-term follow-up. These women are the same individuals as those in the long-term follow-up in *Paper I* with the addition of one participant with reassigned menopausal status. Participants in *Paper III* were from the short-term follow-up and included all the participants from *Paper I* and four additional participants who were not excluded from this substudy. In *Paper IV*, all participants were from the short-term follow-up and, based on their baseline menopausal status, divided into two groups: early menopausal transition (EarlyMT, n = 8) and late menopausal transition (LateMT, n = 17).

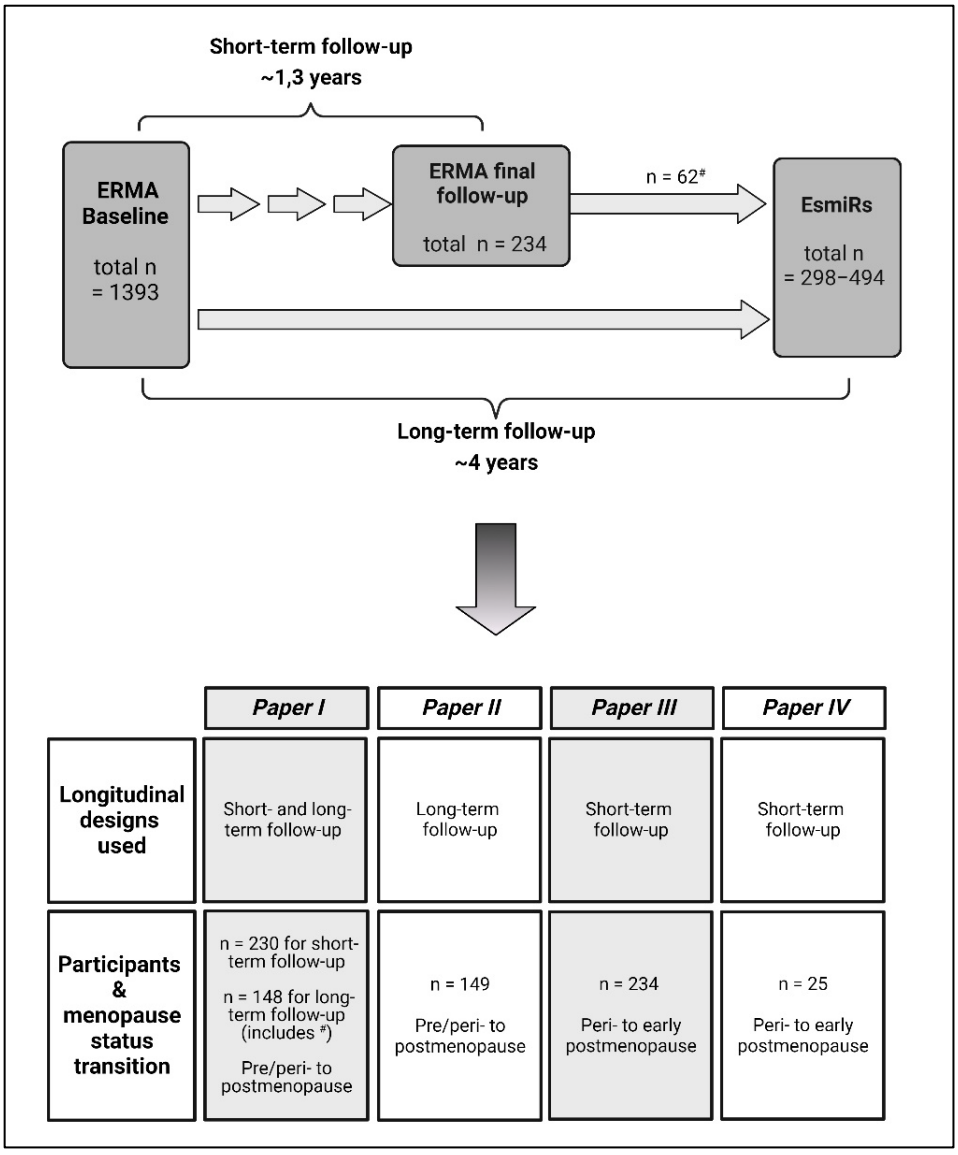

FIGURE 4 Study flow of the short- and long-term follow-up studies and descriptions of the designs of *Papers I-IV*. ERMA, Estrogenic Regulation of Muscle Apoptosis; EsmiRs, Estrogen, MicroRNAs and the Risk of Metabolic Dysfunction. # Participants who continued from the short-term to long-term follow-up. Figure created with Biorender.com.

4.2 Ethics

The studies were approved by the ethical committee of the Central Finland Health Care District (ERMA 8U/2014 and EsmiRs 9U/2018). All participants gave their written informed consent and were informed on the potential risks and personal benefits of the study. Risks presented included minor pain from venepuncture, radiation dose from DXA and CT, and pain and discomfort from muscle biopsy. Participants were not offered any financial benefits outside possible traveling costs, and they were informed that withdrawing from the measurements and discontinuation was possible at any stage of the study. The study was conducted in accordance with the Declaration of Helsinki.

4.3 Measurements

Summary of the used variables and statistics in *Papers I-IV* are presented in Table 3.

TABLE 3 Summary table of the methods used in the main analyses.

Method	***Paper I*** **"Adiposity"**	***Paper II*** **"Metabolic health indicators"**	***Paper III*** **"Lean and muscle mass"**	***Paper IV*** **"Muscle RNA signaling"**
Body anthropometrics and composition	• Total FM • Total fat% • Trunk FM • Android FM • Gynoid FM • Gluteofemoral FM • Gluteofemoral fat% • Leg FM • Android-to-gynoid ratio • Mid-thigh SAT area • Muscle compartment AT area • Muscle density	• Total FM • Android FM • Waist circumference • Waist-to-hip ratio	• LBM • LBMI • ALM • ALMI • Leg LM • Absolute muscle area • Relative muscle area	• LBM • ALM • Total FM • Android FM • Gynoid FM • Gluteofemoral FM • Leg FM • Absolute muscle area • Mid-thigh SAT • Muscle density
Serum analysis	• Adiponectin • Leptin • Resistin	-	-	-
Muscle biopsy analysis	• Fiber type • Lipid accumulation index • Lipid droplet area • Oxidative and glycolytic capacity	-	• Fiber type • Fiber area	• DE-, over-representation and interaction analysis of mRNA, lncRNA and miRNA

continues

TABLE 3 continues

Method	*Paper I* "Adiposity"	*Paper II* "Metabolic health indicators"	*Paper III* "Lean and muscle mass"	*Paper IV* "Muscle RNA signaling"
Lifestyle habits	Physical activity • SR-PA • ACC-PA DQS Exogenous hormone use: • 4-class variable at all measurement points	Physical activity • ACC-PA • SR-PA Exogenous hormone use: • 4-class variable at both measurement points	Physical activity • SR-PA • ACC-PA Exogenous hormone use: • Baseline progestogen use • Duration of HT use	Physical activity • ACC-PA • SR-PA No exogenous hormone users included
Main statistics	• Linear mixed model • Spearman correlations	• Linear mixed model	• Paired t-test and Wilcoxon rank test • Generalized estimating equations	• Spearman correlations • DESeq2

ACC-PA, accelerometer-measured physical activity; ALM, appendicular lean mass; ALMI, appendicular lean mass index; AT, adipose tissue; DE, differential expression; DQS, diet quality score; FM, fat mass; HT, menopausal hormone therapy; LBM, lean body mass; LBMI, lean body mass index; LM, lean mass; SAT, subcutaneous adipose tissue; SR-PA, self-reported physical activity

4.3.1 Body anthropometrics (*I-IV*)

Body mass was measured with a digital scale and body height with a stadiometer. WC was measured midway between the superior iliac spine and the lower rib margin, and HC at the level of the greater trochanters (Snijder et al., 2003). WHR was calculated by dividing WC by HC. All measurements were done in a fasted state and in light underwear.

4.3.2 Body composition (*I-IV*)

Fat mass. For the total and regional FM analyses, total, trunk, gynoid, android, gluteofemoral and right leg FM, and total and gluteofemoral fat percentages were measured with DXA (LUNAR Prodigy, GE Healthcare, Chicago, IL, USA). The gluteofemoral area was outlined manually using the iliac crest line as the upper limit and the knee joint as the lower limit of a rectangle (Peppa et al., 2013). The android-to-gynoid-ratio was calculated by dividing android FM (kg) by gynoid FM (kg). All measurements were done after overnight fasting. To accompany the leg DXA scans, the right mid-thigh was scanned at the level of the muscle biopsy with a qCT (Siemens Somatom Emotion scanner, Siemens, Erlangen, Germany) from those women, who at baseline did not use progestogen preparations or had conditions affecting natural menstrual bleeding pattern. From the cross-sectional image, the muscle and adipose tissue areas were separated from the femur using a machine learning algorithm or manually if needed. For the mid-thigh fat area, the areas of subcutaneous and muscle compartment adipose tissue were measured using appropriate thresholds in Python Software (version 3.6). Muscle

density was calculated and expressed using Hounsfield Units (HU). Images were analyzed using ImageJ software (v.1.52, NIH, USA) and Python.

Lean and muscle mass. For the total and regional lean mass and muscle area analysis, the variables of LBM, ALM (summed lean mass of arms and legs) and LM of the right leg were measured with DXA. The LBM index (LBMI) was calculated by dividing LBM (kg) by height squared (m^2). The ALM index (ALMI) was calculated by dividing ALM (kg) by height squared (m^2). Mid-thigh muscle area was measured using the same CT scans as for the adipose tissue area analysis and the muscle area inside the muscle fascia was measured.

4.3.3 Lifestyle habits (*I-IV*)

Physical activity. PA was evaluated with a structured questionnaire (Kujala et al., 1998) and hip-worn accelerometer (E. K. Laakkonen, Kulmala, et al., 2017). The questionnaire included four questions on the frequency, intensity and duration of leisure time PA bouts and the average time spent in active commuting in a normal everyday life. Based on the answers, metabolic equivalent of a task (MET) hours per day for leisure-time PA were calculated. These values were used for analysis in *Papers I, III* and *IV*, and for reporting participant characteristics also in *Paper II*. Device-measured PA was assessed with seven consecutive days of accelerometer wear (ActiGraph GT3X+ or wGT3X+, Pensacola, FL, USA). The amount of time spent at different PA intensities was evaluated using triaxial vector magnitude cutoff points for light, moderate and vigorous PA (E. K. Laakkonen, Kulmala, et al., 2017; Sasaki et al., 2011) in *Papers I, III* and *IV* and for reporting participant characteristics also in *Paper II*. Average minutes of summed MVPA were adjusted to 16 hours of daily wear time, including both work and leisure time. For *Papers II* and *IV*, PA level was also assessed using mean amplitude deviations (MAD) from the accelerometer data. MAD reflects the directly measured acceleration in the X, Y and Z directions and captures activity volume across the whole intensity range. MAD values have been validated against oxygen consumption (Vähä-Ypyä et al., 2015). For analysis, mean MAD values for five second epochs per measurement period were calculated.

Diet quality. Diet quality was assessed using a food frequency questionnaire and quantified using a diet quality score (DQS). The food-frequency questionnaire listed 45 typical food items of the Finnish food culture and 6 response options. The DQS was calculated based on 11 components that are characteristic of a healthy diet, as described in the Nordic Nutrition Recommendations 2012 (The Nordic Council of Ministers, 2014). Regular use of vegetables, fruits, berries, dark or crispbread, low-fat dairy, fish, nuts, and seeds was considered beneficial. Moreover, limited intake of refined baked products, processed meats and grain products, sugar-sweetened beverages, fast food, and sweet or salty snacks was also favored. Each component was scored 0 or 1, and the maximum possible score was 11 points. A higher DQS score indicated a healthier diet. DQS used was based on previously validated DQS (Masip et al., 2019).

Use of exogenous hormones. Exogenous hormone use was evaluated based on self-reports and when necessary, complemented with nurse's interview. Participants were categorized as non-users, progestogen-only users, estrogen-only users or progestogen and estrogen users. Hormone preparations including estrogen or progestogen for contraceptive and HT use, such as pills, intra-uterine device, patches, and transdermal gels were included. Intravaginal estrogen therapies, such as creams or tablets, were not considered as HT due to their mainly local effects. Participant bleeding status was evaluated using data from questionnaires and nurse's interviews. After hysterectomy, and often during the use of progestogen preparations, menstrual bleeding does not occur and thus determination of the length and regularity of the normal menstrual cycle is complicated, which also affects the exact determination of menopausal status. Women with an intact uterus and no regular use of progestogen-based preparations, including an intra-uterine device, were regarded as having natural bleeding status. At the follow-up measurements, all participants reporting the use of estrogen and/or progestogen irrespective of hysterectomy status were regarded as hormone users.

4.3.4 Background variables (*I-IV*)

The background lifestyle habits of smoking and use of alcohol were assessed with questionnaires. Smoking was categorized as current smoker or non-smoker. Weekly alcohol use was reported as units per week, in which one unit corresponds to one 33cl bottle of regular beer or cider, 12cl of wine or 4cl of strong spirits (12 grams of alcohol). Educational level was obtained from questionnaires and categorized as primary (primary school classes from 1–10), secondary (upper secondary school or post-secondary vocational college diploma) and tertiary (polytechnic or university degree).

4.3.5 Acquisition of biological samples (*I-IV*)

Blood. Fasted blood samples were taken from an antecubital vein in the supine position between 7–10 a.m. At baseline, participants were asked to visit the laboratory during menstrual cycle days 1–5 if the cycle was predictable. Serum was incubated for 15–30 minutes and centrifuged for 10 minutes at 2700 x g. Samples for E2 and FSH were stored at -20°C before analysis. Samples for adipokine analysis were stored at -80 °C before analysis.

Muscle biopsy. Muscle biopsies were collected from a subpopulation of women who did not use any type of hormonal contraception or HT and had natural bleeding status at baseline. Biopsies were taken from the middle portion of the m. vastus lateralis using a modified Bergström needle technique under local anesthesia. All visible connective and adipose tissue was removed, and the sample was quickly divided into three parts. The parts for protein and RNA analysis were snap-frozen in liquid nitrogen. The third part was embedded

transversely on a cork with TissueTek on the base and frozen in isopentane cooled in liquid nitrogen. All samples were stored at -150 °C until analysis.

4.3.6 Hormone assessments (*I-IV*)

Hormones. Serum E2 and FSH were measured with IMMULITE 2000 XPi (Siemens Healthineers, Erlangen, Germany). E2 analysis kit's lower limit of quantification (LLQ) was 0.073 nmol/L and intra-assay coefficient of variation (%CV) 6.7 and inter-assay %CV 9.7. For FSH kit, LLQ was 0.1 IU/L, intra-assay %CV was 3.4 and inter-assay %CV 5.4. For the serum adipokine analysis (*Paper I*) leptin, adiponectin and resistin levels were measured with a Quansys Multiplex-kit (custom kits HCUM190820-ID and HA2M200303-ID, Quansys Biosciences, Utah, US) according to the manufacturer's instructions. Each sample was measured in singlets. LLQ was custom kit lot specific and varied for adiponectin between 44–140 ng/ml, for leptin 0.25–0.65 ng/ml and for resistin 33.4–44.8 pg/ml. Measured mean intra- and inter-assay %CV for adiponectin were 5.6 and 15.8, for leptin 3.5 and 24.6, and for resistin 3.8 and 18.0, respectively. Inter-assay variation was taken into consideration using normalization based on three reference samples in all plates.

4.3.7 Muscle tissue histology (*I, III*)

Immunohistochemistry and lipid droplet staining (Paper I). Reagents used for staining are listed in detail in Supplemental Table 1 and referred in the text as superscripted numbers. In *Paper I,* the aim was to investigate fiber type-specific characteristics in lipid accumulation and enzyme content. Transverse, 10 µm sections were cut with a cryostat, airdried and fixed in 4% paraformaldehyde (PFA). The samples were then incubated with 100 mM glycine and blocked in 5% goat serum (GS). Primary and secondary antibodies were added in 5% GS. MHC distribution was analyzed with primary antibodies against type I[1] (2 µg/ml) and type IIX[2] (5 µg/ml). Anti-laminin[3] (1:250) was used to detect myofiber plasma membrane. Primary antibodies were incubated at +4 °C overnight and attachment was visualized with fluorescent secondary antibodies [4, 5, 6] (all 1:500). To stain neutral lipids, LD540[7] (0.1 µg/ml) was incubated on sections for 20 minutes at room temperature. After thorough washing with phosphate-buffered saline (PBS), sections were mounted with Mowiol-Dabco and imaged with confocal microscopy. For image analysis, the laminin signal was enhanced, and broken cells were excluded in ImageJ with the Trainable Weka Segmentation plugin and later by human inspection. Cell segmentation and the measurement of the lipid droplet (LD) area, number and area fraction were performed as previously reported (Fachada et al., 2022). A lipid accumulation index (LAI) for each fiber type was calculated as $\frac{mean\ total\ lipid\ droplet\ area\ in\ fibers}{mean\ fiber\ size} x\ 100$ (Goodpaster, Theriault, et al., 2000).

Enzyme histology (Paper I). For the *m. vastus lateralis* enzyme quantification analysis, serial 12 µm transverse sections were cut. Enzyme activities of SDH and GPD were measured with histological staining. The SDH incubation medium consisted of 1mg/ml NBT[8] and 27mg/ml sodium succinate[9] in 0.2M phosphate buffer, pH 7.4. Sections were incubated in prewarmed solution for 90 minutes at +37.2 °C, washed with MQ-water and mounted with Mowiol. The GPD medium consisted of 1.2mM NBT[8], 2.3mM menadione[10] and 9.3mM alfa-glycerophosphate[11] in 0.05M Tris-Buffer, pH 7.4. Sections were incubated in prewarmed solution for 40 minutes at +37.2°C, rinsed with tap water and cleared with acetone series. Lastly, sections were rinsed with MQ-H_2O and mounted with Mowiol. Samples were imaged with an Olympus BX50 (10x/0.30) (Olympus, Tokyo, Japan). In ImageJ (v. 1.53c), images were transformed into an 8-bit greyscale format. A minimum of 55 cells per sample was manually cropped, and mean grey value was calculated. Enzyme staining was paired with MHC-staining with serial sections. First, sections were airdried and blocked in 10% GS. Primary and secondary antibodies were added in 10% GS. The fiber types were analyzed with antibodies against type I[1] (2 µg/ml) and type II[12] (2 µg/ml) fibers. Anti-laminin[3] was used to detect fiber borders. Sections were incubated in primary antibodies at room temperature for 1 hour, washed with PBS and incubated with secondary antibodies [6,13] (both 1:500) at room temperature for 1 hour. After PBS washing, sections were mounted with Mowiol-Dabco. Images were analyzed with ImageJ. Corresponding fibers were localized manually between histological and immunohistological images.

Myofiber type distribution and area measurement (Paper III). To study skeletal muscle fiber distribution and single fiber area, 10 µm serial transverse sections were cut, air dried and fixed in 4% PFA in PBS (pH 7.4) for 15 minutes. The samples were incubated in 100mM glycine for 10 minutes (aldehyde group-binding) and treated with 0.2% Triton X-100 for 10 minutes (permeabilization). Samples were blocked with 5% GS and primary antibodies were added in 1% GS. MHC antibodies against type I[1] and type II[12] fibers in 5 µg/ml and 6 µg/ml, respectively, were used to investigate fiber distribution. Antibody for laminin[3] was used in 1:250 to detect myofiber plasma membrane for area quantification. Primary antibodies were incubated at +4 °C overnight. The next morning, after thorough washing, fluorescent secondary antibodies [13, 14] were added 1:500 in 1% GS and samples incubated for 1 hour in room temperature. After washing steps, sections were mounted and counterstained[15] and imaged with confocal microscopy (LSM 700, Axio Observer, Zeiss, Oberkochen, Germany). Images were analyzed manually with ImageJ software (v.1.52, NIH).

4.3.8 Myosin heavy chain isoform separations with SDS-PAGE (*III*)

Reagents used for staining are listed in detail in Supplemental Table 1 and referred in the text as superscripted numbers. Muscle samples assigned for protein analysis (weight ~ 4–12 mg) were homogenized in 1:100 myosin extraction buffer, which consisted of 0.1 M KCl[16], 0.1 M KH_2PO_4[17], 0.05 M

$K_2HPO_4 \cdot 3H_2O$[18], 0.01 M EDTA[19], 0.02 M NaPPi [20], BME[21], Pepstatin A[22] and inhibitor [23]. Ingredients were diluted in MQ-water and pH was set to 6.5 with potassium hydroxide. Working solution was prepared 1:1 to 10% Triton-X. Homogenization was performed with TissueLyser II (Qiagen, Hilden, Germany) and continued with 24-h shaking at +4 °C. After that, samples were centrifugated for 10 minutes at 10 000 × g at +4 °C (Eppendorf 5424, FA-45-24-11, Hamburg, Germany). 20 µL of obtained supernatant was mixed with 80 µl working Laemmli sample buffer (95% Laemmli buffer, 5% BME) and 30 µl glycerol. Samples were heated for 4 minutes at +100 °C and then frozen to -20 °C. 200–300 ng of total protein was loaded into the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system, consisting of 3% stacking gel and 6.7% separating gel with 30% glycerol. Electrophoresis was run in Bio-Rad Protean II Xi Cell (Bio-Rad, Hercules, CA, US) for 42–44 h at 70–90 V at +4 °C. After the run, gels were fixed in ethanol-acetic-acid-solution for one hour and washed with water. A sensitizer (0.02% sodiumthiosulphate-5-hydrate [24] in water) was applied to gels for 1 minute. Gels were washed and incubated in cold 0.1% silver nitrate solution with formaldehyde. After staining, the gels were again washed and developed with 3% sodiumcarbonate solution with formaldehyde, until the staining was visible. Development was terminated in 5% acetic acid solution. Gels were imaged with ChemiDoc MP (v.2.2.0.08, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and images analyzed with Image Lab (v.6.0.1, Bio-Rad Laboratories, Inc.).

4.3.9 RNA extraction and sequencing (*IV*)

RNA extraction. Total RNA extraction was performed using the Qiagen miRNeasy Mini Kit (217004, Qiagen, Hilden, Germany). Muscle biopsy samples (weight ~ 10–60 mg) were placed in Qiazol Lysis Reagent and homogenized with a metal bead in TissueLyser II (2 min, 25hz). After 5 minutes tabletop incubation, chloroform was added, and the sample was shaken manually. The solution was centrifuged for 15 minutes at 12 000xg in +4 °C. Upper layer containing total RNA was separated and mixed with 100% ethanol. The mixture was then transferred to a spin-column and centrifuged. Column and attached RNA were rinsed and finally eluated into RNAse-free water. Samples were stored at -80 °C.

Sequencing. 15 µl of RNA-samples were sent in dry ice to Novogene's UK laboratory for next-generation sequencing (NGS) of mRNAs, small RNAs (sRNA) and lncRNAs. First, sample RNA integrity, concentration and sample purity were assessed with Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA) and agarose gel electrophoresis. Despite lower quality results in one sample, all samples were qualified for library preparation.

To construct the mRNA and lncRNA libraries, ribosomal RNA was removed, and remaining RNA was fragmentated. Via reverse transcription, single complementary DNA strands were synthesized. A mixture of dNTPs, RNAse H and DNA polymerase I was further added to initiate second-strand synthesis. After series of end repair, A-tailing and use of U-adaptor, PCR

amplification was performed resulting in final double stranded cDNA library. Correct size libraries analyzed with agarose gel were selected for sequencing.

For sRNA sequencing, 3 µg of total RNA per sample was used as input material. Sequencing libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. After adapter ligation, first-strand cDNA was synthesized. PCR amplification was performed and the products were purified on an 8% polyacrylamide gel. DNA fragments corresponding to 140~160 bp (the length of sRNA plus the 3' and 5' adaptors) were recovered and dissolved in 8 µl elution buffer. Library quality was assessed on the Agilent Bioanalyzer 2100 system. Sequencing for all samples was done with Illumina NovaSeq 6000 in Novogene's laboratory (Cambridge, UK). For small RNA, 50bp single-end reads were generated and for longer RNAs, paired-end reads of 150bp were generated.

4.3.10 Bioinformatics (*IV*)

For mRNA and lncRNA bioinformatic analysis, raw data were first processed through Novogene in-house scripts. Reads containing adapter, poly-N sequences or reads of low quality were removed. Q20, Q30 and GC content of the clean data were calculated. Reference genome (Hg38) and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly. Clean paired-end reads were mapped to the reference genome using HISAT2 software. To identify lncRNA, the mapped reads of each sample were assembled by StringTie and merged through cuffmerge. To identify lncRNAs, low expression level transcripts were filtered out, the exon number was set to >2 and the transcript length set to >200nt. To identify novel lncRNAs (TCONS), the transcripts were analyzed for their protein-coding potential. Coding-Non-Coding-Index, Coding Potential Calculator and Pfam were used predict the coding potential of the transcripts. Transcripts predicted with coding potential by at least one of the three tools were filtered out. Novel genes were predicted by using StringTie and Cuffcompare.

For sRNA (18–35bp) analysis, raw data was cleaned by removing reads with poly-N, 5′ adapter contaminants, without 3′ adapter or the insert tag, containing poly-A/-T/-G or -C and low-quality reads. Remaining reads were mapped to reference sequence by Bowtie. miRBase20.0 was used as reference, modified mirdeep2 and srna-tools-cli were used to obtain the potential miRNAs. Novogene custom scripts were used to obtain the miRNA counts.

Before DE analysis, a filter to exclude biologically non-significant sequences was applied. To be included in the analysis, the mRNA, lncRNA and miRNA transcript had to have more than 1 count per million library counts (CPM>1), to be expressed in at least 21% of the samples (in three samples in the EarlyMT and in seven in the LateMT group). Additionally, for mRNA to be accepted, the transcript needed to have an Ensembl ID and to be recognized as "protein coding" by Ensembl database. For lncRNA, the additional requirements for transcript acceptation were Ensembl ID, Ensembl gene ID and Ensembl biotype "lncRNA".

Accepted mRNA and lncRNA transcripts were collected to gene-level using R package tximport (Soneson et al., 2016). mRNA, lncRNA and miRNA normalization and DE analysis were performed with paired-samples DESeq2.

Over-representation analysis. Over-representation analyses were conducted with gprofiler (Raudvere et al., 2019) and databases of GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) were used as sources. The main transcripts (highest expression level) of all the prefiltered genes were imported into GSEA (Gene Set Enrichment analysis). Analysis was performed with the fgsea R package (Korotkevich et al., 2021) using t-test statistic as the ranking metric and GO Biological Processes and Reactome as databases (MSigDB) (Liberzon et al., 2011). For the GO and KEGG analyses, a DE threshold of $p_{adj} < 0.1$ was used.

Interactions between RNA species. Interactions between mRNA and lncRNA genes and miRNAs were investigated using the QIAGEN Ingenuity Pathway Analysis (IPA) (Krämer et al., 2014). Canonical pathways analysis identified the pathways from the IPA library that were most significant in the data set. Molecules from the data set that met the inclusion criteria (log_2 fold change (LFC) > ± 1.5 and $p < 0.05$) were included for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways: 1) the ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway was calculated; and 2) a right-tailed Fisher's Exact Test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

For My Pathway analysis DE mRNA genes (LFC > ± 1.5 and $p_{adj}<0.05$) and the top 20 expressed miRNAs and lncRNAs in both groups were included. Molecules were represented as nodes, and the biological relationship between two nodes was represented as a line. All the connections were supported by at least one reference from the QIAGEN Knowledge Base. Dashed lines represent an indirect connection, while a solid line represents direct connections. Molecules without interactions to menopausal hormones or downstream functions were removed from the final representation of the results.

4.3.11 Statistics (*I-IV*)

In *Papers I* and *III*, statistical analyses were performed with IBM SPSS Statistics versions 24 or 26 (Chicago, IL, USA). In *Paper II*, analyses were carried out in R, whereas in *Paper IV* both R and SPSS (v.28) were used. All data were checked for normality and parametric tests were used whenever data fulfilled the criteria. Model assumptions were tested with residual plots, Q–Q plots, and correlation analysis. Statistical significance was set to $p \leq 0.05$. For *Paper IV*, $p \leq 0.1$ and multiple testing corrections were also used, whenever appropriate. All data are presented as mean ± SD, unless otherwise stated.

Paper I. The independent samples t-test, chi-squared test and Mann-Whitney U tests were used to compare baseline characteristics between the pre- and perimenopausal groups. The paired t-test, Wilcoxon signed-rank test and marginal homogeneity tests were used to test for differences in baseline characteristics, adipokines and the adiposity variables between baseline and follow-ups. The Wilcoxon signed-rank test was used to test for differences in annual changes within participants at three body composition measurement points. The Friedman test was used to compare lipid droplet size and the lipid accumulation index between the three fiber types. The Wilcoxon signed-rank test was used to assess the difference in relative changes during follow-up in the cell variables. Spearman correlations were calculated and visualized with GraphPad Prism (v.9.1.1) to examine associations between cell and selected adipose tissue variables. Linear mixed-effect models were created to examine the associations between the adiposity measurements and covariates during the follow-ups. Duration of follow-up in years was selected to represent time owing to variation in follow-up times and menopausal status. Therefore the results of the models are annual changes. Models were controlled for education, baseline mean-centered age, mean-centered PA (either self-reported or accelerometer measured), mean-centered DQS, the use of exogenous hormones and the interaction between time and PA (fixed effects). The models were constructed using an unstructured longitudinal correlation matrix, and intercept and time were used as random effects. Longitudinal associations between adipokines and adiposity variables were investigated with similar linear mixed-effect models but without the interaction between time and PA. All variables and covariates were evaluated for outliers (above 3rd quartile + 3*interquartile range or 1st quartile – 3*interquartile range). Based on their extreme values in body adiposity (n = 3), SR-PA (n = 5), ACC-PA (n = 3) and adipokines (n = 3), participants were removed from the analyses. Multicollinearity between covariates was assessed with Variance Inflation Factor analyses. Due to the observational nature of this study with predetermined associations of interest, the results are presented without multiple comparison corrections.

Paper II. The main analyses were conducted using linear mixed-effect models with random intercept. For the outcome variables, the fixed effects were time, menopausal group, ACC-MAD, and interactions between time and menopausal group as well as time and ACC-MAD. The interactions were included in the models to study how the change in PA was associated with the change in the outcome variables during the follow-up. Covariates included as fixed effects were mean-centered baseline age and the use of exogenous sex hormones. Only the use of exogenous sex hormones was controlled for as potentially confounding variable in the models as it could affect menopausal status assignment. Missing data (total amount 741/13 708, equaling 5%) occurred due to invalid or missing measurements, or unclear or incomplete questionnaire responses and was assumed to occur at random. Multiple imputation was used to improve the data

analysis with the "mice" package in R. After complete case analysis, no notable difference was observed in the results.

Paper III. Paired t-test and Wilcoxon rank test were used to test for differences in lean and muscle mass variables between baseline and final follow-up. Generalized estimating equations (GEE) modelling was used to examine associations between the change in lean and muscle mass measurements and covariates in more detail during the follow-up. Models were controlled for baseline progestogen use, duration of HT use, and follow-up time. To further investigate whether PA and age were also significant predictors, they were individually included in the model.

Paper IV. DE analysis was conducted by an experienced bioinformatist using R-package DESeq2 (Love et al., 2014). Spearman correlations were used to investigate the associations of changes in RNA transcript/gene expression with body composition and PA. The changes were calculated for the most abundantly expressed transcript of the studied gene.

5 RESULTS

5.1 Participant characteristics (*I-IV*)

At baseline in the short-term follow-up study, the participants' mean age was 51.6 ± 1.9 years, mean height 1.65 ± 0.06 m, and on average they were slightly overweight (BMI 25.6 ± 3.9 kg/m^2). About half were normal weight at both baseline and follow-up (Table 4). At baseline, 63% had natural bleeding status, 5% had had hysterectomy and the rest were exogenous hormone users (only progesterone at baseline). The bleeding status proportions remained relatively unchanged. Nearly all had at least secondary level education. As expected, the women experienced a decrease in E2 levels and an increase in FSH levels during the study. Mean follow-up time was 1.3 ± 0.7 years.

At baseline in the long-term follow-up the participants' mean age was 51.4 ± 1.7 years, mean height 1.66 ± 0.05 m, and they were also slightly overweight (BMI of 25.4 ± 3.9 kg/m^2). Approximately half of them were overweight or obese at baseline and at follow-up (Table 4). About 60% of the participants had natural bleeding status, 10% had had hysterectomy and 30% were using progesterone at baseline. The bleeding status proportions remained relatively unchanged. Of the participants, nearly all had at least secondary level education. The decrease in E2 and increase in FSH levels was even more apparent in the long-term than in the short-term follow-up. The mean follow-up time in the long-term follow-up was 3.9 ± 0.2 years.

Both cohorts were a representative sample of middle-aged Finnish women (Kekäläinen et al., 2021).

TABLE 4 Participant characteristics in the short- and long-term follow-up studies.

	Short-term follow-up			**Long-term follow-up**		
	Baseline	**Follow-up**	***Change***	**Baseline**	**Follow-up**	***Change***
	n = 230			**n = 148**		
Body mass, kg [a]	69.7±11.1	70.3±11.5	*0.7±2.7* ***	69.6±10.7	71.8±12.1	*2.2±3.6* ***
BMI, kg/m^2 [a]	25.6±3.9	25.8±4.1	*0.3±1.0* ***	25.4±3.9	26.2±4.4	*0.8±1.4* ***
Underweight (<18.5)	0% (0)	0% (0)		0% (0)	1% (1)	
Normal (18.5–24.9)	52% (119)	47% (109)		52% (72)	42% (59)	
Overweight (25–29.9)	34% (77)	37% (86)		36% (50)	37% (52)	
Obese (30->)	15% (34)	15% (35)		12% (17)	19% (27)	
Bleeding status						
Natural status	63% (144)	60% (137)		59% (87)	57% (85)	
Hysterectomy	5% (12)	5% (11)		10% (14)	12% (17)[#]	
Hormone user[b]	32% (74)	36% (82)		32% (47)	31% (46)	
Education						
Primary	3% (6)			1% (1)		
Secondary	52% (120)			55% (82)		
Tertiary	45% (104)			44% (65)		
Sex hormones						
E_2, nmol/L	0.34±0.27	0.24±0.18	*-0.10±0.33* ***	0.48±0.50	0.20±0.21	*-0.28±0.54* ***
FSH, IU/L	36.1±21.7	66.7±28.1	*30.5±31.7* ***	24.1±21.8	80.4±32.0	*56.3±36.4* ***

Values presented as mean ± SD. (n) = number of participants. BMI, body mass index; E2, estradiol; FSH, follicle-stimulating hormone. [a] n = 9 missing from long-term follow-up, [b] includes progestogen use at baseline and both estrogen or/and progestogen use at follow-up, [#]includes n = 1 with surgical menopause. *p < 0.05, **p < 0.01, ***p < 0.001.

Biopsied sub-populations. Altogether 25 participants in the short-term and seven in the long-term follow-up consented to baseline and follow-up muscle biopsies. Five participants consented to muscle biopsies at both the short- and long-term follow-ups, i.e., at three time points in total. In the short-term follow-up study baseline, the biopsied subgroup were on average 52.2 ± 2.1 years old and did not differ from the non-biopsied follow-up participants in any of the studied variables, except for bleeding status and use of exogenous hormones as only participants with natural bleeding status and no estrogen or progesterone use were recruited for biopsies (Supplemental Table 2). Among the long-term follow-up participants (age at baseline 51.3 ± 2.5 years), the biopsied subgroup at baseline had higher FSH levels ($p < 0.05$) than the non-biopsied group but did not differ in any other variables. During both follow-up studies, the changes in background characteristics and sex hormone levels in the biopsied participants did not differ from those of the others in their group (Supplemental Table 2).

5.2 Changes in body composition and metabolic health indicators (*I-III*)

5.2.1 Adiposity and indicators of metabolic health (*I, II*)

During the short-term follow-up, the relative increases in DXA FM variables varied between ~2 and 4%, the most significant relative increase occurring in android and trunk FM (4.3 and 3.8%, respectively) (Table 5). In the mid-thigh, a significant increase in subcutaneous fat area (2.3%) was observed. During the long-term follow-up, the relative increases in the DXA variables varied between ~7 and 14% with the largest in trunk and android FM (both 13.6%) (Table 5). A significant increase in subcutaneous and muscle compartment fat area was also observed in mid-thigh in the long-term follow-up measurement (6.6 and 16%). Muscle density decreased by 7% during the long-term follow-up.

The change in adiposity was also investigated with adjusted models for self-reported and accelerometer PA, separately. The results of *Paper I* analyses are presented in Table 6. The annual increases in total and android FM were ~0.65 and 0.09 kg respectively. These results corresponded well to the results obtained from *Paper II* analyses using a slightly different model (change in total FM during ~ four years +2.87 kg (SR-PA model) and +1.72 kg (ACC-MAD model; change in android FM during ~ four years +0.39 kg (SR-PA model) and +0.26 kg (ACC-MAD model)). The annual increase in android FM was higher than in gynoid area (0.09kg vs. ~0.065 kg, respectively). The increase in trunk FM was also more pronounced than the increase in gluteofemoral area (~0.44 kg/year vs. ~0.26 kg/year). Also leg FM and subcutaneous fat area were found to increase and muscle density decrease around FMP also after adjustment. The results on annual changes in the models with different PA measures were relatively similar.

TABLE 5 Unadjusted changes in fat mass variables during the short- and long-term follow-ups. Relative changes (%) shown in brackets.

	Short-term follow-up			Long-term follow-up		
	Baseline	**Follow-up**	***Change (%)***	**Baseline**	**Follow-up**	***Change (%)***
Total and regional fat	**n = 219**			**n = 132**		
Total FM, kg	25.7±8.8	26.4±9.0	*0.8±2.5*** *(3.1)*	24.4±8.8	27.1±9.6	*2.6±2.8*** *(10.7)*
Total fat percentage	35.8±7.8	36.6±7.5	*0.8±2.4****	34.2±8.0	36.7±7.9	*2.5±2.3****
Trunk FM, kg	13.1±5.4	13.6±5.7	*0.5±1.7*** *(3.8)*	12.5±5.3	14.2±6.0	*1.7±2.0*** *(13.6)*
Gynoid FM, kg	5.0±1.4	5.1±1.4	*0.1±0.5*** *(2.0)*	4.7±1.4	5.0±1.5	*0.3±0.5*** *(6.4)*
Android FM, kg	2.3±1.0	2.4±1.0	*0.1±0.3*** *(4.3)*	2.2±1.0	2.5±1.1	*0.3±0.4*** *(13.6)*
Leg FM, kg	4.5±1.5	4.6±1.5	*0.1±0.5*** *(2.2)*	4.2±1.5	4.5±1.6	*0.3±0.5*** *(7.1)*
Gluteofemoral FM, kg	10.5±3.5	10.9±3.5	*0.3±1.0**** *(2.9)*	10.0±3.4	11.0±3.8	*1.0±1.2**** *(10.0)*
Gluteofemoral fat percentage	36.7±6.8	37.7±6.6	*0.9±2.1**** *(2.5)*	35.1±7.0	37.5±6.7	*2.4±2.2**** *(6.8)*
Android-to-gynoid-ratio	0.45±0.14	0.46±0.14	*0.01±0.04**** *(2.2)*	0.45±0.15	0.50±0.15	*0.05±0.05**** *(11.1)*
Mid-thigh fat	**n = 76**			**n = 17**		
Subcutaneous fat area, cm^2 [a]	64.2±15.8	65.7±17.0	*1.5±3.8*** *(2.3)*	65.0±17.5	69.3±18.7	*4.3±4.1*** *(6.6)*
Muscle compartment AT area, cm^2	9.4±3.0	9.6±3.1	*0.1±1.1* *(1.1)*	8.1±2.2	9.5±1.4	*1.3±1.9** *(16)*
Muscle density, HU	53.1±3.7	53.4±3.9	*0.3±1.7* *(0.6)*	53.7±4.0	50.0±3.1	*-3.7±3.7*** *(-6.9)*

Values presented as mean ± SD. AT, adipose tissue; FM, fat mass; HU, Hounsfield units. [a] n = 1 missing from short-term follow-up. *p < 0.05, **p < 0.01, ***p < 0.001.

TABLE 6 Linear model results for the annual changes in adiposity around menopause.

FM measure	Annual change around menopause[a] (SR-PA)	Annual change around menopause[a] (ACC-MVPA)
Total FM, kg	0.68***	0.63***
Android FM, kg	0.09***	0.09***
Trunk FM, kg	0.45***	0.42***
Gynoid FM, kg	0.07***	0.06***
Gluteofemoral FM, kg	0.27***	0.25***
Leg FM, kg	0.07***	0.06***
Android-to-gynoid-ratio	0.01***	0.01***
Subcutaneous fat area, cm^2	1.05***	1.28***
Muscle compartment fat area, cm^2	-0.14	0.11
Muscle density, HU	-0.23	-0.65***

ACC-MVPA, accelerometer-measured moderate-to-vigorous physical activity; FM, fat mass; HU, Hounsfield unit; SR-PA, self-reported physical activity. [a]Variable estimate associated with the progression of menopausal status during one year. Models adjusted for centered baseline age, education level, follow-up time, hormone use, diet quality, physical activity (SR-PA/ACC-MVPA) and interaction of time and physical activity. ***p < 0.001.

Unadjusted changes in the metabolic health indicators were investigated in the short- and long-term follow-ups (Table 7). In both follow-ups, a small, but significant increase in WC and WHR was observed. In adipokines, in both follow-ups an increase was observed in serum leptin and adiponectin and a decrease in resistin levels.

TABLE 7 Unadjusted changes in indicators of metabolic health in short- and long-term follow-ups.

	Short-term follow-up			Long-term follow-up		
	Baseline	**Follow-up**	***Change***	**Baseline**	**Follow-up**	***Change***
Anthropometrics	**n = 228**			**n = 137**		
WC, cm	83.8±10.6	84.5±10.9	*0.8±4.0* **	82.9±10.3	84.2±11.2	*1.3±3.9* ***
WHR	0.83±0.07	0.84±0.06	*0.01±0.03* ***	0.82±0.07	0.84±0.05	*0.01±0.04* ***
Adipokines	**n = 110**			**n = 68**		
Leptin, ng/ml	42.4±30.5	50.4±38.2	*8.0±18.2* ***	40.8±30.2	54.3±39.6	*13.5±23.2* ***
Adiponectin, ng/ml	16644±6232	18475±7730	*1831±4285* ***	16510±6908	19669±8979	*3159±6241* ***
Resistin, pg/ml	18842±7958	17243±7556	*-1599±5723* **	20481±9053	17946±7575	*-2536±7353* *

Values presented as mean ± SD. WC, waist circumference; WHR, waist-to-hip ratio. *p < 0.05, **p < 0.01, ***p < 0.001.

The results of the adjusted linear-mixed models for WC, WHR and adipokines are presented in Table 8. WC increased on average by 1.49 cm during transition from pre- or perimenopause to postmenopause, when the model was adjusted with SR-PA. For WHR, after adjusting for confounders, postmenopausal status was no longer a significant contributor. For leptin, the menopausal progression was positively associated with an annual increase of 1.4 ng/ml in serum levels also when adjusted for total, gynoid and gluteofemoral FM, but not android FM (in Table 8 results shown only for models adjusted with total FM). For adiponectin, the progression of menopausal status was associated with an increase in adiponectin levels in all the FM-adjusted models, and in total FM adjusted model the annual increase was 623 ng/ml. In contrast, resistin levels were negatively associated with the menopausal progression in all the FM adjusted models and the annual decrease was on average 628 pg/ml, when the linear model was adjusted with total FM.

TABLE 8 Adjusted results for changes in metabolic health indicators in women transitioning from pre/perimenopause to postmenopause.

Metabolic health indicator	Annual change around menopause[a]	Change due to pre/peri- to postmenopause transition[b] (SR-PA)	Change due to pre/peri- to postmenopause transition[b] (ACC-MAD)
Waist circumference, cm	-	1.49**	0.44
Waist-to-hip ratio	-	0.60	-0.75
Leptin, ng/ml	1.4*	-	-
Adiponectin, ng/ml	623***	-	-
Resistin, pg/ml	-628***	-	-

ACC-MAD, accelerometer-measured mean amplitude deviations; SR-PA, self-reported physical activity. [a] Models adjusted for total fat mass, centered baseline age, education level, follow-up time (in years), exogenous hormone use, diet quality, and SR-PA. [b] Models adjusted for menopausal group, physical activity (SR-PA/ACC-MAD), age at baseline, exogenous hormone use, time (measurement point), time x group, and time x physical activity interactions. - not studied. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The body composition of altogether 60 women was measured in the both in short- and long-term follow-up. Of them, one was later excluded as outlier, while CT data was available from only 14 participants. Figure 5 presents the mean values of the adiposity variables in the three timepoints (B, F1 and F2) and the calculated median annual change between the transition from perimenopause to early postmenopause (FU1) and from early postmenopause to later menopause (FU2). The rate of change was similar between FU1 and FU2 in all other adiposity variables except for muscle density, where the decrease (lipid accumulation to the muscle) was significantly higher during FU2.

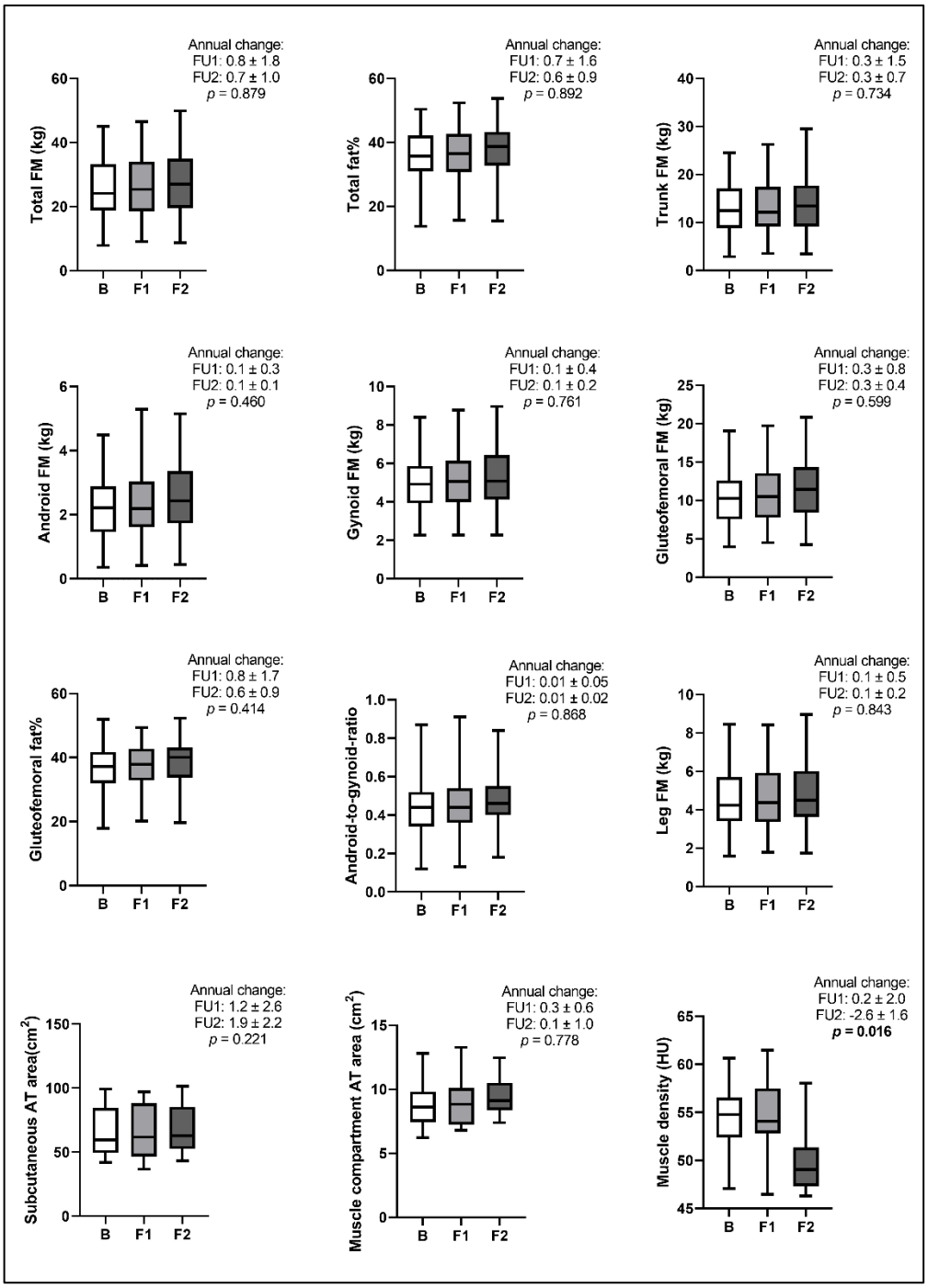

FIGURE 5 Body adiposity variables of participants assessed at three measurement points (n = 59 for the three upper row and n = 14 for the bottom row variables). Box plots show the mean (vertical line), interquartile ranges and max- and min-values (upper and lower limits). Annual changes are presented as median ± SD. AT, adipose tissue; B, baseline measurement; F1, short-term final follow-up measurement; F2, long-term follow-up measurement; FM, fat mass; FU1, follow-up from perimenopause to early postmenopause (time between B and F1); FU2, follow-up from early postmenopause to later postmenopause (time between F1 and F2); HU, Hounsfield unit. Significant p-values ($p < 0.05$) between annual changes are highlighted.

5.2.2 Lean and muscle mass (*III*)

During both follow-ups, a significant decline was observed in all the DXA-measured LM variables (unpublished data from long-term follow-up) as presented in Table 9. The largest decrease was observed in the appendicular region, where ALM, ALMI and leg LM decreased by 1.1%, 1.1% and 1.5% during the short-term follow-up and 2.7%, 3.0% and 2.9% during the long-term follow-up, respectively. In the mid-thigh muscle measures, a decrease was also observed in both absolute and relative muscle area, the most significant of which was in the absolute muscle area (-4.3%) during the long-term follow-up.

TABLE 9 Unadjusted changes in lean and muscle mass measures during the short- and long-term follow-ups. Relative changes (%) shown in brackets.

	Short-term follow-up			**Long-term follow-up**		
	Baseline	**Follow-up**	***Change (%)***	**Baseline**	**Follow-up**	***Change (%)***
Total and regional lean mass	**n = 219**			**n = 132**		
LBM, kg	41.7±4.4	41.5±4.4	*-0.2±1.3* (-0.5)*	42.5±4.1	42.1±4.3	*-0.4±1.6** (-1.0)*
LBMI, kg/m²	15.3±1.3	15.2±1.3	*-0.1±0.5* (-0.7)*	15.5±1.3	15.3±1.4	*-0.2±0.6** (-1.3)*
ALM, kg	18.0±2.2	17.8±2.2	*-0.2±0.8*** (-1.1)*	18.4±2.1	17.9±2.1	*-0.5±0.9*** (-2.7)*
ALMI, kg/m²	6.6±0.6	6.5±0.6	*-0.1±0.3*** (-1.1)*	6.7±0.7	6.5±0.6	*-0.2±0.3*** (-3.0)*
Gluteofemoral LM, kg	17.5±2.1	17.4±2.1	*-0.2±0.5*** (-1.1)*	17.7±2.0	17.6±2.0	*-0.2±0.7** (-1.1)*
Leg LM, kg	6.8±0.9	6.7±0.8	*-0.1±0.4** (-1.5)*	6.9±0.8	6.8±0.8	*-0.2±0.4*** (-2.9)*
Mid-thigh muscle	**n = 76**			**n = 17**		
Absolute muscle area, cm²	166.8±9.5	165.1±10.0	*-1.6±3.9*** (-1.0)*	168.0±11.4	160.8±8.5	*-7.2±5.8*** (-4.3)*
Relative muscle area (%) [a]	69.6±5.6	68.9±6.1	*-0.7±1.5*** (-1.0)*	69.8±6.1	67.4±6.2	*-2.3±1.7*** (-3.3)*

Values presented as mean ± SD. ALM, appendicular lean mass; ALMI, appendicular lean mass index; LBM, total lean body mass; LBMI, total lean body mass index; LM, lean mass.
[a] n = 1 missing participant in short-term follow-up. *p < 0.05, **p < 0.01, ***p < 0.001.

In the short-term follow-up, the longitudinal change in LM measures was studied using a GEE model. Table 10 presents the results of adjusted models, with either self-reported or accelerometer-measured PA as the PA measure. The progression of menopausal transition from peri- to postmenopause was a significant negative contributor to all the studied LM variables. For example, the progression from peri- to postmenopause was associated with 0.20 kg decrease in LBM and more than 1 cm² decrease in absolute muscle area.

TABLE 10 Adjusted generalized estimating equation model variable estimates for the change in menopausal status in models investigating associations with lean and muscle mass measures in the short-term follow-up study.

Lean and muscle mass measure	Change due to peri- to postmenopause transition (SR-PA)	Change due to peri- to postmenopause transition (ACC-MVPA)
LBM, kg	-0.203*	-0.231*
LBMI, kg/m^2	-0.073*	-0.086*
ALM, kg	-0.238***	-0.292***
ALMI, kg/m^2	-0.088***	-0.108***
Leg LM, kg	-0.091**	-0.114***
Absolute muscle area, cm^2	-1.6**	-1.8**
Relative muscle area (%)	-0.7***	-0.7**

ALM, appendicular lean mass; ALMI, appendicular lean mass index; LBM, total lean body mass; LBMI, lean body mass; LM; lean mass. Models adjusted for menopausal status, estrogen therapy use time, baseline use of progestogen (except for absolute and relative muscle areas), follow-up time, age at baseline and either self-reported (SR-PA) or accelerometer-measured moderate-to-vigorous physical activity (ACC-MVPA). *p < 0.05, **p < 0.01, ***p < 0.001.

Biopsied sub-populations. The baseline values of the body composition and metabolic health indicators did not differ between the non-biopsied and biopsied subgroups in the short-term follow-up. In the long-term follow-up, only the absolute muscle area was observed to differ between the biopsied subgroup and the non-biopsied subgroups (non-biopsied subgroup: 168.0 ± 11.4 cm^2; biopsied subgroup: 183.8 ± 3.8 cm^2, $p < 0.05$). During the follow-ups, the changes in the adiposity variables in the biopsied and non-biopsied study groups did not differ from each other in either of the studies. The relative changes in the LM variables in the short-term follow-up did not differ between the whole and biopsied population. In the long-term follow-up, a larger decrease in absolute muscle area was observed in the biopsied subgroup (non-biopsied group: -7.2 ± 5.8 cm^2; biopsied subgroup: -14.8 ± 3.3 cm^2, $p < 0.05$). The changes in adipokines, WC, and WHR were similar in the biopsied and non-biopsied subgroups.

5.3 Skeletal muscle tissue analysis (*I, III, IV*)

5.3.1 Morphological changes (*I, III*)

5.3.1.1 Histological analysis of fiber size and type (*I, III*)

Skeletal muscle fiber distribution was investigated in the short- and long-term follow-ups. Table 11 presents the results of the short-term follow-up, where the fiber types were classified into two subtypes (type I and type II) based on

immunohistochemical staining. At both in baseline and follow-up, the ratio was approximately 50:50 and no change was found in type-specific fiber size during the follow-up. However, at both baseline and follow-up, type I fibers were larger than type II fibers ($p < 0.001$).

TABLE 11 Distribution and size of type I and II muscle fibers in the short-term follow-up.

	Short-term follow-up		
	Baseline	**Follow-up**	***Change***
	n = 7		
Type I (μm²)	3526±1334	3525±1618	*-1±1297 (NS)*
% of all fibers	53.3±10.9	51.1±14.4	*-2.2±7.7 (NS)*
Type II (μm²)	2098±948	2399±1218	*301±469 (NS)*
% of all fibers	46.7±10.9	48.9±14.4	*2.2±7.7 (NS)*
P-value for difference in size between cell types	<0.001	<0.001	

Values presented as mean ± SD. NS, difference not significant.

Fiber type distribution was also investigated using a immunohistochemical staining to separate all three main fiber types (type I, type IIA and type IIX) at the short-term follow-up study baseline (n = 10), short-term follow-up study follow-up measurements (n = 8) and long-term follow-up study follow-up measurements (n = 7) (unpublished data). In these samples, type I fibers constituted ~70% of all fibers, type IIA ~24% and type IIX ~6% of all fibers. No change between baseline and follow-up was observed in this distribution during the two follow-ups.

5.3.1.2 Electrophoretic analysis of fiber type (*III*)

In the short-term follow-up, myosin isoform distribution was also studied in muscle homogenates with SDS-PAGE (n = 25 sample pairs for baseline and follow-up). At both baseline and at follow-up, the main isoform was found to be type I MHC (~52%), with 41% type IIA and ~7% MHC IIX. No change was observed in the distributions during the follow-up.

5.3.1.3 Metabolic capacity and lipid droplet accumulation (*I*)

Muscle fiber oxidative and glycolytic capacities were investigated in muscle tissue sections in both follow-up studies. The enzymatic staining results were paired with serial sections stained against type I or II fibers. At all time points (n = 10 for baseline, n = 8 for short-term follow-up study follow-up measurement and n = 7 for long-term follow-up study follow-up measurement), a significant difference was observed between the fiber types in their metabolic capacities. Type I fibers, as expected, had higher oxidative capacity, whereas type II fibers had higher glycolytic capacity ($p < 0.05$). When the longitudinal changes in metabolic capacities were investigated (n = 8 for the short-term and n = 7 for the long-

term follow-up), no change was observed in the relative oxidative capacity of the two fiber types (Figure 6A), whereas type II fibers showed a small increase in glycolytic capacity at the long-term follow-up (Figure 6B).

LD area and LAI were investigated in sections also stained for fiber type (types I, IIA and IIX). Stainings were conducted for samples from the short- and long-term follow-ups. At baseline, the lipid droplets were significantly larger in type I fibers than in type IIA and IIX fibers (I: 0.79 ± 0.35; IIA: 0.69 ± 0.19; IIX 0.60 ± 0.34 μm^2, $p < 0.05$), but in other timepoints no difference was observed. At all individual time points, LAI was always largest (approximately two to three times larger than in type IIX with the lowest LAI) in type I fibers ($p < 0.01$). When the relative changes during the follow-ups were investigated, fiber type-specific LD area (Figure 6C) and LAI (Figure 6D) remained unchanged.

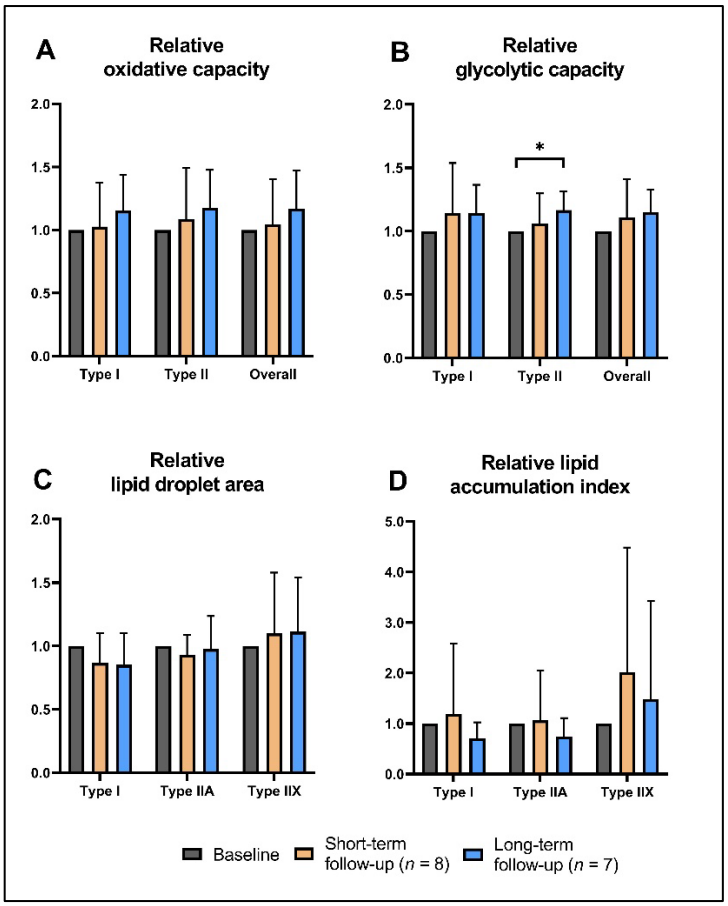

FIGURE 6 Relative oxidative and glycolytic capacities and lipid measures of different muscle fiber types and muscle tissue overall during the short- and long-term follow-ups. Baseline value is set to one and the follow-up values are compared to that. *p<0.05.

5.3.1.4 Correlations between muscle fiber characteristics and total body composition (*I*)

Correlations between tissue-level body composition variables with cellular metabolic and lipid accumulation variables were compared at different

timepoints: at the short-term follow-up baseline (Baseline), short-term follow-up study follow-up (F1), and at long-term follow-up study follow-up (F2). At baseline, significant positive correlations were found for all the fiber adiposity variables with at least one studied DXA variable (Figure 7). At F1 and F2, correlations with the body composition variables were found only for LD area in type I fibers, LAI I and LAI IIA. Significant correlations with metabolic capacities were found at single time points (data not shown). At F1, leg FM correlated positively with the glycolytic capacity of type II cells and total tissue (for both r_s ~ 0.8, $p < 0.05$).

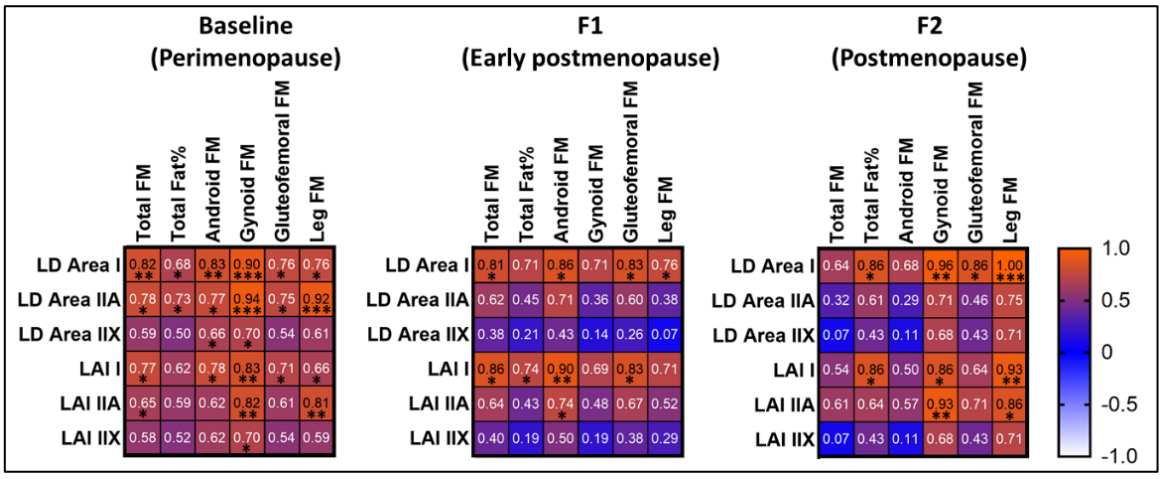

FIGURE 7 Correlations between cell and body adiposity variables in perimenopause, early postmenopause and postmenopause. FM, fat mass; LAI, lipid accumulation index; LD, lipid droplet. Orange color indicates high positive correlation, whereas blue color indicates smaller positive correlation. * p< 0.05, ** p<0.01, ***p<0.001 (image originally published in *Paper I*).

5.3.2 Muscle transcriptome (*IV*)

5.3.2.1 Sample quality

Before sequencing, RNA sample quality was assessed by using integrity value. After sequencing, the sample quality was estimated using the mean Q30-value. Both of these measures indicated good or high-quality data. The sequence length distribution of sRNA was checked for each sample to ensure enrichment of miRNAs. Due to its distorted sequence length distribution and lowest integrity value, one sample pair was excluded from both the sRNA and long-RNA analysis. Thus, the final n of the samples for further analysis was 48, comprising 24 longitudinal sample pairs.

5.3.2.2 Characteristics of transcriptome data

mRNA and lncRNA. Altogether 88 966 different transcripts were regarded as mRNAs. After filtering, 12 503 different mRNA genes were observed in the data. Of these, 314 were only expressed in the EarlyMT and 174 only in the LateMT groups. The majority of the exclusive genes were relatively low-expression level

genes (basemean per PERI or POST group < 100). Of the most abundantly expressed mRNA genes, the top 20 included, for example, *MYH7*, *ACTA1*, *MT-CO1*, *CKM* and *DES*. Additionally, the normalized expression level of hormone receptors and steroidogenic enzymes were investigated, and mRNA for *ESR1, ESR2, GPER1, AR*, *PGR, HSD17B1, HSD17B4, HSD17B5* and *SRD5A1* were observed. In addition, very low-expression level (CPM < 1 and/or present only in few samples) reads for *FSHR* and steroidogenic enzymes *HSD3B2*, *HSD17B2*, *CYP19A1* were found.

Of all the long RNA reads, ~27 500 reads were assigned to be lncRNAs. After filtering, altogether 8 279 genes were left for further analysis. Of these, 710 were only expressed in EarlyMT, while 452 genes were unique to LateMT. As in the mRNAs, the uniquely expressed genes were low expression. The 20 most abundantly expressed lncRNAs in both groups included, for example, NORAD, MALAT1, XIST, NEAT1, MIR1-1HG, H19, SNHG14, KCNQ1OT1, SNHG5, SNHG16, MIR133A1HG, MIR193BHG, and five genes (e.g., AC093010.2 and AC087190.3), whose function is yet not well understood.

sRNA. Altogether 1 599 miRNAs were found from the data. After filtering, 397 miRNAs were left for further analysis. Of these, 28 miRNAs were only expressed in EarlyMT and seven exclusively in LateMT. The 20 most abundant miRNAs in both groups constituted 97% of all miRNA reads and included, for example, several let-7-family members, miR-21-5p, -26a-5, -30d-5p, -99a-5p, -126-3p, -148a-3p, -378a-3p, -378c, -378d and four myo- or muscle enriched miRNAs (miR-1-3-p, -133a-3p, -206 and -486-5p).

5.3.2.3 Differential expression analysis

In total, 49 DE mRNA genes were found, 30 in EarlyMT and 19 in LateMT (for all p_{adj} <0.05 and LFC ≥ ± 1.5) (Table 12). The DE genes in EarlyMT included, for example, *ELN, PRKCA*, steroidogenic enzyme *SRD5A1*, and apoptosis-linked *PIDD1*. The DE genes in LateMT included molecular switch regulator *NUDT4, ECM1*, and the negative regulators of apoptosis *NAA35* and *BIRC6,* among others. In addition, many transcription regulators were also observed, including *GTFG2F2, E2F3, SLFN11, MAFK, ZEB1* and zinc fingers *ZNF84* and *ZNF611*. The DE genes also included *MKNK1, JAK2 MYD88*, which are components of the important signaling pathways related to growth and protein synthesis in muscle.

TABLE 12 Differentially expressed mRNA genes (post vs. peri) in the EarlyMT and LateMT groups.

EarlyMT

Upregulated					**Downregulated**				
Gene	**LFC**	**PERI**	**POST**	**p_{adj}**	**Gene**	**LFC**	**PERI**	**POST**	**p_{adj}**
ELN	12.4	11 ± 27	121 ± 179	0.012	***EXTL3***	-9.6	1123 ± 760	282 ± 458	0.004
GTF2F2	11.9	61 ± 149	122 ± 193	0.024	***KIAA0355***	-9.2	756 ± 182	361 ± 421	0.002
PRKCA	8.5	167 ± 256	276 ± 175	0.035	***VPS28***	-7.7	49 ± 31	21 ± 36	0.004
PYCR1	8.5	31 ± 54	70 ± 64	0.018	***ZNF84***	-6.6	133 ± 72	47 ± 65	0.005
SESN2	8.4	24 ± 38	63 ± 47	0.019	***PIDD1***	-5.7	44 ± 43	1 ± 1	0.005
INTU	6.7	57 ± 68	120 ± 67	0.041	***NLRC5***	-5.0	93 ± 91	3 ± 5	0.024
TMEM39B	6.3	22 ± 35	77 ± 39	0.034	***AC093512.2***	-4.9	455 ± 867	38 ± 59	0.004
SRD5A1	6.3	46 ± 58	126 ± 20	0.004	***ALMS1***	-4.2	474 ± 377	173 ± 374	0.004
CHPF2	6.0	63 ± 102	146 ± 129	0.047	***C1QTNF9***	-3.9	71 ± 38	20 ± 30	0.035
MTHFSD	5.5	29 ± 47	143 ± 60	0.004	***SGSH***	-2.9	54 ± 26	10 ± 10	0.024
MYD88	5.4	2 ± 4	23 ± 14	0.007	***MGLL***	-2.8	884 ± 994	31 ± 12	0.037
APC	4.7	1 ± 1	662 ± 771	0.010	***GBP5***	-2.6	76 ± 81	59 ± 130	0.037
TOM1L2	4.5	37 ± 34	586 ± 588	0.043	***MAFK***	-2.3	677 ± 303	218 ± 305	0.009
TMEM120B	4.0	178 ± 261	643 ± 364	0.008	***RBMS1***	-1.8	349 ± 101	145 ± 134	0.038
ATP5MC2	1.9	325 ± 209	890 ± 450	0.007					
JAK2	1.7	307 ± 183	1104 ± 716	0.024					

LateMT

Upregulated					**Downregulated**				
Gene	LFC	PERI	POST	p_{adj}	**Gene**	**LFC**	**PERI**	**POST**	**p_{adj}**
NUDT4	14.6	430 ± 984	639 ± 1278	0.002	***E2F3***	-12.2	82 ± 108	32 ± 94	0.022
SLFN11	11.1	103 ± 180	160 ± 167	0.005	***NAA35***	-9.6	260 ± 228	216 ± 324	<0.001
AL136295.3	11.0	53 ± 116	115 ± 125	0.005	***ZNF611***	-6.9	79 ± 76	32 ± 54	<0.001
MKNK1	8.9	80 ± 111	173 ± 122	<0.001	***SLC22A17***	-4.4	19 ± 16	5 ± 13	0.005
ARHGAP19	8.6	48 ± 77	117 ± 115	0.033	***TLK2***	-3.1	126 ± 157	51 ± 116	0.017
ZNF761	6.2	44 ± 56	86 ± 39	<0.001	***BIRC6***	-2.9	300 ± 249	77 ± 131	0.003
LYPLA1	5.4	53 ± 107	130 ± 177	0.016	***ZEB1***	-2.7	1271 ± 938	550 ± 719	0.005
KANK3	5.0	29 ± 71	75 ± 113	0.036	***GORASP1***	-1.7	160 ± 117	48 ± 45	0.004
PTPN20	4.8	27 ± 48	41 ± 48	0.002					
ECM1	2.4	12 ± 16	34 ± 22	0.019					
ULK3	1.7	74 ± 84	173 ± 117	0.012					

Expression presented as group basemean ± standard deviation. EarlyMT, participants in the early menopausal transition at baseline; LateMT, participants in the late menopausal transition at baseline; LFC, log2 fold change; PERI, perimenopause; POST, postmenopause.

No DE genes were found for lncRNAs and miRNAs. However, at the transcript level, ten DE lncRNA transcripts were observed ($p_{adj} < 0.05$ and LFC $\geq \pm 1.5$). In EarlyMT, transcripts from OSER1-DT, MALAT1 and AC025171.1 were downregulated. In LateMT, transcripts from BAIAP2-DT and LINC02541 were upregulated and AC083798.2, AL050309.1, LINC00667, IQCH-AS1 and ENTPD1-

AS1 were downregulated when comparing postmenopausal samples to perimenopausal samples.

5.3.2.4 Overrepresentation analysis of differentially expressed mRNAs

The potential functions of the DE mRNAs were investigated using GO, KEGG, GSEA and IPA analyses. Since the number of DE genes was relatively small, to describe the potential functions more widely, a less stringent p-value was used for these analyses. This resulted in including 96 genes for EarlyMT and 39 genes for LateMT for GO and KEGG-analyses. Genes imported from EarlyMT were enriched ($p_{adj} < 0.1$) in GO-terms "protein binding", "macromolecule catabolic process", "catalytic complex", "cytosol", "cytoplasm" and "transferase complex". Genes imported from LateMT were enriched in the terms "prostaglandin receptor activity", "prostanoid receptor activity" and "cytosol".

In GSEA analysis, EarlyMT genes were enriched ($p_{adj} < 0.1$) in GO terms "external encapsulating structure organization", "cell substrate adhesion", "receptor mediated endocytosis", "collagen biosynthetic process" and "regulation of cell substrate adhesion" and in Reactome in terms "ECM organization", "interferon signaling", "degradation of the ECM" and "regulation of insulin like growth factor transport and uptake by insulin like growth factor binding proteins". In LateMT, genes were enriched in GO terms "cell substrate adhesion" and "external encapsulating structure organization", whereas in Reactome results, genes belonging to terms "ECM organization", "syndecan interactions" and "ECM proteoglycans" were enriched ($p_{adj} < 0.1$).

IPA was conducted to predict possible associations between menopausal hormones, DE genes, the top 20 regulatory RNAs and muscle downstream functions. To explore the potential pathways more widely, all the RNA molecules with unadjusted $p < 0.05$ and LFC ± 1.5 were imported for the core analysis. Thus, 267 RNA molecules for EarlyMT and 133 for LateMT were included. In EarlyMT, the top 15 significant and relevant canonical pathways included, e.g., oxidative phosphorylation, renin-angiotensin signaling, androgen signaling, estrogen receptor signaling, mitochondrial dysfunction and sirtuin signaling pathways (Figure 8A). Renin-angiotensin signaling, IL-13 signaling, estrogen receptor signaling and, for example, white adipose tissue browning were found to be activated, whereas sirtuin signaling was predicted to be downregulated. For LateMT (Figure 8B), the pathways of e.g., lipoate salvage and modification, asparagine biosynthesis, apoptosis signaling, and D-myo-inositol-5-phosphate metabolism were enriched ($p < 0.05$).

For the My Pathway analysis, the 49 DE genes from Table 12 were the target of interest. Regarding the upstream regulation of these genes, E2 was predicted to regulate *E2F3, APC, PRKCA, JAK2* and *MGLL*, while P4 was predicted to regulate *E2F3* and *MYD88*. E2- and P4-regulated genes were found to be associated with, for example, muscle cell apoptosis, cell death and proliferation, muscle hypertrophy and glucose metabolism. When the top 20 expressed regulatory RNAs were included in the analysis, miR -21 and -26 were found to be downstream targets of estrogenic regulation and had known causal effects on

muscular hypertrophy, muscle cell death and apoptosis. miR-21 was found to regulate *MYD88*, while miR-26 was downstream regulated by *E2F3* in the IPA analysis. While miR-1, -133 and -378 and lncRNAs MALAT1, SNHG14 and KCNQ1OT1 did not have previously known associations with the upstream regulator hormones, they were connected to muscle cell proliferation, muscle apoptosis, muscle cell death and muscular hypertrophy. In EarlyMT, muscle cell apoptosis and cell death were predicted to be activated, while glucose metabolism disorder and muscle cell proliferation were predicted to be inhibited (Figure 8C). The measured data from LateMT predicted that muscle cell proliferation, cell death and glucose metabolism disorder were inhibited, while muscle cell apoptosis was expected to be activated (Figure 8D).

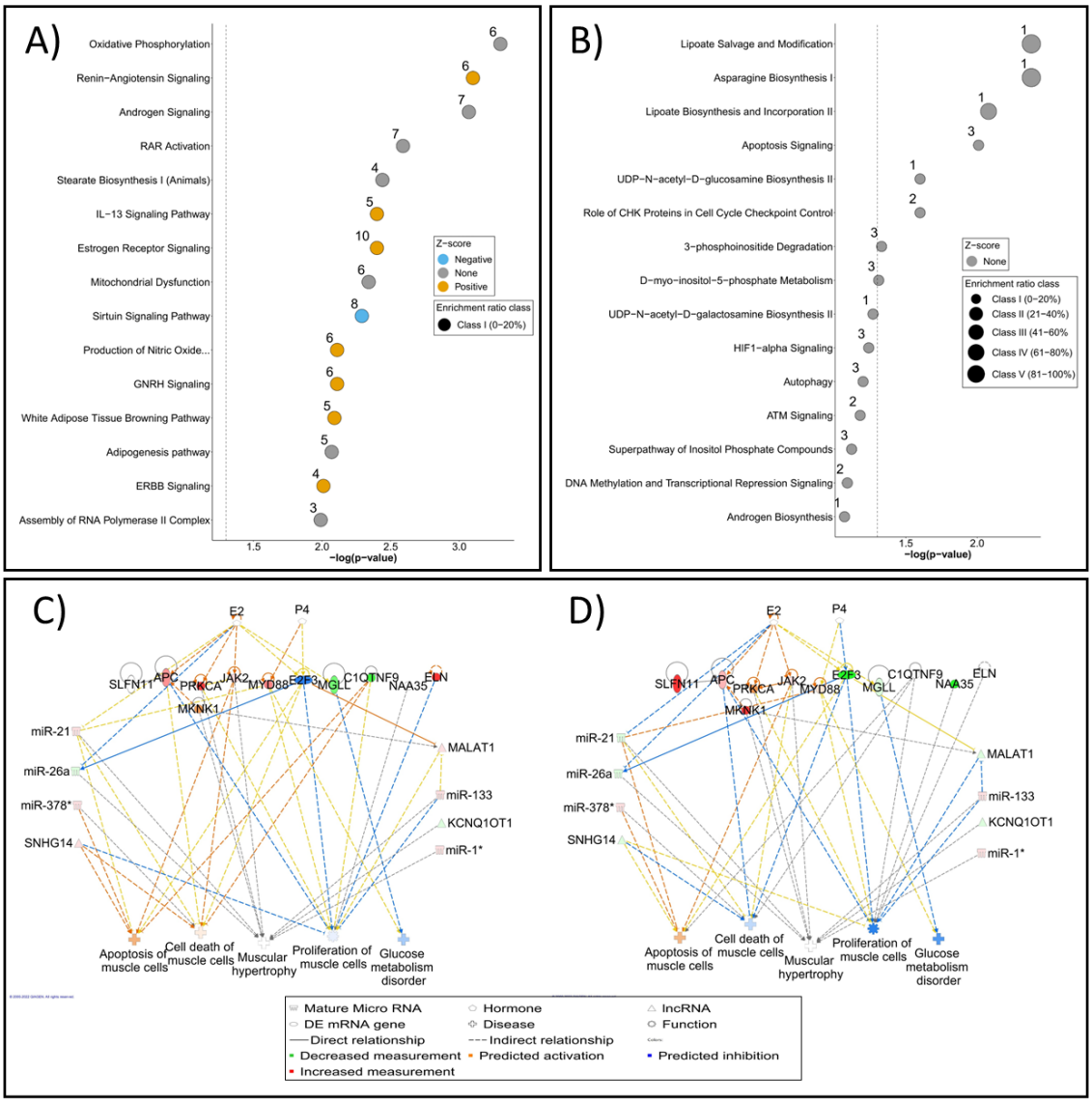

FIGURE 8 Results of the Ingenuity Pathway Analysis. Top 15 enriched canonical pathways in EarlyMT (A) and LateMT (B) and results of My Pathway analysis for predicted upstream regulatory hormones, differentially expressed genes, top 20 regulatory miRNAs and lncRNAs and their downstream functions using EarlyMT (C) and LateMT (D) measured data.

5.3.2.5 Correlation analysis of RNAs with adiposity and lean and muscle mass variables

mRNAs. Several correlations were found for the changes (Δ= POST - PERI) in mRNA expression and the body composition variables (Table 13). In EarlyMT, Δ*PIDD1*, Δ*INTU,* and Δ*C1QTNF9* were found to correlate positively with ΔAndroidFM and ΔLegFM, whereas Δ*GTF2F2* and Δ*C1QTNF9* correlated positively with the changes in LBM, LegLM and ALM. Δ*MAFK* and Δ*ALMS1* were found to correlate negatively with the changes in at least two adiposity variables. In LateMT, the change in *ZEB1* was found to correlate positively with the change in android and gynoid FM. The change in *KANK3* correlated positively with muscle area and negatively with gynoid FM, while the change in *SLFN11* correlated positively with ΔSAT and negatively with ΔMuscle density.

TABLE 13 Associations between the changes in differentially expressed mRNA gene expression levels and body composition variables in EarlyMT and LateMT.

	EarlyMT		**LateMT**	
Variable	**Positive association**	**Negative association**	**Positive association**	**Negative association**
Total FM	-	MAFK	-	
Android FM	PIDD1	ALMS1	ZEB1	-
Gynoid FM	-	EXTL3	ZEB1	KANK3
Gluteofemoral FM	-	MAFK	-	-
Leg FM	C1QTNF9 INTU	ALMS1	-	-
Mid-thigh SAT area	-	MAFK	SLFN11	-
Muscle density	-	-	-	SLFN11
Total LBM	GTF2F2	-	-	-
ALM	GTF2F2 C1QTNF9	TMEM39B	-	-
Leg LM	GTF2F2 C1QTNF9	TMEM39B	-	-
Mid-thigh muscle area	-	-	KANK3	-

ALM, appendicular lean mass; EarlyMT, participants in the early menopausal transition at baseline; FM, fat mass; LateMT, participants in the late menopausal transition at baseline; LBM, lean body mass; LM, lean mass; SAT, subcutaneous adipose tissue. In all shown associations $p < 0.05$ and $r_s < -0.49$ or > 0.49.

lncRNAs. Although no DE lncRNA genes were found, the associations between the change in top 20 lncRNA gene main transcript expression and body composition variables were still investigated (Table 14). In EarlyMT, increased adiposity was associated with increases in ΔMIR1-1HG, ΔNORAD and ΔAC006064.5, whereas negative correlations with increased adiposity were

observed with ΔAC087190.3, ΔMIR193BHG and ΔSNHG14. Decreased lean and muscle mass was associated with decreases in AC068700.1 and MALAT1 and with an increase in AC093010.2. In LateMT, increased adiposity was associated with increased expression of AC068700.1, AC069360.1 and MALAT1. The decrease in lean and muscle mass variables was associated with decreases in AC087190.3, KCNQ1OT1, MIR133A1HG and NUTM2A-AS1. SNHG5 was the only lncRNA gene correlating negatively with body composition variables in LateMT (ΔLM and ΔALM).

miRNAs. The correlations between the changes in the top 20 expressed miRNAs and body composition variables were also investigated (Table 14). In EarlyMT, increased adiposity was associated with decreased expression of miR-21-5p, -26a-5p, -99a-5p, -126-3p, let-7a-5p, let-7g-5p and let-7i-5p. Decreased lean and muscle mass was associated with decreased expression of miR-1-3p, -21-5p, -30d-5p, -206 and -378a-3p, and with increased expression of miR-26a-5p. In LateMT, all the correlations found were positive for both fat and lean mass variables. Increased adiposity correlated with changes in miR-1-3p, -26a-5p, -30d-5p, -99a-5p, let-7g-5p and let-7a-5p. ΔmiR-148a-3p correlated positively with ΔLBM.

TABLE 14 Associations between the changes in the long non-coding and microRNA expression levels and body composition variables in EarlyMT and LateMT.

	EarlyMT		LateMT	
Variable	**Positive association**	**Negative association**	**Positive association**	**Negative association**
Total FM	MIR1-1HG	AC087190.3 miR-126-3p	MALAT1 AC069360.1 AC068700.1 miR-26a-5p miR-30d-5p	-
Android FM	-	miR-26a-5p miR-126-3p	AC069360.1 **miR-1-3p** miR-26a-5p	-
Gynoid FM	NORAD	let-7a-5p miR-99a-5p let-7g-5p let-7i-5p miR-21-5p	AC068700.1 AC069360.1 miR-30d-5p	-
Gluteofemoral FM	MIR1-1HG	AC087190.3 miR-21-5p	MALAT1 **miR-1-3p** miR-99a-5p	-
Leg FM	AC006064.5	SNHG14	MALAT1	-
Mid-thigh SAT area	MIR1-1HG	AC087190.3 MIR193BHG miR-99a-5p	let-7a-5p let-7g-5p	-
Total LBM	**miR-1-3p** miR-378a-3p **miR-206** miR-30d-5p miR-21-5p	-	miR-148a-3p	-
ALM	AC093010.2 miR-378a-3p **miR-206**	-	AC087190.3	SNHG5
Leg LM	AC093010.2 miR-378a-3p **miR-206** miR-30d-5p	AC068700.1	KCNQ1OT1 NUTM2A-AS1	SNHG5
Mid-thigh muscle area	-	MALAT1 miR-26a-5p	MIR1331HG	-

ALM, appendicular lean mass; EarlyMT, participants in the early menopausal transition at baseline; FM, fat mass; LateMT, participants in the late menopausal transition at baseline; LBM, total lean body mass; LM, lean mass; SAT, subcutaneous adipose tissue. In all shown associations $p < 0.05$ and Rs <-0.49 and >0.49. Bolded microRNAs are myomiRNAs.

5.4 Associations of lifestyle habits with body composition, metabolic health indicators and muscle RNA signaling (*I-IV*)

The participants in both longitudinal studies were relatively active, as at group mean level they fulfilled the national recommendations for weekly MVPA (150 minutes of moderate or 75 minutes of vigorous PA) (Table 15). While no change was observed in the level of self-reported PA, a modest decrease in ACC-MVPA was observed during the long-term follow-up. Diet quality score remained unchanged in both studies. Thirty-seven of the participants in the short-term follow-up and 27 participants in the long-term follow-up started using estrogen containing HT during the follow-up period. None of the biopsied participants in the follow-ups started using HT.

TABLE 15 Physical activity, diet quality and the use of exogenous hormones characteristics of the participants in the short- and long-term follow-ups.

	Short-term follow-up			**Long-term follow-up**		
	Baseline	**Follow-up**	***Change***	**Baseline**	**Follow-up**	***Change***
Lifestyle habits	**n = 230**			**n = 148**		
SR-PA, MET-h/day[a]	4.5±4.0	4.7±3.7	*0.2±3.0*	5.3±4.6	4.9±3.8	*-0.4±3.7*
ACC-MVPA, min/day[b]	51.9±29.6	49.8±23.9	*-2.1±24.7*	54.4±32.6	48.4±28.9	*-6.0±28.2* *
ACC-MAD, mg[c]	28.6±10.2	27.9±8.6	*-0.7*±8.4	29.7±11.2	27.8±8.2	*-1.9±7.9*
Diet quality score[d]	6.0±2.2	5.9±2.2	*-0.1±1.6*	5.8±2.5	5.8±2.3	*-0.01±1.9*
Use of exogenous hormones						
None	68% (156)	64% (148)		68% (100)	66% (98)	
E	0% (0)	1% (2)		0% (0)	3% (4)	
P	32% (74)	20% (45)		32% (48)	16% (23)	
E + P	0% (0)	15% (35)		0% (0)	16% (23)	

Values presented as mean ± SD. (n) = number of participants. ACC-MAD, accelerometer measured physical activity in mean amplitude deviations; ACC-MVPA, accelerometer-measured moderate-to-vigorous physical activity; E; estrogen; MET, metabolic equivalent of a task; mg, milligravity (0.001g); P, progestogen; SR-PA, self-reported physical activity in MET-hours per day. [a] n = 3 participants missing from the short-term and 7 from the long-term follow-up, [b] n = 61 missing from the short-term and 21 from the long-term follow-up, [c] n = 71 missing from the short-term and 23 from the long-term follow-up, [d] n = 6 missing from the short-term and 8 from the long-term follow-up. *p < 0.05, **p < 0.01, ***p < 0.001.

The associations of PA with body composition, metabolic health and mRNA expression were studied using both self-reported and accelerometer-measured PA. For body composition and metabolic health variables, associations were obtained using linear or GEE models. For mRNA, associations were calculated using correlation analyses between the changes in the variables. The main

findings are presented in Table 16 (for all $p \leq 0.05$). SR-PA was positively associated with both the total body and appendicular LM variables and muscle density, and negatively with most of the studied adiposity measures. WC and WHR were also negatively associated with SR-PA. ACC-MVPA and ACC-MAD were positively associated with the LM measures and negatively with the FM and metabolic health indicators, as also found for SR-PA. At the RNA level, the results were more variable, as all of the observed associations were unique between the different PA parameters. Interestingly, however, four associations among the mRNAs were found, where the association of higher PA level was opposite to the association of menopause. Genes *APC*, *MYD88* and *ATP5MC2* were upregulated in postmenopause (Table 12), but correlated negatively with increased SR-PA, ACC-MADs or ACC-MVPA, respectively. Similarly, *GORASP1* was downregulated in postmenopause (Table 12), whereas increased SR-PA was associated with increased *GORASP1* expression.

TABLE 16 Associations between the changes in physical activity and adiposity variables, metabolic health indicators, lean and muscle mass variables and messenger RNA molecules.

Physical activity measure	***Paper I* "Adiposity"**	***Paper II* "Metabolic health indicators"**	***Paper III* "Lean and muscle mass"**	***Paper IV* "Muscle RNA signaling"**
SR-PA	All fat variables, except muscle compartment AT area ↓, muscle density↑	Total FM, android FM, WC and WHR ↓	LBM, ALM, ALMI, leg LM, relative muscle area ↑	GORASP ↑ APC ↓
ACC-MVPA	Trunk and android FM ↓	Not studied	LBM, ALM, leg LM ↑	ATP5MC2, MAFK, ALMS1 and MGLL ↓
ACC-MAD	Not studied	Total FM, android FM, WC and WHR ↓	Not studied	INTU ↑ MYD88 ↓

ACC-MAD, accelerometer-measured mean amplitude deviation; ACC-MVPA, accelerometer-measured moderate-to-vigorous physical activity; ALM, appendicular lean mass; ALMI, appendicular lean mass index; AT; adipose tissue; FM, fat mass; LBM; total lean body mass; LM, lean mass; SR-PA, self-reported physical activity; WC, waist circumference; WHR, waist-to-hip ratio. ↑ Positive association, ↓ negative association between the variables.

The associations between muscle mass, adipose tissue mass and metabolic health indicators with diet quality and exogenous hormone use were investigated in adjusted models. The main findings ($p \leq 0.05$) are presented in Table 17. Healthier diet quality was associated with less adiposity in total, and in the trunk, android, gynoid and gluteofemoral regions and with higher muscle density. Perimenopausal progestogen use was associated higher LBM, ALM and leg LM. The combined use of estrogen and progestogen during the menopausal transition

was associated with lower android FM and higher gynoid FM. In addition, leptin levels were positively associated with combined HT use.

TABLE 17 Associations of diet quality and exogenous hormone use with adiposity, metabolic health indicators and lean and muscle mass variables.

Variable	***Paper I*** **"Adiposity"**	***Paper II*** **"Metabolic health indicators"**	***Paper III*** **"Lean and muscle mass"**	***Paper IV*** **"Muscle RNA signaling"**
Diet quality score	Total, trunk, android, gynoid, leg and gluteofemoral FM ↓ muscle density ↑ (SR-PA model) Total, trunk, android, gynoid, leg and gluteofemoral FM ↓ (ACC-MVPA model)	Not studied	Not studied	Not studied
Exogenous hormone use	Progestogen + estrogen: Gynoid FM ↑, android-to-gynoid-ratio ↓ (ACC-MVPA model) Leptin ↑ (SR-PA model)	Progestogen + estrogen: Android FM ↓ (SR-PA model)	Progestogen: LBM, LBMI and ALMI ↑ (SR-PA model) LBM, LBMI, ALM, ALMI, leg LM↑ (ACC-MVPA-model)	Not studied

ACC-MVPA, accelerometer-measured moderate-to-vigorous physical activity; ALM, appendicular lean mass; ALMI, appendicular lean mass index; FM, fat mass; LBM, total lean body mass; LBMI, lean body mass index; LM, lean mass; SR-PA, self-reported physical activity. ↑ Positive association, ↓ negative association between the variables.

6 DISCUSSION

This dissertation research conducted among two longitudinal cohorts of middle-aged Finnish women showed that the menopausal transition and menopause are associated with changes in body composition, metabolic health indicators and muscle RNA signaling. More specifically, body adiposity was found to increase at the total body level and especially in the metabolically harmful abdominal area. LM decreased at the total body level and especially in the appendicular area. Of the metabolic health parameters, WC, WHR, and systemic adiponectin and leptin levels increased and resistin levels decreased during the transition to postmenopause. The changing hormonal environment was associated with 49 DE mRNA genes, but not with regulatory lncRNA genes or miRNAs in muscle. The affected transcriptome was linked to, e.g., extracellular matrix remodeling, energy metabolism, and muscle cell apoptosis, all processes which contribute to body and muscle composition. A higher PA level and healthier diet quality were associated with lower adiposity, more beneficial fat distribution, and higher lean and muscle mass. Associations between changes in PA and changes in muscle RNA expression were also found. The use of exogenous hormones was associated with higher muscle mass and a metabolically healthier body fat distribution.

6.1 Menopause, adiposity and metabolic health indicators

In this study, the annual increase in total FM was on average 0.6 kg during the four years around menopause. Previous longitudinal studies of middle-aged women have reported increases between 0 and 0.9 kg per year (Abdulnour et al., 2012; Greendale et al., 2019; Guo et al., 1999; Lovejoy et al., 2008; Marlatt et al., 2020), whereas an older study, which also included younger women, reported a higher annual increase of total FM 1.4 kg in women approaching midlife (M.-F. Sowers et al., 1996). Some studies have also compared different menopausal groups, and found the highest increment in body adiposity in women who

became postmenopausal during the study period (Greendale et al., 2019; Lovejoy et al., 2008). Moreover, the same is not observed by all groups (Abdulnour et al., 2012). The findings of this thesis are largely consistent with those of previous research, although it seems clear that the characteristics of the study population, such as genetic background (Greendale et al., 2019, 2021; Marlatt et al., 2020), play a part in the final results. In the current study, when the changes in adiposity between the menopausal transition and further progress in menopause were compared in a smaller subgroup of 60 women, the only difference observed was in muscle density, which showed a significantly higher decrease right after than before menopause. The two years prior to and after FMP have especially been reported to be a time of accelerated increase in adiposity (Abdulnour et al., 2012; Greendale et al., 2019; Lovejoy et al., 2008; Marlatt et al., 2020). It is possible that the time interval between the three samples in the current study was too short to observe measurable changes or to detect clearer trends. The finding of a decrement in muscle quality, especially after menopause, is logical, as loss of E2 has also previously been associated with reduced muscle quality (Pöllänen et al., 2011).

The results of this thesis also indicated that relatively more (approximately twice as much) adipose tissue was accumulated in the android region compared to the gynoid or gluteofemoral region. An increase was also observed in the unadjusted measures of WC and WHR. Mid-region adiposity has been associated with higher risks for cardiometabolic conditions (H. Kwon et al., 2017; M. Zhang et al., 2015) and is thus of concern. To my best knowledge, only one previous study has compared increases on the regional body level (Greendale et al., 2021), although several studies have reported significant increases in different abdominal variables (e.g.,Franklin et al., 2009; Lee et al., 2009; Lovejoy et al., 2008; Marlatt et al., 2020). In line with the current result, Greendale and colleagues also reported a higher increase in the android than gynoid area. Unfortunately, the change in VAT specifically could not be confirmed in the current study. In fact, although the measurement of WC correlates relatively well with VAT adiposity (Camhi et al., 2011), two longitudinal studies have reported a significant increase in VAT but no change in WC (Abdulnour et al., 2012; Franklin et al., 2009). This highlights the need for further inspection of the different adipose tissue depots.

Increased adiposity, and especially an increase in the WHR has been previously linked to decreases in metabolic health, in which the adipose tissue-secreted adipokines play a part (Fasshauer & Blüher, 2015). In both of the longitudinal studies reported in this thesis, an increase was observed in leptin and adiponectin levels, and a decrease in resistin levels. All these changes remained significant even when the models were adjusted for FM, indicating that menopause *per se* contributed to the changes. Similar increases in leptin have not been reported previously in longitudinal designs (Kanaley et al., 2001; C. G. Lee et al., 2009), although both E2 and FSH levels have been found to associate positively with leptin levels in various study designs (Di Carlo et al., 2002; Fungfuang et al., 2013; Geber et al., 2012; Kristensen et al., 1999; M. R. Sowers et al., 2008). However, the associations in middle-aged women using estrogen-

based HT vary (Gower et al., 2000; Konukoglu et al., 2000). These two longitudinal studies reported that the change in leptin was associated more closely with the increase in FM rather than change in the hormonal milieu. Interestingly, Lee et al. reported that the change in leptin correlated specifically with the change in intra-abdominal fat (C. G. Lee et al., 2009). However, in the current study, when the leptin values were adjusted for android FM, the significance of the association with menopausal status was no longer significant. Thus it is possible, that in contrast with earlier studies (Rissanen et al., 1999; Samaras et al., 2010), VAT and also overall mid-region fat play a large role in leptin production. Leptin has been shown to have tissue-dependent effects on insulin sensitivity, as in muscle it may increase glucose uptake, while in adipose tissue both increases and decreases in insulin sensitivity have been observed (Barzilai et al., 1997; J. Wang et al., 1999). As leptin is also known to positively control appetite and increase metabolism (Mantzoros et al., 2011), the overall effect of its increase in menopausal women requires further investigation.

The results of this thesis on adiponectin are in line with those of previous studies, which have also reported an increase in adiponectin levels in postmenopause (C. G. Lee et al., 2009; M. R. Sowers et al., 2008). Moreover, in postmenopausal women, higher E2 levels have been associated with lower adiponectin levels (Im et al., 2006; Kunnari et al., 2008). Interestingly, Sowers et al. observed a significant decrease in serum adiponectin in perimenopause when compared to pre- and postmenopause (M. R. Sowers et al., 2008). Adiponectin is regarded as an anti-inflammatory adipokine, one major source of which is thought to be gluteal fat. Although an increase in gluteal adiposity was observed in the present data, an increase in adiposity also occurred in the waist area. The increase in VAT, especially, which has previously shown a negative association with adiponectin levels (C. G. Lee et al., 2009), seems to conflict with finding of an increase in adiponectin in the estrogen-deficient state. One factor that may help to explain this apparent contradiction is that although the women in the current study gained fat in the waist region, they also gained fat in the gluteal region, and hence their WHR or android-to-gynoid ratio remained relatively unchanged. This simultaneous increase in HC may thus suggest that overall their adipose tissue remained healthy despite the increase in FM. The participants in this study also had relatively healthy lifestyle habits, which may have contributed to a metabolically healthier phenotype. In rodents, the loss of estrogens has been associated with a decrease in adiponectin receptors, thus possibly contributing to hyperadiponectinemia (Chattopadhyay et al., 2022). Whether a similar mechanism explaining the increased adiponectin occurs in menopausal women remains to be determined.

Adipose tissue macrophage-secreted resistin (Patel et al., 2003), which was observed to decrease in this study, is considered to be an inflammatory adipokine due to its insulin-desensitizing properties and lipolytic actions (N. Chen et al., 2014). Only three previous studies have investigated the effects of menopausal status on resistin, and while two reported no associations (Chalvatzas et al., 2009; Hong et al., 2007), the third (M. R. Sowers et al., 2008), which was the only

longitudinal study, reported an decrease in postmenopause similar to the present results. Resistin levels have been associated previously with increasing metabolically harmful adiposity (Norata et al., 2007), and higher expression levels have been observed in the abdominal region in both the subcutaneous and visceral areas (McTernan et al., 2002). Thus, again, the decrease in resistin in postmenopause seems counterintuitive, as postmenopause has been widely associated with increased inflammation and decreased metabolic health (Janssen et al., 2008; Q. Wang et al., 2018). Whether the characteristics of resistin change or healthy lifestyle habits also contribute to lowered resistin levels in menopause warrant further experimental studies.

Overall, the results of this thesis on adiposity are in line with that of the previous longitudinal studies (e.g., Greendale et al., 2019; Lovejoy et al., 2008), that the menopausal transition increases adiposity independently of aging. Surprisingly, although adiposity also increased in the waist area and worsened systemic metabolic health indicators have been reported in the same cohort earlier (Hyvärinen et al., 2021; Karppinen, Törmäkangas, et al., 2022), the present adipokine profile indicates a lower inflammatory profile in the body that might have been expected. Healthier lifestyle habits, or enhanced adipose tissue steroidogenesis in postmenopause contributing to local hormone levels, and hence adipokine secretion (Paatela et al., 2016), may contribute to these results (Camhi et al., 2015; Steiner & Berry, 2022).

6.2 Menopausal changes in skeletal muscle

In this thesis, small but significant decreases were observed in lean and muscle mass during both the short-term (-0.5 to -1.5% in ~1.3 years) and long-term (-1.0 to -4.3% in ~3.9 years) follow-ups. More specifically, during both follow-ups LBM decreased on average by 0.10–0.15 kg/year. Several earlier studies on menopausal-related hormonal changes have reported decreases of ~0.2% (-0.04 to -0.08 kg) per year in LBM in several cohorts (Dehghan et al., 2021; Greendale et al., 2019; M. Sowers et al., 2007), whereas other studies have reported no changes, even during longer follow-ups (Abdulnour et al., 2012; Franklin et al., 2009; Marlatt et al., 2020). In contrast, longer studies on aging have reported an average annual loss of 0 to 0.5 kg of FFM in women, and the largest decrease owing to muscle loss during and after midlife (Gába & Přidalová, 2014; Janssen et al., 2000; Kyle et al., 2006). Thus, the results of this thesis are relatively well in line especially with the previous studies, highlighting a rapid lean and muscle mass loss in midlife. One reason for the slightly varying results between cohorts may lie in the different methodologies used. Lean or muscle mass is most often estimated using BIA, DXA, CT or MRI. Moreover, LM, FFM, ALM or muscle area all vary slightly in their surrogacy potential for muscle mass estimation. For example, DXA LBM comprises not only skeletal muscle, but also organs, skin and tendons (Buckinx et al., 2018). Thus small changes in skeletal muscle mass may be masked by small increases in organ or intestine size, which may, at least partly,

correlate with increasing weight (Q. He et al., 2009). ALM or individual limb measurement on the other hand is often considered a more accurate muscle mass surrogate, as this area excludes organs. In fact, in this study the largest relative decreases were observed in ALM and single leg-level and with using CT imaging, which to my knowledge has not been used earlier in longitudinal menopause studies. Thus, the confirmed limb-level decreases in LM strengthen the observed validity of the decrease in LBM.

The results of this thesis on the longitudinal effects of the menopausal transition on skeletal muscle morphology are also first of their kind, whereas in earlier studies only middle-aged HT users and non-users (Qaisar et al., 2013; Widrick et al., 2003), and middle-aged pre- and postmenopausal women have been compared (Pérez-Gómez et al., 2021). On the topic of fiber size, in the current study type I fibers were found to have the largest cross-sectional area, whereas no change was observed in the cross-sectional area of either type I or type II fibers between the peri- and postmenopausal states, a finding which is in line with the results of the corresponding cross-sectional studies (Pérez-Gómez et al., 2021; Qaisar et al., 2013; Widrick et al., 2003). In the current study, decreases were observed in leg muscle mass and area, thus the lack of size change in fiber level was an unexpected finding. However, the small sample size and large intraindividual variation in fiber size most probably contribute to this result. At all time points, histological staining showed the present participants to have a slightly higher proportion of type I fibers (~52% and ~70%) compared to type II fibers, as similarly reported previously in pre- and postmenopausal women and in HT-discordant twins (Pérez-Gómez et al., 2021; Qaisar et al., 2013), although Widrick et al. (2003) reported a higher proportion of type II than type I fibers. In addition to methodological variation, biopsy depth and large interindividual variance, especially in small scale studies, affect the results, and thus may explain the difference observed in this study. When the MHC isoform proportions obtained from the electrophoretic analyses were compared, no change was observed in the current study in the fiber-type ratios between estrogen-discordant stages, as similarly reported in a HT-discordant population (Widrick et al., 2003).

The investigation of muscle metabolic capabilities and lipid accumulation in menopausal women is, to my best knowledge, the first of its kind. This thesis confirmed that type I fibers are more oxidative and accumulate more lipids than type II fibers also in a cohort of middle-aged women. A novel finding was that these properties did not change in any of the fiber types, despite increased adiposity, during the menopausal transition. This was of interest, as muscle oxidative capacity has been found to decrease in individuals with reduced muscle density and obesity (J. He et al., 2001; J. A. Simoneau et al., 1995). However, as exercise has been shown to increase oxidative metabolism (Goodpaster et al., 2001; Pileggi et al., 2022), it is possible that since the women in our study were fairly active, they did not experience a deterioration at the muscle metabolism level despite their increased adiposity. Interestingly, several significant correlations between the cell variables and total body composition

were found, which have not been reported from menopausal women before. For example, single lipid droplet area and the lipid accumulation index positively correlated with several total body adiposity variables. Similar correlations for adiposity have also been observed in other populations (Goodpaster, Theriault, et al., 2000; J. He et al., 2004; Malenfant et al., 2001). These studies have also reported associations between fiber lipid content and deteriorated insulin sensitivity. Unfortunately, this study was not able to further investigate the participants' insulin sensitivity, but slightly higher blood glucose and insulin levels in postmenopause from the same cohort have been reported earlier (Hyvärinen et al., 2021; Karppinen, Juppi, et al., 2022). Nevertheless, in both studies the values remained non-pathological, and could not be used to diagnose decreased insulin sensitivity. Overall, decreased insulin sensitivity during midlife may be related to aging rather than menopause (Thurston et al., 2018).

One recently established theory on menopause-related muscle loss relates to the increase in FSH rather than decrease in E2. While FSH has been found to increase lipid biosynthesis in muscle (X. Cui et al., 2016), increased ectopic fat has been shown to increase inflammation and thus potentially contribute to cellular damage (K. A. Britton & Fox, 2011; Sachs et al., 2019). Thus, the rapid increase in FSH during the menopausal transition could, in addition to aging, contribute to the accelerated muscle loss.

Overall, the results of this thesis support earlier observations that menopause and related hormonal changes are associated with muscle loss at the whole muscle level, but not necessarily at the muscle fiber cross-sectional level also in relatively healthy and active women.

6.3 Menopause associated findings of muscle RNA signaling

DE mRNAs. The current study found 49 DE mRNA genes, only one of which has, to my knowledge, earlier been reported to be regulated by menopause. *ZNF84* has previously been linked in human muscle to postmenopause progression (Pöllänen et al., 2007). However, whereas Pöllänen and group (2007) reported upregulation of ZNF84 in later postmenopause, the opposite was observed in the current study. ZNF84 is predicted to function in transcriptional regulation and has also been found to upregulate the expression of p21, a regulator of the senescence process in vitro (Strzeszewska-Potyrała et al., 2021). In breast cancer cells, E2 has been found to decrease *ZNF84* mRNA expression (Dip et al., 2009). These mechanisms in the muscle require detailed investigation.

In the current study, the DE genes were associated with regulation of the extracellular matrix (e.g., *ECM1* and *ELN*), cell-cell-interactions (e.g., *APC, JAK2* and *PRKCA*), energy metabolism (e.g., *SESN2*), apoptosis signaling (*PIDD1* and *BIRC6*), muscle hypertrophy (e.g., *JAK2* and *MKNK1*) and, for example, mitochondrial dysfunction (e.g., *ATP5MC2)*. Similar over-represented functions have also been reported by others. In postmenopausal twins discordant for HT, genes related to cellular and environmental interactions and anatomical structure

were found to be differentially expressed (Ronkainen et al., 2010). Another study found that postmenopausal HT use affected the expression of genes related to the post-translational modifications of proteins, proteo- and peptidolysis and, for example, cell proliferation, whereas postmenopausal aging was associated with genes related to RNA splicing, protein folding and, for example, proteolysis (Pöllänen et al., 2007). In animal studies, ovariectomy has been associated with the upregulation of atrophy- and apoptosis-linked genes and downregulation of antioxidant genes in muscle (Baltgalvis et al., 2010; E.-J. Cho et al., 2021; Karvinen et al., 2021). Owing to the similarity between the results of this study and others in the pathways affected by the loss of ovarian hormones, the finding of only one common gene was unexpected. Possible factors contributing to the differences in results may include more dramatic hormone-level differences in previous study set-ups, the dynamic nature of transcriptome (Baudrimont et al., 2017), and also the simultaneous effect of aging (Pöllänen et al., 2007). In addition, the DE genes identified in the current study were found to be linked to mitochondrial function, one of the main regulators of skeletal muscle metabolism. Pathways of oxidative phosphorylation and mitochondrial dysfunction were found to be over-represented and renin-angiotensin signaling was also found to be activated. Earlier animal studies have reported a reduction in muscle oxidative capacity, mitochondrial biogenesis and respiratory function due to loss of estrogens (Barbosa et al., 2016; Campbell et al., 2003; Torres et al., 2018). Additionally, increased renin-angiogenin signaling has been found to promote mitochondrial reactive oxygen species production and thus contribute to muscle wasting (Powers et al., 2018). Thus, the findings of this thesis can be considered to add to and confirm the previous findings on the potential signaling mechanisms affecting muscle mass loss and metabolism in menopause.

At the individual gene level, several of the identified DE genes have previously been associated with muscle properties. For example, *E2F3, ZEB1, APC, JAK2*, and *MYD88* have been found to regulate muscle cell and tissue regeneration, proliferation and cellular death (Gallot et al., 2018; H.-R. Kim et al., 2019; Parisi et al., 2015; Siles et al., 2019; Song et al., 2015; K. Wang et al., 2008). E2F3 and ZEB1 are known to have specifically positive effects on muscle mass, and E2 and P4 are known to upregulate their expression in other tissues than muscle (R. Liu et al., 2011; Mazur et al., 2015; Qiao et al., 2011; R.-F. Wu et al., 2018). The findings of this study on the downregulation of *E2F3* and *ZEB1* in postmenopause may indicate that similar regulation also occurs in skeletal muscle. Surprisingly, in contrast to earlier findings on a reduction in the satellite cell pool (Collins et al., 2019), APC, JAK2 and MYD88, which have important roles in satellite cell differentiation, proliferation and tissue repair were found in the current study to be upregulated in postmenopause. However, since the satellite cell pool is also regulated by several other factors, such as Wnt7a and collagen (Dumont et al., 2015), the predicted dysregulation of genes in this study at the mRNA level may be only part of the whole entity. In tumor and animal models, *APC, JAK2* and *MYD88* have been found to be upregulated by E2 and P4

(N. L. Cho et al., 2007; N. Gupta et al., 2012; Jeong et al., 2005; Monroe et al., 2005), which is the opposite of what was observed in this study.

PRKCA and *MGLL*, which have previously been associated with adiposity and metabolic health, were among the observed DE genes. In the current study, *PRKCA* was upregulated in postmenopause. As PRKCA phosphorylates several protein targets and has been observed to induce hypertrophy in cardiac muscle (Braz et al., 2002), but to inhibit glucose uptake in skeletal muscle (Letiges et al., 2002), the observed upregulation may contribute to muscle insulin sensitivity. In cancer cells, E2 has been found to upregulate *PRCKA* (Boyan et al., 2003), whereas our results in muscle suggest the opposite. MGLL is responsible for the last step of cellular lipolysis, converting monoacylglycerides into free fatty acids and glycerol. In addition, MGLL plays a role in the endocannabinoid system and inflammation (Gil-Ordóñez et al., 2018). Surprisingly, total body knockout of MGLL protects from obesity and insulin resistance, whereas overexpression in the small intestine leads to fat accumulation (Chon et al., 2012; Yoshida et al., 2019). *MGLL* expression has been observed to be upregulated by E2 in tumor cells (Ariazi et al., 2011), but no previous data on skeletal muscle were found. In the current study, *MGLL* was downregulated in postmenopause, which may indicate decreased lipolysis in the muscle tissue.

In addition to the abovementioned genes, the correlation analysis also revealed some new potential contributors to skeletal muscle mass and adiposity. For LM especially, the changes in *GTF2F2*, *C1QTNF9* and *TMEM39B* were found to have multiple correlations. GTF2F2 is a transcription factor, which in the current study was positively associated with several LM variables. In humans, GTF2F2 has not been studied in regards to body composition, but in pig muscle it has been found to participate in the regulation of feed efficiency traits, including the percentage of LM and intramuscular fatness (Ramayo-Caldas et al., 2019). However, the details of this regulation are not clear. Additionally, *GTF2F2* has been found to be upregulated by E2 and P4 in vitro and in vivo (Boverhof et al., 2008; Tamm et al., 2009), whereas in the current study the opposite was observed. Very little previous data on skeletal muscle is found for C1QTNF9 and TMEM39B. C1QTNF9 is a likely new adipokine, which has been found to improve insulin signaling, mitochondrial content and to reduce apoptosis, but also proliferation in cardiac and smooth muscle cells (Kambara et al., 2012; Uemura et al., 2013; Wei et al., 2014). In the current study, *C1QTNF9* was found to be downregulated in postmenopause and to correlate positively with LM. The detailed effects of CIQTNF9 in skeletal muscle remain unknown, but the present results suggest that it could potentially have a positive effect on muscle mass. Lastly, *TMEM39B* has interestingly been found to be upregulated in the muscles of older men and women (Thalacker-Mercer et al., 2010), as also in the present data. The increase in *TMEM39B* in the current study was associated with a decrease in regional LM, indicating that it may be a negative regulator of muscle mass. Based on target gene prediction, *TMEM39B* is a target of ERα (Mathelier et al., 2014), and thus subject to estrogenic regulation.

In the adiposity correlation analyses, the changes in *ALMS1*, *MAFK* and *ZEB1*, especially, were found to have significant correlations. For *ALMS1* and *MAFK*, no previous data exist on muscle tissue. ALMS1 has a function in maintaining cellular shape and intracellular transport, while defects in *ALMS1* have been associated with young-onset obesity syndrome (Collin et al., 2002; L. Yu et al., 2021). In the current study, downregulation of *ALMS1* was similarly associated to increased adiposity. In epithelial cells, E2 has been observed to decrease *ALMS1* expression (Winuthayanon et al., 2014), while in this study a downregulation was observed in the estrogen-deprived state. *MAFK*, which correlated negatively with FM, gluteofemoral FM and thigh SAT, has been associated earlier with, for example, neuronal degeneration (Katsuoka & Yamamoto, 2016). In vivo, progestogens have been shown to upregulate MAFK expression (Vallejo et al., 2014), as also observed in present skeletal muscle samples. ZEB1, which had positive correlations with android FM and gynoid FM, is required in muscle for regeneration (Siles et al., 2019), whereas its functions in adiposity are unknown. Overall, the generalizability of the previous literature to the findings of the current study regarding muscle mass and adiposity are very speculative, but may nevertheless encourage further in-depth research.

Unexpectedly, it was found the DE mRNA genes in women followed from early perimenopause to early postmenopause were different from those in the women followed from late perimenopause to early postmenopause. The EarlyMT women showed a larger decrease in E2 and increase in FSH than the LateMT women. The EarlyMT women were followed on average for slightly longer than the LateMT women (1.5 ± 0.9 years vs. 1.0 ± 0.6 years), and when the effect of time was investigated using repeated samples correlations or in a DESeq2 model, the two groups were not found to be differentiated by the interval between their biopsies. Thus, these results suggest that differences in hormonal changes, or in crossing the threshold levels for E2, may underlie the differences in the DE mRNA genes. Perimenopause has been found to be a time of many non-linear changes that may not become visible if comparisons are limited solely to pre- and postmenopausal women (Abdulnour et al., 2012; Matthews et al., 2009; M. R. Sowers et al., 2008).

Regulatory RNAs. In the current study, no DE lncRNA genes were observed. The results of this thesis thus conflict somewhat with the previous animal studies, in which several DE lncRNAs in the rodent OVX model and in fish after E2 supplementation have been observed (Chai et al., 2019; J. Wang et al., 2017). The DE lncRNAs found in these studies were related to pathways regulating the citrate cycle, p53 signaling, adipocytokine signaling, estrogen signaling and, for example, fatty acid synthesis, indicating important regulatory roles for the ovarian hormones in cellular lncRNA signaling. Additionally, in human cell studies E2 has been found to regulate the expression of, for example, H19 and MALAT1 (Sedano et al., 2020). Although no DE lncRNA genes were found in the present study, ten DE lncRNA transcripts were observed, of which one was a downregulated transcript of MALAT1. In skeletal muscle, MALAT1 promotes myoblast proliferation (Watts et al., 2013) and decreases during aging (Ruan et

al., 2022), and hence the observed downregulation may contribute to the regenerative capacity of the muscle. The roles of the other DE lncRNA transcripts in relation to menopause-related hormones or muscle tissue properties remains to be discovered.

During the current study, the expression levels of miRNAs also remained unchanged during natural menopause. Previous in vitro studies have reported E2 and P4 to regulate the expression of, for example, miR-21, -26, -133, -148a and -let-7a (Bhat-Nakshatri et al., 2009; Klinge et al., 2010; Pan et al., 2017; Tan et al., 2014; Tao et al., 2014; Xie et al., 2014), whereas previous in vivo and human studies have observed changes in miRNAs after E2 treatment in cattle, in mouse OVX and ER KO models and in HT-discordant twins and (Collins et al., 2019; Karvinen et al., 2021; Martignani et al., 2019; Olivieri et al., 2014). Although the current study is the first longitudinal study to be conducted on menopausal humans, the findings are somewhat unexpected when compared to earlier ones. It is possible, that due the continuing fluctuation of hormones during perimenopause and early postmenopause, miRNA signaling also fluctuates, unlike in the OVX or HT models, where hormone levels are kept relatively stable by exogenous preparations and thus differences are not observed.

Since to date only a few studies have reported on associations between muscle ncRNA expression and body composition variables, correlation analyses between changes in RNA expression and in FM and LM variables were conducted. Among the top 20 expressed lncRNA genes and DE lncRNA transcripts, MALAT1, LINC02541 and MIR1-1HG, for example, were found to correlate positively with several FM variables. Similar positive correlations have been previously reported between adipose tissue and MALAT1 (J. Han et al., 2021; Piórkowska et al., 2022), while downregulation of LINC02541 has previously been associated with metabolically unhealthy obesity (Prashanth et al., 2021). MIR1-1HG, which serves as a host gene for myomiRNA miR-1, was also found to be positively associated with the FM variables in this study but has not previously been investigated in muscle or other tissues. For the lean mass variables, all the reported correlations in this thesis are, to my best knowledge, novel. Only KCNQ1OT1 has previously been investigated in skeletal muscle cells, where it was shown to reduce the expression of cell-cycle inhibitor p57 (Andresini et al., 2019). In the current study it was also associated positively with the lean mass variables.

The miRNA results of the current study showed that miR-30d-5p, -206 and -378a-3p were especially found to correlate positively with the LM variables. Similar results have been found in humans for miR-30d-5p, while miR-206 has been observed to attenuate muscle atrophy in mice and miR-378a-3p to promote myoblast proliferation (Gagan et al., 2011; Q.-K. Huang et al., 2016; C. J. Mitchell et al., 2018). For adiposity variables, several correlations were observed, especially in the changes in miR-1, miR-21-5p, -26a, -30d, -99a and -126-3p. In humans, serum miR-1 has been linked to increased risk for type 2 diabetes (Al-Kafaji et al., 2021), whereas in rodents, miR-1 expression is increased in obesity (D. E. Lee et al., 2016), although, conversely, increased muscular miR-1

levels have also been associated with improved insulin sensitivity (Rodrigues et al., 2021). miR-21 has also been proposed to be an inflammatory marker and to contribute to muscle atrophy (Borja-Gonzalez et al., 2020; W. A. He et al., 2014). In adipose tissue, miR-26 and -30 have been found to promote adipogenesis, and miR-99a to associate negatively with inflammation (Jaiswal et al., 2019; Karbiener et al., 2014; Zaragosi et al., 2011). In vivo, muscle miR-126 levels have been observed to be decreased in obesity (Gomes et al., 2017). Although several of the earlier results are not specifically from skeletal muscle, the findings for muscle miRNAs of this thesis can be considered relatively consistent with those of previous studies.

As with to the differences in DE mRNAs between EarlyMT and LateMT, several of the correlations of the miRNAs and lncRNAs with the body composition variables also differed between the groups. Possible reasons for this include the effect of the relatively small sample size, especially of the EarlyMT group. Additionally, since several of the adiposity and muscle mass variables correlated with each other, correlation patterns may emerge. Third, since the groups differed from each other in mRNAs, it seems reasonable to assume that the same regulators that affect mRNA transcription, may also regulate non-coding RNA transcription.

Several of the observed DE RNAs in the muscle of this study have been found to be regulated by especially E2 and P4, but data from muscle tissue is rare. Although DE mRNA genes were observed, due to the observational nature of the study, it cannot be concluded completely that the changes are solely due to menopause. Observations of the current study may function as a start for further studies, as skeletal muscle has been studied somewhat little in this context.

6.4 Lifestyle habits, body composition, metabolic health indicators and muscle transcriptome

Physical activity. Hormonal aging and its consequences cannot be completely prevented, but several aging-related changes can be attenuated through lifestyle habits (Cartee et al., 2016; Galland, 2010). In the current study, PA was investigated using three different variables, and it was found, that a higher PA level in midlife was associated with lower adiposity and metabolic health indicators WC and WHR, and higher LM and muscle density. Similar results have also been reported from observational studies by other groups (Kanaley et al., 2001; Sternfeld et al., 2004, 2005). In addition, several intervention studies have confirmed that PA in the recommended weekly amounts during midlife continues to be effective in improving body composition, and increasing muscle power and physical performance (e.g., Coll-Risco et al., 2019; Dam et al., 2020; Sipilä et al., 2001). In the current study, no associations were observed between adipokines and PA. No previous studies have investigated these associations in a similar context. Earlier controlled PA studies on middle-aged women have

shown that while resistance exercise can decrease leptin and resistin levels, the effects on adiponectin levels are mixed (K. M. Park et al., 2019; Ward et al., 2020). In the current study, the results between the different PA variables regarding body composition and metabolic health indicators were aligned with each other, thereby supporting the observed associations on the positive effects of PA.

The associations between changes in the muscular transcriptome and PA variables were also investigated. Only a few previous studies could be found reporting associations for the same RNAs as those observed in this study. In the current study, negative associations were observed between the changes in *APC, ATP5MC2, MYD88, MAFK* and *MGLL,* and PA levels. Of two of these, APC and MYD88, the present results contrast with previous findings. Previous research has found that PA increases *APC* expression (Coyle et al., 2007), whereas for MYD88 the results vary across species. In humans, *MYD88* expression in skeletal muscle decreased after physical inactivity (O. S. Kwon et al., 2015), whereas in rats it decreased after PA (Shirvani et al., 2021). As both APC and MYD88 are also targets of ovarian hormone regulation (N. L. Cho et al., 2007; El Sabeh et al., 2021; Matias et al., 2021), the simultaneous change in hormone levels may contribute to these changes. The present findings for MGLL resemble earlier observations (Schönke et al., 2020). For ATP5MC2 and MAFK, previous studies have reported more ambiguous results. *ATP5MC2* encodes a subunit of mitochondrial ATP synthase (complex V). In this study *ATP5MC2* was found to be upregulated in postmenopause, while one previous study suggests that estrogen deprivation is not associated with protein levels of complex V (Nyberg et al., 2017). Overall, long term PA increases mitochondrial density (reviewed in Nilsson & Tarnopolsky, 2019), and thus exercise could also be linked with increased complex V expression. However, two studies on menopausal women reported a blunted response to exercise of mitochondrial properties (Abildgaard et al., 2013; Nyberg et al., 2017). MAFK is a co-activator of Nrf2, which is known to have antioxidative properties and to be induced by exercise, but it is also known that this response is diminished during aging (Mallard et al., 2020). Perhaps, owing to aging, exercise either fails to promote or begins to downregulate MAFK expression, which further leads to reduced Nrf2 response.

Diet quality. The results of this thesis suggest that healthier diet quality is associated with lower adiposity and higher muscle quality in middle-aged women. These observations resemble those of previous studies, in which a Mediterranean diet, especially, has been found to be beneficial for maintaining or improving middle-aged women's body composition (Flor-Alemany et al., 2020; Lombardo et al., 2020; L. Wu et al., 2014). Our study was not designed to measure aspects of Mediterranean diets but instead reflected the Nordic Nutrition Recommendations (The Nordic Council of Ministers, 2014). Since menopause-related hormones alone not only have wide-ranging effects on appetite control (Buffenstein et al., 1995), but also affect, for example, leptin response (Clegg et al., 2006), it could be beneficial for women to receive nutritional guidance in midlife to ensure optimal dietary choices.

Exogenous hormones. Previous studies investigating the effects of HT on body composition and metabolic health have reported both beneficial (e.g., Ahtiainen et al., 2012; Kristensen et al., 1999; Salpeter et al., 2006; Sipilä et al., 2001) and mixed results (e.g., Ambikairajah et al., 2019; Javed et al., 2019; Kunnari et al., 2008). In the current study, combined estrogen and progestogen use was associated with higher gynoid FM and leptin levels, and lower android FM and a lower android-to-gynoid ratio during mid-life. In line with the results in the current study, also a large meta-analysis found HT to be protective against mid-region adiposity, although HT was also found to increase total body fat percentage (Ambikairajah et al., 2019). Possibly due to increased SAT, increases in leptin have also been reported earlier (Konukoglu et al., 2000), whereas contradictory results have been reported in non-FM-adjusted studies (Gower et al., 2000; Kristensen et al., 1999). Due to leptin's high link to adiposity, interpretation of further studies would benefit from consistent FM adjustments before final conclusions on the relationship between leptin and HT. Additionally, in the current study, baseline progestogen use was associated with higher LM on both the total body and regional body level. In premenopausal women, the use of a progestogen containing intrauterine device has been associated with slightly larger relative muscle mass compared to non-users, but also with an increase in body fatness (Suuronen et al., 2019). In postmenopausal women, P4 supplement has been associated with an increased protein fractional synthesis rate, although whether this translated to total body level was not reported (G. I. Smith et al., 2014). The results of this study are thus in line with those of the earlier studies, although the current study was not originally designed to investigate the effects of HT and thus the use of different HT preparations was not controlled.

6.5 Methodological considerations

This study utilized data from two longitudinal cohorts of middle-aged women undergoing menopause. The invited population consisted of 82% of the whole age cohort of 47- to 55-year-old women living in the Jyväskylä region. Of the responders, only those with relatively good health, without severe inflammatory or metabolic conditions and BMI < 35 kg/m^2 were included in the laboratory measurements. Although the selection process limits generalization of the results mainly to women in good physical health and with no conditions affecting daily functioning, the sample represented the Finnish middle-aged female population relatively well (Kekäläinen et al., 2021).

The study design of short- and long-term follow-ups with two (three for a smaller subpopulation) measurement points allowed the monitoring of body composition around the FMP but did not allow the exact measurement of annual changes or observation of potentially non-linear change rates in earlier premenopause or at later postmenopause. From the 60 women, whose body composition were measured in perimenopause, early postmenopause and at later postmenopause, estimation of the annual changes before and after

menopause was possible, but this analysis revealed differences only in one variable.

Menopausal status at all time points was assigned using data from both self-reported menstrual cycle and the measurement of systemic FSH. Due to the pulsative nature of FSH, especially during the menopausal transition and early menopause, the FSH measurements were repeated after a short interval and averaged to ensure better accuracy and to avoid the effect of daily fluctuation. A longer menstrual data collection period before the baseline and end measurements might have increased the precision the assignment of menopausal status; however, due to the retrospective nature of this study, a period of at least six months was considered sufficient. With respect to menopausal hormones, E2 was measured using immunoassays, the sensitivity of which may be lower at lower serum concentrations (Rosner et al., 2013). However, E2 measurement was only measured to describe the participants, and not used to characterize menopausal status or used in the statistical models. Additionally, due to high material cost, serum adipokine measurements were conducted in singlets instead of duplicates. This may have had a slight effect on accuracy. However, as the results of this thesis were relatively in line with the previous results, the results can be considered reliable.

The amount of fat and lean mass and muscle area was measured using DXA and CT. These methods are considered to be gold standards for measuring body composition and to have high reproducibility (Cordero-MacIntyre et al., 2002; Lohman et al., 2009; Strandberg et al., 2010), although the ability of DXA to detect the smallest changes in regional muscle mass in longitudinal designs has been debated (Delmonico et al., 2008; Tavoian et al., 2019). However, I consider that this limitation has been addressed in this thesis by using both total body and regional LM variables, complementary CT imaging and a relatively large sample population. Unfortunately, I was unable to differentiate waist area SAT from VAT as I did not have access to a newer version of the DXA analysis program or MRI. This additional measure would have benefited the study greatly. All the body composition measurements were done by trained and experienced personnel.

In the current study muscle biopsies of *m. vastus lateralis* were used. The biopsies were both taken and handled by an experienced physician and laboratory staff. The location of the biopsy was measured, and the successive biopsies were taken in close proximity to the first. Muscle biopsies are susceptible to various sources of error. For example, it has been stated that myosin isoform expression varies between the different muscle layers as well as along the longitudinal axis (Horwath, Envall, et al., 2021; Lexell & Taylor, 1991). Thus, if repeat biopsies are taken from slightly varying layers, this can affect the analysis of fiber type and size. In addition to the sampling location, the orientation of the fibers in the section (cross-sectional or longitudinal) as well as freezing artefacts may affect the results. I addressed this possibility by manually pre-examining all the sections before the image analysis. Due to the time-consuming nature of the staining protocols, I was able to handle only a limited number of samples per

analysis. This affected the power of statistical analysis and may contribute to the current results. Although the stainings of the muscle samples were conducted using recognized protocols drawn from earlier publications and from the database of our own laboratory, I sought to optimize them further whenever needed.

For the RNA analysis, all the possible biopsies available were used. The pre- and after-sequencing quality of the samples was estimated using, for example, RNA integrity values and Q30 values. The data were also inspected visually in several ways, and thus these results can be considered trustworthy. In the absence of universal guidelines for handling sequencing data with bioinformatics, decisions on this were made based on the existing literature and after consulting experts in the field. It was observed that even small changes in setting a filter affected the results, and thus the final parameters used are reported as in precise detail. With regards to the over-enrichment and IPA analyses, both included manual decision making and thus the results are subject to alteration after different imported settings. The results of the correlation analyses in *Paper IV* were reported without correcting for multiple comparisons. I am aware that this increases the risk of false-positive results, but due to the novelty value and exploratory nature of the study, the current way of reporting was selected.

For the measurement of lifestyle habits, PA, especially, was comprehensively estimated using two different methods and three variables. As it is widely recognized that self-reported and accelerometer-measured PA measure somewhat differing aspects of PA, using both in the study improves the reliability of the conclusions. The DQS used has been shown to correlate with negatively with body fat percentage in our own studies (data not published); however, due to its discrete nature, it does not capture small changes in diet or food quantity. Unfortunately, it also does not offer information on the dietary caloric content, which would have been especially interesting in this study. The use of exogenous hormones during the study was investigated using questionnaires and nurse interviews. As only a small fraction of the women reported using either progestogens at baseline or started using HT during the study, and since some of the reporting was done retrospectively and without details on dosages, prevents the drawing of very strong conclusions from this subpopulation. Nevertheless, the observations of the HT users in this study are still in line with those reported in previous studies.

6.6 Future perspectives

The findings presented in this dissertation, along with previous research, suggest that the hormonal changes that occur during the menopausal transition impact body composition, metabolic health indicators and skeletal muscle RNA signaling. Further study is needed to understand in detail why it is that not all women experience similar changes during menopause. This dissertation could be a useful starting point for research on this important issue.

Despite the progress made in understanding the hormonal regulation of fat and muscle tissue, many questions remain unresolved, such as ascertaining the precise molecular mechanisms that impact muscle mass and metabolic health during menopause. The results obtained from muscle biopsies in the present study provide initial insights into the intramuscular factors that influence muscle homeostasis and contribute to metabolic health. The DE genes identified in this thesis should be further validated through protein-level analysis. If changes are observed at the protein level, knockout models, overexpression, or inhibitors could be used to further investigate the role of these molecules. As skeletal muscle tissue is known to secrete signaling molecules that impact several other tissues (Leal et al., 2018), it would be interesting to confirm whether the identified functional proteins also function as measurable myokines. More research is also needed to understand the specific roles played by P4 and FSH in regulating body composition and muscle function, as the levels of these hormones also change dramatically during menopause. 3D or multi-tissue models later accompanied with a suitable in vivo model could potentially provide more knowledge.

This thesis examined the associations between lifestyle habits and health parameters through observational data. Currently, the amount of research on the specific effects of PA, diet quality and the use of exogenous hormones on health during menopause is limited, and the extent to which the negative changes due to menopause can be mitigated by lifestyle changes remains unclear. Perhaps the most reliable way to study the effects of lifestyle on menopausal health would be through randomized controlled trials. In these studies the intervention period should be long enough (at least one to two years), and focus could be on different PA patterns (such as resistance vs. resistance + endurance exercise, moderate or high-intensity etc.), structured diet (e.g., Mediterranean or high protein diet) or on investigating more carefully the metabolic pathways of different HT preparations. Previous studies have been successfully conducted (Simkin-Silverman et al., 2003; Sipilä et al., 2001), but more research among different populations and with multiple intervention groups is needed. The menopausal transition and postmenopause are times of varying symptoms that can impair a woman's ability to maintain healthy lifestyle habits, and hence more research should focus on addressing these symptoms, such as poor sleep, hot flashes, and lack of energy, to better support healthy lifestyle choices. In addition, more specific and sustainable instructions based on scientific evidence should be developed for menopausal women to assist them maintain motivation.

While human physiology during menopause is most suitably investigated using human models, the effects of chronological aging are simultaneously present. Studies on gonadotropin antagonists can be, and have been, used to investigate non-permanent ovarian suppression (Gavin et al., 2020; Shea et al., 2015). However, this treatment also lowers systemic FSH levels, which may have an impact on the results. Middle-aged women, who go through prophylactic ovary removal to reduce cancer risk, as in Karia et al. (2021), are one interesting study group for modeling hormonal changes. These women experience a rapid drop in ovarian hormone levels but maintain normal pituitary response.

Investigating this group of women in more detail in a longitudinal design could provide more information on the effects of hormonal changes.

In conclusion, based on the results of this thesis, further research on the reasons behind menopausal changes in health could enable more targeted interventions that help women to maintain optimal health during and after mid-life. While aging cannot be fully prevented at present or in the near future, delaying its effects would likely lead to healthier and more enjoyable later years.

7 MAIN FINDINGS AND CONCLUSIONS

The main findings of this thesis are:

1. Increases in body adiposity, especially in the waist area, and decreases in lean and muscle mass during midlife are associated with menopause and related hormonal changes. However, while in our cohort the total and central body adiposity were increased and lean and muscle mass was lost, the changes in women's serum adipokines were somewhat opposite to what was expected and did not indicate clear decrement of metabolic health, possibly due to the women's overall healthy lifestyle habits.

2. During the menopausal transition, muscle mRNA expression changes and this change is specific to the baseline menopausal transition stage. The genes that were found to respond to hormonal changes have the potential to contribute to several aspects of skeletal muscle tissue homeostasis and metabolism, but also total body composition. In the current study, the muscle lncRNA gene or miRNA expression did not change during the menopausal transition, possibly indicating that mRNA expression may be more sensitive to changes in ovarian and gonadotropin hormones than miRNA or lncRNA gene expression. Moreover, muscle fiber type, size, lipid accumulation or enzymatic capacity did not change significantly.

3. Higher PA, measured with both self-reports and accelerometer data is associated with lower adiposity and higher lean and muscle mass in middle-aged women. Healthier diet quality is also associated with lower adiposity at both the total body and regional levels. In the current study, exogenous hormone use, including both progestogen only or in combination with estrogen, was associated with a more beneficial body fat distribution and higher lean mass. Moreover, correlations were found with changes in PA and muscle mRNA levels. Thus, especially PA and healthier diet quality, and possibly also the use of exogenous hormones, are efficient for maintaining a healthier body composition in middle-aged women.

YHTEENVETO (SUMMARY IN FINNISH)

Vaihdevuosien siirtymävaiheen vaikutukset kehonkoostumukseen: hormonaalisten muutosten, lihaksen RNA signaloinnin ja elintapamuuttujien yhteydet.

Moni nainen alkaa keski-ikää lähestyessään kokea erilaisia oireita, kuten epäsäännöllisiä kuukautisia, heikentynyttä unenlaatua, kuumia aaltoja ja esimerkiksi kehonkoostumuksen muutoksia. Tämä ajanjakso eli vaihdevuosien siirtymävaihe ennen menopaussia johtuu pääasiassa munasarjojen toiminnan heikkenemisestä hormonaalisen ikääntymisen myötä. Menopaussin eli viimeisten kuukautisten jälkeen nainen ei enää voi tulla raskaaksi. Vaihdevuosien siirtymävaiheen hormonimuutosten ajankohta, muutosten mukanaan tuomat oireet ja niiden kesto vaihtelevat yksilöllisesti. Hormonimuutoksista merkittävimpiä ovat naishormoni estradiolin määrän väheneminen ja aivolisäkkeen erittämän follikkelia stimuloivan hormonin määrän kasvaminen verenkierrossa. Erityisesti estradiolilla tiedetään olevan vaikutuksia useisiin elimistön kudoksiin, kuten rasva-, luurankolihas-, luu- ja hermokudokseen. Menopaussin ohittamisen jälkeen naisten riski osteoporoosiin, sydän- ja verisuonitauteihin ja tyypin II diabetekseen sairastumiseen kasvaa selvästi ja jää koholle loppuelämäksi. Vaikka aihetta on tutkittu paljon, yksityiskohdat hormonimuutosten merkityksestä ovat vielä selvittämättä.

Tämän tutkimuksen tarkoituksena oli selvittää pitkittäisaineistossa, vaikuttaako vaihdevuosien siirtymävaiheen ja menopaussin läpikäyminen naisten kehon rasvoittumiseen, aineenvaihdunnallisen terveyden markkereihin, lihasmassan määrään sekä lihaskudoksen ominaisuuksiin. Lisäksi selvitettiin, ovatko keski-iän fyysinen aktiivisuus, ruokavalion laatu ja ehkäisy- ja hormonikorvaushoitovalmisteiden käyttö yhteydessä kehonkoostumukseen, aineenvaihdunnallisen terveyden markkereihin ja lihaksen RNA-molekyylien ilmentymiseen.

Tässä väitöskirjassa käytettiin aineistona kahta pitkittäistutkimusta, jotka käsittivät yhteensä yli kolmesataa Jyväskylän seudulla asuvaa keski-ikäistä naista. Estrogeeni, vaihdevuodet ja toimintakyky (ERMA) -tutkimus koostui 234:stä tutkimuksen alussa vaihdevuosien siirtymävaiheessa (perimenopaussissa) olevasta naisesta, joita seurattiin yksilöllisesti yli menopaussin varhaiseen postmenopaussiin asti. Estrogeeni, mikro-RNA:t ja metabolisten toimintahäiriöiden riski (EsmiRs) -tutkimus oli neli-vuotisseurantamittaus ERMA-tutkimuksen alkumittauksille, jossa tutkittiin 149 naista, jotka siirtyivät joko vaihdevuosien siirtymävaihetta edeltävästä vaiheesta eli premenopaussista tai perimenopaussivaiheesta postmenopaussiin. Vaihdevuosivaihe määritettiin tutkimuksen eri vaiheissa käyttäen STRAW+10 kategorisointia, joka perustuu kuukautisten säännöllisyyden ja follikkelia stimuloivan hormonin pitoisuuksien määrittämiseen.

Väitöskirjassa hyödynnettiin tutkimuksista kerättyjä kehonkoostumus-mittausten tuloksia, veri- ja lihasnäytteitä sekä kyselylomakkeilla ja aktiivisuus-mittarilla kerättyä tietoa naisten kuukautisista, ruokavaliosta, hormoni-valmisteiden käytöstä sekä fyysisestä aktiivisuudesta. Kehon rasvan ja lihaksen määrä

mitattiin kaksienergiaisella röntgenabsorptiometrialla sekä oikean reiden koostumus kvantitatiivisella tietokonetomografiakuvantamisella. Verinäytteistä mitattiin estradiolin, follikkelia stimuloivan hormonin, sekä rasvakudoksen erittämien adipokiinien (adiponektiini, leptiini ja resistiini) pitoisuudet. Reidestä otetusta lihasnäytteestä määritettiin lihassolujen koko, tyyppi, aineenvaihdun-nallinen aktiivisuus ja solun sisäisen rasvan määrä. Lisäksi lihasnäytteistä tutkittiin eri proteiineja koodaavien ja koodaamattomien RNA-molekyylien pitoisuuksia sekvensoinnin ja bioinformatiikan avulla. Kyselylomakkeilla kerättyjen tietojen perusteella laskettiin ruokavalion laatu, vapaa-ajalla ja työmatkalla harrastetun fyysisen aktiivisuuden määrä sekä määritettiin mahdollinen ehkäisy- tai hormonikorvaushoitovalmisteiden käyttö. Lantiolle sijoitetulla aktiivisuusmittarilla kerättiin tietoa koko hereilläoloajan fyysisen aktiivisuuden määrästä.

Tämä tutkimus osoitti, että vaihdevuosien aikainen kehon rasvan kertyminen on yhteydessä elimistön hormonimuutoksiin ja että rasvaa kertyy erityisesti keskivartalon alueelle. Vaihdevuosien siirtymävaiheen läpikäyminen oli yhteydessä myös muuttuneisiin adipokiinitasoihin, mutta joiden perusteella naisten aineenvaihdunnallinen terveys ei heikentynyt merkittävästi. Lisäksi vaihdevuosien siirtymävaiheen läpikäyminen oli yhteydessä lihasmassan vähenemiseen ja tiettyjen lihaskudoksessa esiintyvien lähetti-RNA-molekyylien määrien muutoksiin. Geenitason muutoksilla havaittiin yhteyksiä myös kehonkoostumuksen muutoksiin. Tutkittaessa elintapamuuttujia havaittiin, että korkeampi fyysisen aktiivisuuden määrä, korkeampi ruokavalion laatu ja sukupuolihormonivalmisteiden käyttö keski-iässä olivat yhteydessä pienempään rasvamassan määrään, aineenvaihdunnallisesti suotuisampaan rasvanjakautumiseen sekä korkeampaan lihasmassan määrään. Fyysisen aktiivisuuden määrän muutoksissa ja geenien ilmentymisessä havaittiin myös yhteyksiä.

Koska naiset voivat eliniänodotteen kasvettua elää jopa kolmasosan elämästään postmenopaussissa, on tärkeää ymmärtää paremmin tekijöitä, jotka altistavat terveyden heikkenemiselle. Väitöskirjani tulokset vahvistavat aiemmin tehtyjä havaintoja vaihdevuosien merkityksestä keskivartalolihavuuden synnylle ja lihasmassan vähenemiselle sekä tarjoavat uusia molekyylitason kohteita tarkempien mekanismien selvittämiseen. Tulokset korostavat erityisesti fyysisen aktiivisuuden ja terveellisen ruokavalion, sekä mahdollisesti myös ehkäisy- ja hormonikorvaushoitovalmisteiden käytön merkitystä terveyden ylläpidossa keski-iässä ja sen jälkeen.

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APPENDICES

SUPPLEMENTAL TABLE 1 Reagents used in muscle tissue staining and in the myosin heavy-chain separation and identification protocol (*Papers I* and *III*)

	Reagent	Source	Catalog number
1	A4.951, mouse	Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, USA	
2	6H1, mouse	DSHB	
3	Anti-laminin, rabbit	Sigma-Aldrich, St. Louis, MO, USA	#L9393
4	Alexa Fluor 405, goat anti-mouse	Invitrogen, Carlsbad, CA, USA	#A31553
5	Alexa Fluor 647, goat anti-mouse,	Invitrogen	#A21238
6	Alexa Fluor 488, goat anti-rabbit	Invitrogen	#A11034
7	LD540	Department of Chemistry, University of Jyväskylä	
8	Nitrotetrazolium blue chloride	Sigma	#N-6876
9	Sodium succinate	Sigma	#S-2378
10	Menadione	Sigma	#47775
11	Alfa-glycerophosphate	Sigma	#G6501
12	A4.74, mouse	DSHB	
13	Alexa Fluor 546, goat anti-mouse	Invitrogen	#A11003
14	Alexa Fluor 488, goat anti-rabbit	Invitrogen	#A11008
15	Prolong Gold with Dapi	Invitrogen	#P36931
16	Potassium chloride	Merck, Darmstadt, Germany	#4936
17	Potassium phosphate monobasic	Fluka, Charlotte, NC, US	#60218
18	Di-potassiumhydrogenphospate trihydrate	Aldrich, St. Louis, MO, US	#22,131-7
19	EDTA	WVR, Radnor, PA, US	#20301.186
20	Sodium pyrophosphate decahydrate	Sigma-Aldrich	#221368
21	Beta-mercaptoethanol	Sigma	#M3148
22	Pepstatin A	Sigma	#P5318
23	Halt Proteinase and Phosphatase Inhibitor	ThermoFisher Scientific, Waltham, MA, USA	#1861281
24	Sodiumthiosulphate-5-hydrate	Riedel–de Haen, Charlotte, NC, US)	#31459

SUPPLEMENTAL TABLE 2 Longitudinal characteristics of the biopsied participants

	Short-term follow-up (n = 25)		Long-term follow-up (n = 7)	
	Baseline	Follow-up	Baseline	Follow-up
Background characteristics				
Height, m	1.65 ± 0.1		1.66 ± 0.04	
Body mass, kg	70.0 ± 12.8	70.4 ± 13.2	70.9 ± 15.1	72.0 ± 11.6
Body mass index, kg/m²	25.8 ± 4.5	26.0 ± 4.6	25.8 ± 5.1	26.2 ± 3.9
Normal (18.5–24.99)	52% (13)	56% (14)	57% (4)	43% (3)
Overweight (25–29.99)	28% (7)	24% (6)	29% (2)	43% (3)
Obese (> 30)	20% (5)	20% (5)	14% (1)	14% (1)
Natural bleeding status	100% (25)	100% (25)	100% (7)	86% (6)
Hysterectomy	0% (0)	0% (0)	0% (0)	14% (1)
Sex hormones				
E_2, nmol/L	0.32 ± 0.24	0.21 ± 0.14	0.33 ± 0.27	0.12 ± 0.05
FSH, IU/L	42.6 ± 24.0	73.2 ± 21.7***	41.0 ± 30.7	94.6 ± 35.6*
Lifestyle habits				
SR-PA, MET-h/day	4.8 ± 3.3	4.5 ± 2.9	8.7 ± 8.8	7.0 ± 3.2
ACC-PA, min/day	50.2 ± 27.4	48.3 ± 24.1	78.1 ± 55.6	71.2 ± 43.6
ACC-MAD, mg [a]	28.2 ± 8.2	27.2 ± 7.2	37.6 ± 21.8	31.3 ± 15.4
Diet quality score, points	6.4 ± 2.4	6.1 ± 2.3	8.3 ± 1.6	7.6 ± 0.8
Current smoker	4% (1)	4% (1)	0% (0)	0% (0)
Non-smoker	96% (24)	96% (24)	100% (7)	100% (7)
Alcohol use, portions/week	5.2 ± 5.9	4.9 ± 4.9	4.7 ± 4.4	3.4 ± 3.3
Total and regional fat and lean mass				
Total FM, kg	25.6 ± 8.8	26.4 ± 9.3	24.4 ± 9.5	26.5 ± 7.7
Total fat percent	35.9 ± 7.1	36.8 ± 7.0*	33.7 ± 5.3	36.6 ± 4.5*
Gynoid FM, kg	4.8 ± 1.3	5.0 ± 1.3	4.6 ± 1.3	4.9 ± 0.8
Android FM, kg	2.4 ± 1.0	2.5 ± 1.1	2.3 ± 1.1	2.6 ± 0.9
Leg FM, kg	4.3 ± 1.3	4.4 ± 1.3	4.1 ± 1.2	4.4 ± 1.0
Gluteofemoral FM, kg	10.3 ± 3.3	10.6 ± 3.4	9.9 ± 4.1	10.8 ± 3.3
Gluteofemoral fat percent	36.6 ± 6.0	37.6 ± 5.7*	34.2 ± 5.0	37.1 ± 4.4*
Total body lean mass, kg	41.5 ± 5.1	41.2 ± 5.3	43.3 ± 4.7	42.4 ± 4.3
LBMI, kg/m²	15.3 ± 1.5	15.2 ± 1.5	15.8 ± 1.5	14.5 ± 1.2
Appendicular lean mass, kg	17.8 ± 2.6	17.6 ± 2.8	19.2 ± 2.5	18.6 ± 2.1
ALMI, kg/m²	6.57 ± 0.74	6.48 ± 0.81	6.99 ± 0.79	6.76 ± 0.57
Leg lean mass, kg	6.7 ± 1.0	6.6 ± 1.1	7.3 ± 0.9	7.0 ± 0.9*
Mid-thigh fat and muscle				
SAT area, cm² [b]	59.9 ± 15.2	60.4 ± 15.4	47.6 ± 5.0	51.6 ± 7.5
Muscle comp. AT area, cm² [c]	9.6 ± 3.6	9.8 ± 3.8	9.1 ± 1.4	9.5 ± 1.1
Muscle density, HU [c]	53.6 ± 3.3	54.3 ± 3.6*	55.3 ± 1.9	51.5 ± 3.0
Abs. muscle area, cm² [c]	168.9 ± 11.4	167.1 ± 11.7	183.8 ± 3.8	169.1 ± 2.3
Metabolic health indicators				
Waist circumference, cm	84.9 ± 12.0	86.4 ± 13.2	85.3 ± 18.2	85.3 ± 12.8
Waist-to-hip-ratio	0.84 ± 0.07	0.85 ± 0.08	0.84 ± 0.09	0.85 ± 0.04
Leptin, ng/ml	35.5 ± 16.8	41.1 ± 17.8*	29.4 ± 18.5	41.1 ± 16.4
Adiponectin, ng/ml	15271 ± 6429	16171 ± 6656	16819 ± 7109	19431 ± 7340*
Resistin, pg/ml	17229 ± 6842	15340 ± 5713	19288 ± 11097	16842 ± 6277

Values presented as mean ± SD. ACC-MAD, accelerometer measured physical activity in mean amplitude deviations; ACC-PA, accelerometer-measured moderate-to-vigorous physical activity; ALMI; appendicular lean mass index; AT; adipose tissue; E2, estradiol; FM; fat mass; FSH, follicle-stimulating hormone; HU, Hounsfield unit; LBMI; lean body mass index; MET, metabolic equivalent of a task; mg, milligravity (0.001g); SAT, subcutaneous adipose tissue; SR-PA, self-reported physical activity in MET-hours per day;
[a] n = 1 missing from the short-term follow-up, [b] n = 4 missing from the short-term follow-up, n = 4 missing from the long-term follow-up, [c] n = 3 missing from the short-term and n = 4 missing from the long-term follow-up. *p<0.05, **p<0.01, ***p<0.001.

ORIGINAL PAPERS

I

TOTAL AND REGIONAL BODY ADIPOSITY INCREASES DURING MENOPAUSE—EVIDENCE FROM A FOLLOW-UP STUDY

by

Juppi, H.-K., Sipilä, S., Fachada, V., Hyvärinen, M., Cronin, N., Aukee, P., Karppinen, J. E., Selänne, H., Kujala, U. M., Kovanen, V., Karvinen, S., & Laakkonen, E. K. 2022

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RESEARCH ARTICLE

Total and regional body adiposity increases during menopause—evidence from a follow-up study

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Abstract

For women, menopausal transition is a time of significant hormonal changes, which may contribute to altered body composition and regional adipose tissue accumulation. Excess adiposity, and especially adipose tissue accumulation in the central body region, increases women's risk of cardiovascular and metabolic conditions and affects physical functioning. We investigated the associations between menopausal progression and total and regional body adiposity measured with dual-energy X-ray absorptiometry and computed tomography in two longitudinal cohort studies of women aged 47–55 (n = 230 and 148, mean follow-up times 1.3 ± 0.7 and 3.9 ± 0.2 years, mean baseline BMI 25.5 kg/m^2). We also examined associations between menopausal progression and skeletal muscle fiber characteristics, as well as adipose tissue-derived adipokines. Relative increases of 2%–14% were observed in regional and total body adiposity measures, with a pronounced fat mass increase in the android area (4% and 14% during short- and long-term follow-ups). Muscle fiber oxidative and glycolytic capacities and intracellular adiposity were not affected by menopause, but were differentially correlated with total and regional body adiposity at different menopausal stages. Menopausal progression and regional adipose tissue masses were positively associated with serum adiponectin and leptin, and negatively associated with resistin levels. Higher diet quality and physical activity level were also inversely associated with several body adiposity measures. Therefore, healthy lifestyle habits before and during menopause might delay the onset of severe metabolic conditions in women.

KEYWORDS
adipokine, body fat distribution, longitudinal studies, obesity, perimenopause, physical activity

Abbreviations: ACC-PA, Accelerometer-measured physical activity; BMI, Body mass index; CT, Computed tomography; DHEAS, Sulfated dehydroepiandrosterone; DQS, Diet quality score; DXA, Dual-energy X-ray absorptiometry; ERMA, Estrogenic Regulation of Muscle Apoptosis - study; EsmiRs, Estrogen, MicroRNAs and the Risk of Metabolic Dysfunction - study; E2, Estradiol; FM, Fat mass; FSH, Follicle-stimulating hormone; GPD, α-glycerophosphate dehydrogenase; HU, Hounsfield units; HT, Hormone therapy; IMCL, Intramyocellular lipid droplet; IUD, Intra-uterine device; LPL, Lipoprotein lipase; MET, Metabolic equivalent of a task; MVPA, Moderate-to-vigorous physical activity; SDH, Succinate dehydrogenase; SHBG, Sex-hormone binding globulin; SR-PA, Self-reported physical activity.

1 | INTRODUCTION

Adipose tissue has an important role in energy storage and hormonal supply (Chait & den Hartigh, 2020). Genetic and environmental factors determine adipose tissue mass and distribution by modulating energy balance and lipid-related enzyme activity. Subcutaneous adipose tissue serves as a long-term lipid storage, while visceral adipose tissue is metabolically more active and acts as an acute-response supplier of systemic fatty acids (Chait & den Hartigh, 2020). Both the mass and the location of adipose tissue are important at the systemic level, as higher gluteofemoral adipose tissue mass has been linked with a better metabolic profile and higher insulin sensitivity, while increased waist adiposity may increase cardiovascular and metabolic risks (Manolopoulos et al., 2010; Peppa et al., 2013). Sex chromosomes and hormones are known to be important mediators of adipose tissue distribution. For example, regional expression of lipoprotein lipase (LPL), the enzyme responsible for releasing fatty acids for storage lipid droplets, differs between the sexes, contributing to the waist-centered adipose tissue accumulation pattern in men and pear-like (legs and gluteal area) shape in women (Wang & Eckel, 2009; Wells, 2007). Moreover, sex hormones such as estradiol (E2) and testosterone regulate the expansion and metabolism of adipocytes, and consequently also depot insulin sensitivity (Newell-Fugate, 2017).

In addition to supplying energy, adipose tissue secretes hormones known as adipokines. Adiponectin, leptin, and resistin are some of the most studied adipokines, and their roles in adipose tissue originated signaling and as indicators of metabolic health have recently been revealed (Recinella et al., 2020). Adiponectin has been shown to act as an insulin sensitizer and is inversely associated with obesity, type 2 diabetes, and metabolic syndrome (Fasshauer & Blüher, 2015). The gluteofemoral adipose depot is a possible source of adiponectin, while lower adiponectin levels are associated with increased visceral adipose tissue (Manolopoulos et al., 2010). Leptin has a role in signaling full energy stores and decreasing appetite, and leptin concentration correlates positively, similar to resistin levels, with insulin resistance and body adiposity (Recinella et al., 2020). Besides metabolic associations, excess adiposity also affects functional capacity. Lower muscle density, reflecting increased adipose tissue infiltration into the muscle compartment, is associated with whole-body adiposity (Goodpaster et al., 2000) and can lead to decreased muscle power and postural balance (Straight et al., 2019).

At the cellular level, intramyocellular lipid droplets (IMCL) have an important role in energy storage during physical activity. Both IMCL concentration and the metabolic capacity of different muscle fiber types are associated with obesity. For example, increased lipid accumulation in muscle fibers, lower oxidative capacity, and a decreased proportion of type I fibers have all been found to positively correlate with overall adiposity and insulin resistance (He et al., 2001; Tanner et al., 2002). Type I muscle fibers are typically rich in lipid droplets and possess high oxidative capacity, while type II fibers contain fewer lipid droplets and have a higher glycolytic capacity (Schiaffino & Reggiani, 2011). Sex also contributes to differences in lipid droplet metabolism, as women typically have more IMCL compared with men of the same body mass index, yet remain more insulin sensitive (Goossens et al., 2021).

Aging is a major contributor to adipose tissue accumulation, but in women, this seems to accelerate during menopause. Yet, due to a concomitant loss of lean mass (Juppi et al., 2020), weight does not necessarily increase (Greendale et al., 2019). Menopause is characterized by a cessation of ovarian function, which results in low E2 levels and high follicle-stimulating hormone (FSH) levels. E2 has been suggested to regulate LPL (Wang & Eckel, 2009), while FSH has been linked to the promotion of lipid biosynthesis and is positively associated with leptin and negatively with adiponectin levels in cellular and animal models (Liu et al., 2015). Studies investigating the roles of aging and menopause in increasing adiposity have reported contradictory results. A recent meta-analysis suggests that aging is the main contributor to increased overall adiposity, while menopause contributes to adipose tissue accumulation in the waist area (Ambikairajah et al., 2019). Although women of reproductive age are more protected from cardiovascular conditions compared with men, menopause seems to remove this advantage concomitantly with the change in adipose tissue distribution. Studies of postmenopausal women using hormone replacement therapy (HT) highlight the beneficial role of female sex hormones, which are associated with lower visceral adipose tissue mass (Papadakis et al., 2018). In addition to hormones, better diet quality and regular physical activity are widely recognized contributors to fat mass and metabolic health management in midlife.

This study examined the longitudinal associations between menopausal transition and the accumulation of total and regional body adiposity, as well as changes in systemic and muscle tissue adiposity markers. We also investigated other potential contributors to the body adiposity changes, including physical activity, diet quality, and the use of external hormones. We hypothesized that menopausal transition would be positively associated with several body adiposity variables from whole body to cellular level, and that the highest relative increase in adiposity would occur in the android region. We also hypothesized that increases in leptin and resistin levels and a decrease in adiponectin levels would occur during menopausal transition.

2 | RESULTS

2.1 | Summary statistics and baseline characteristics

The data used were from the Estrogenic Regulation of Muscle Apoptosis (ERMA) (Kovanen et al., 2018) and the Estrogen, MicroRNAs, and the Risk of Metabolic Dysfunction (EsmiRs) (Hyvärinen et al., 2021) studies (Figure 1). All participants were Caucasian women. To be included in the current study, a participant needed to have undergone menopausal transition either from pre- or perimenopause to postmenopause (baseline $n = 316$). The

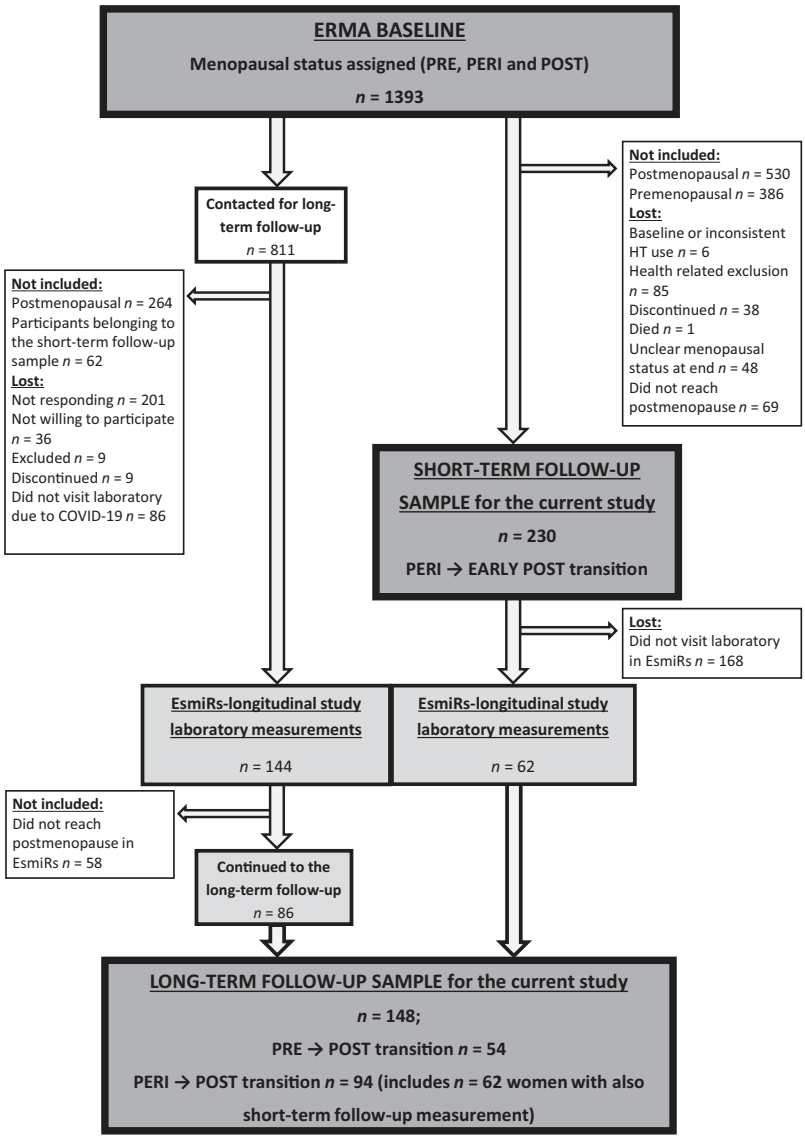

FIGURE 1 Flow chart of the short-term ($n = 230$) and long-term follow-up studies ($n = 148$). ERMA, Estrogenic Regulation of Muscle Apoptosis; EsmiRs, the Estrogen, MicroRNAs and the Risk of Metabolic Dysfunction; HT, hormone therapy; PRE, premenopausal; PERI, perimenopausal; POST, postmenopausal

short-term follow-up sample included 230 perimenopausal women who were followed until early postmenopause (mean follow-up time 1.3 ± 0.7 years). The *long-term follow-up* sample included 148 women who were pre- or perimenopausal at ERMA baseline and postmenopausal at the time of the EsmiRs laboratory measurement (mean follow-up time 3.9 ± 0.2 years). This sample also included a subgroup of women ($n = 62$) who were measured at both the short- and long-term follow-up points.

The baseline characteristics and body composition are described in Tables S1 and S2. Participants were a representative sample of Finnish 47–55-year-old women. At baseline, premenopausal women were about five months younger, had higher E2 and lower FSH, were more likely to use progestogenic contraception (either intra-uterine device (IUD) or mini pills) or to have gone through hysterectomy, and had a lower diet quality score compared with the perimenopausal group (Supplemental Table 1). Although at the group mean level participants were slightly overweight (BMI 25.5 ± 3.9), half of them fulfilled normal weight criteria. For body composition variables, the proportional metrics of total fat mass (FM), total fat-%, gynoid FM, right leg FM, gluteofemoral FM, and gluteofemoral fat-% were lower in the premenopausal than the perimenopausal women (Table S2).

2.2 | Body adiposity increased during short- and long-term follow-ups over the menopause

Short- and long-term follow-up group characteristics are presented in Table 1. Body mass and BMI increased on average by 1% and 3% during both follow-ups respectively ($p < 0.001$ for all). Of the lifestyle habits, accelerometer-measured moderate-to-vigorous

TABLE 1 Participant characteristics and adiposity variables during short- and long-term follow-ups

	Short-term follow-up			Long-term follow-up		
	Baseline	**Follow-up**	***Change from baseline***	**Baseline**	**Follow-up**	***Change from baseline***
Background characteristics	*n* = 230			*n* = 148		
E_2, nmol/L	0.34 ± 0.27	0.24 ± 0.18	*−0.10 ± 0.33****	0.48 ± 0.50	0.20 ± 0.21	*−0.28 ± 0.54****
FSH, IU/L	36.1 ± 21.7	66.7 ± 28.1	*30.5 ± 31.7****	24.1 ± 21.8	80.4 ± 32.0	*56.3 ± 36.4****
Body mass, kg[a]	69.7 ± 11.1	70.3 ± 11.5	*0.7 ± 2.7****	69.8 ± 10.8	72.0 ± 12.1	*2.3 ± 3.6****
BMI, kg/m^2 [a]	25.6 ± 3.9	25.8 ± 4.1	*0.3 ± 1.0****	25.5 ± 3.9	26.3 ± 4.4	*0.8 ± 1.3****
Lifestyle habits	*n* = 230			*n* = 148		
SR-PA, MET-h/day[b]	4.46 ± 3.98	4.69 ± 3.68	*0.23 ± 3.00*	5.27 ± 4.56	4.86 ± 3.81	*−0.42 ± 3.72*
ACC-PA, min/day[c]	51.9 ± 29.6	49.8 ± 23.9	*−2.1 ± 24.7*	54.4 ± 32.6	48.4 ± 28.9	*−6.0 ± 28.2**
Diet quality score[d]	6.01 ± 2.18	5.89 ± 2.16	*−0.12 ± 1.60*	5.86 ± 2.50	5.83 ± 2.29	*−0.03 ± 1.90*
Use of external hormones						
None	*67.8%*	*64.3%****		*67.6%*	*66.2%****	
Estrogen	*0%*	*0.9%*		*0%*	*2.7%*	
Progestogen	*32.2%*	*19.6%*		*32.4%*	*15.5%*	
Estrogen +Progestogen	*0%*	*15.2%*		*0%*	*15.5%*	
Adipokines	*n* = 110			*n* = 68		
Leptin, ng/ml	42.4 ± 30.5	50.4 ± 38.2	*8.0 ± 18.2****	40.8 ± 30.2	54.3 ± 39.6	*13.5 ± 23.2****
Adiponectin, ng/ml	16644 ± 6232	18475 ± 7730	*1831 ± 4285****	16510 ± 6908	19669 ± 8979	*3159 ± 6241****
Resistin, pg/ml	18842 ± 7958	17243 ± 7556	*−1599 ± 5723***	20481 ± 9053	17946 ± 7575	*−2536 ± 7353**
Total and regional fat	*n* = 219			*n* = 132		
Total fat mass, kg	25.7 ± 8.8	26.4 ± 9.0**	*0.8 ± 2.5****	24.4 ± 8.8	27.1 ± 9.6	*2.6 ± 2.8****
Total fat-%	35.8 ± 7.8	36.6 ± 7.5**	*0.8 ± 2.4****	34.2 ± 8.0	36.7 ± 7.9	*2.5 ± 2.3****
Trunk fat mass, kg	13.1 ± 5.4	13.6 ± 5.7**	*0.5 ± 1.7****	12.5 ± 5.3	14.2 ± 6.0	*1.7 ± 2.0****
Gynoid fat mass, kg	5.0 ± 1.4	5.1 ± 1.4**	*0.1 ± 0.5***	4.7 ± 1.4	5.0 ± 1.5	*0.3 ± 0.5****
Android fat mass, kg	2.3 ± 1.0	2.4 ± 1.0**	*0.1 ± 0.3****	2.2 ± 1.0	2.5 ± 1.1	*0.3 ± 0.4****
Right leg fat mass, kg	4.5 ± 1.5	4.6 ± 1.5**	*0.1 ± 0.5***	4.2 ± 1.5	4.5 ± 1.6	*0.3 ± 0.5****
Gluteofemoral fat mass, kg	10.5 ± 3.5	10.9 ± 3.5	*0.3 ± 1.0****	10.0 ± 3.4	11.0 ± 3.8	*1.0 ± 1.2****
Gluteofemoral fat-%	36.7 ± 6.8	37.7 ± 6.6	*0.9 ± 2.1****	35.1 ± 7.0	37.5 ± 6.7	*2.4 ± 2.2****
Android-to-gynoid ratio	0.45 ± 0.14	0.46 ± 0.14	*0.01 ± 0.04****	0.45 ± 0.15	0.50 ± 0.15	*0.05 ± 0.05****
Mid-thigh fat	*n* = 76			*n* = 17		
Subcutaneous adipose tissue area, cm^2[e]	64.2 ± 15.8	65.7 ± 17.0	*1.5 ± 3.8***	65.0 ± 17.5	69.3 ± 18.7	*4.3 ± 4.1***
Muscle compartment adipose tissue area, cm^2	9.4 ± 3.0	9.6 ± 3.1	*0.1 ± 1.1*	8.1 ± 2.2	9.5 ± 1.4	*1.3 ± 1.9**
Muscle density, HU	53.1 ± 3.7	53.4 ± 3.9	*0.3 ± 1.7*	53.7 ± 4.0	50.0 ± 3.1	*−3.7 ± 3.7***

Note: Values are presented as mean ± *SD*.

Abbreviations: E2, estradiol; FSH, follicle-stimulating hormone; BMI, body mass index; SR-PA, self-reported physical activity; MET, metabolic equivalent of task; ACC-PA, accelerometer-measured moderate-to-vigorous physical activity; HU, Hounsfield unit.

[a]Data missing, *n* = 12 from long-term-follow-up.

[b]Data missing, *n* = 3 from short-term and *n* = 7 from long-term follow-up.

[c]Data missing, *n* = 61 from short-term and *n* = 21 from long-term follow-up.

[d]Data missing, *n* = 6 from short-term and *n* = 9 from long-term follow-up.

[e]Data missing, *n* = 1 from short-term follow-up.

$^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

physical activity (ACC-PA) decreased in the long-term follow-up and the use of external hormones increased, shifting from contraceptive use toward HT. In both follow-ups, serum leptin and adiponectin increased and serum resistin concentrations decreased.

All total and regional fat variables measured with dual-energy X-ray absorptiometry (DXA) increased during the follow-ups. The mean relative increments were 2%–4% in the short-term ($p < 0.01$ for all) and 7%–14% in the long-term follow-ups ($p < 0.001$ for all). Computed

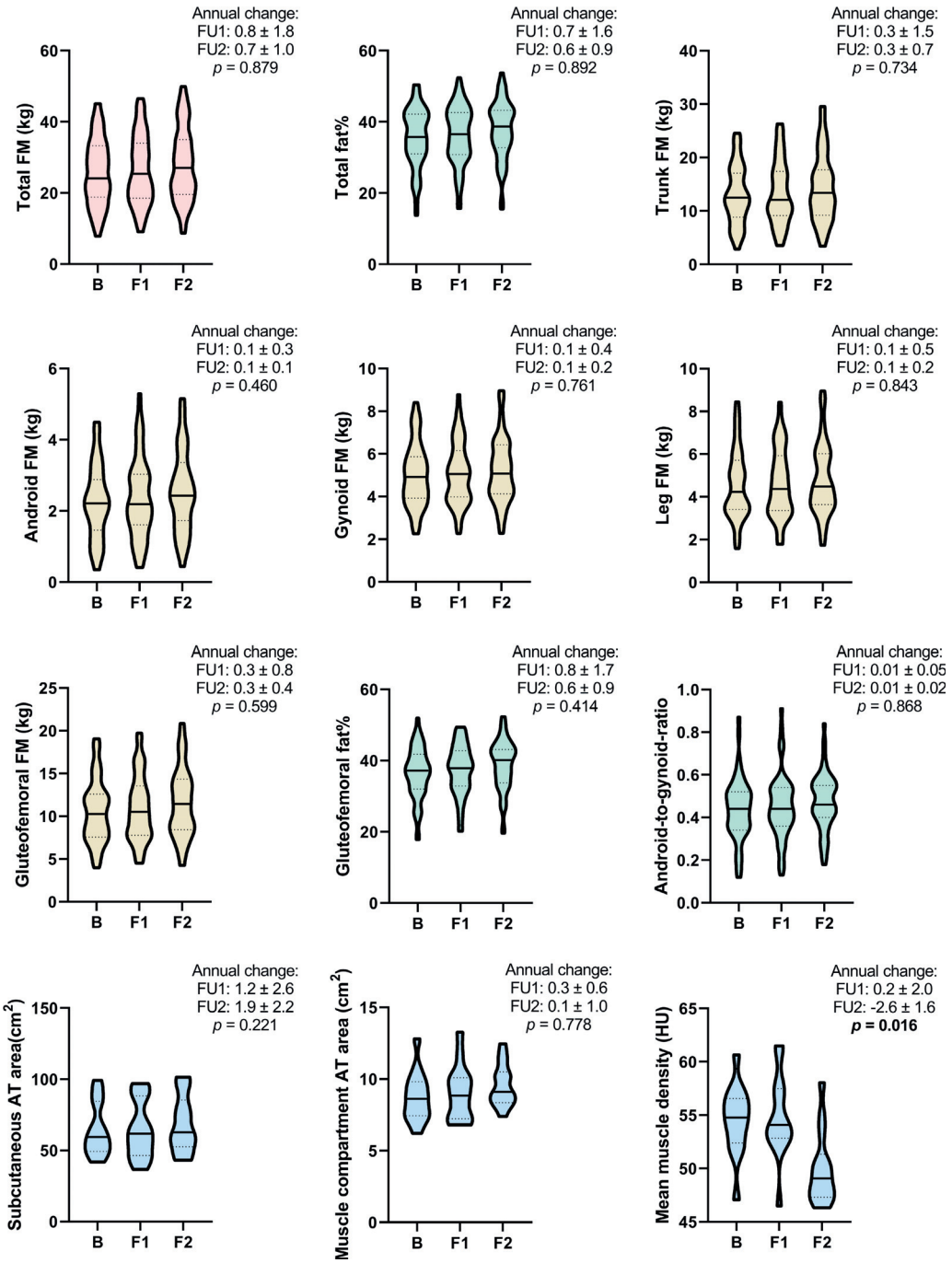

FIGURE 2 Body adiposity variables of participants assessed at three measurement points during the study ($n = 59$ for three upper rows and $n = 14$ for bottom row variables). Violin plots show the median (vertical line), interquartile ranges (dashed lines), and max- and min-values (upper and lower limits). Annual changes are presented as median ± *SD*. B, baseline measurement; F1, first follow-up measurement; F2, second follow-up measurement; FU1, follow-up from perimenopause to early postmenopause (time between B and F1); FU2, follow-up from early postmenopause to later postmenopause (time between F1 and F2); FM, fat mass; AT, adipose tissue; HU, Hounsfield unit. Significant p-values ($p < 0.05$) between annual changes are highlighted

tomography (CT) was used to assess mid-thigh adipose tissue area. Subcutaneous adipose tissue area increased by 2% during the short-term follow-up and 7% in the long-term follow-up ($p < 0.01$ for both). Muscle compartment adipose tissue area increased by an average of 16% ($p < 0.05$) and muscle density decreased by 7% ($p < 0.05$) during the long-term but not the short-term follow-up.

TABLE 2 Linear mixed-effect model results from DXA and CT variables with SR-PA as a physical activity measure (total observations $n = 620$ for DXA and $n = 165–167$ for CT variables)

	Total fat mass (kg)			Trunk fat mass (kg)			Android fat mass (kg)			Gynoid fat mass (kg)			Right leg fat mass (kg)		
	B	95%	CI	B	95%	CI	B	95%	CI	B	95%	CI	B	95%	CI
Univariable model															
Time	**0.69*****	0.57	0.81	**0.46*****	0.38	0.55	**0.09*****	0.07	0.11	**0.08*****	0.05	0.10	**0.07*****	0.05	0.09
Multivariable model[a]															
Time	**0.68*****	0.55	0.80	**0.45*****	0.37	0.54	**0.09*****	0.07	0.11	**0.07*****	0.05	0.09	**0.07*****	0.04	0.09
Hormone use															
E+P4	-4×10^{-3}	−0.75	0.74	−0.07	−0.59	0.45	−0.05	−0.16	0.05	0.08	−0.05	0.22	2×10^{-3}	−0.15	0.15
E	−0.12	−2.34	2.09	0.02	−1.51	1.55	−0.03	−0.34	0.27	−0.12	−0.52	0.29	−0.06	−0.49	0.36
P4	−0.19	−0.90	0.52	−0.24	−0.72	0.24	−0.02	−0.12	0.07	−0.01	−0.14	0.12	−0.06	−0.20	0.08
DQS	**−0.23****	−0.37	−0.09	**−0.16****	−0.26	−0.07	**−0.02****	−0.04	−0.01	**−0.04****	−0.06	−0.01	**−0.03***	−0.06	−0.01
SR-PA	**−0.17*****	−0.25	−0.08	**−0.11*****	−0.17	−0.06	**−0.02*****	−0.03	−0.01	**−0.03*****	−0.04	−0.01	**−0.03****	−0.04	−0.01
Time x SR-PA	-3×10^{-4}	−0.04	0.04	4×10^{-4}	−0.02	0.02	1×10^{-3}	-4×10^{-3}	0.01	3×10^{-3}	-3×10^{-3}	0.01	2×10^{-3}	-5×10^{-3}	0.01

Abbreviations: DXA, dual-energy X-ray absorptiometry; CT, computed tomography; SR-PA, centered self-reported physical activity in MET-hours/day; Note: DXA, dual-energy X-ray absorptiometry; CT, computed tomography; SR-PA, centered self-reported physical activity in MET-hours/day; MET, metabolic equivalent of a task; HU, Hounsfield unit; B, unstandardized regression coefficient; CI, confidence interval; Time: follow-up time in years; Hormone use: reference group is non-hormone users; E, estrogen; P4, progestogen; DQS: centered diet quality score.

[a]Model adjusted with centered baseline age and education level. Significant ($p < 0.05$) B values in bold.

$^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

2.3 | Subgroup analysis of participants with three measurement points

Figure 2 presents adiposity characteristics for the women who were measured three times during the study (DXA, $n = 59$; CT, $n = 14$). In this subpopulation, the mean follow-up time between baseline (B) and the first follow-up point (F1) was 1.4 (0.4–3.6) years, and between F1 and the second follow-up point (F2) 2.5 (0.7–3.7) years. Median annual changes in adipose tissue variables were calculated for both follow-ups to compare the rate of change between the transition from perimenopause to early postmenopause (FU1, from B to F1) and progression through postmenopause thereafter (FU2, from F1 to F2). Only the rate of change in muscle density differed significantly between FU1 and FU2 (0.2 ± 2.0 HU/year vs -2.6 ± 1.6 HU/year, $p = 0.016$). Changes in sex hormones, body mass, BMI, physical activity, and adipokines were similar to the respective follow-ups in the full sample (data not shown).

2.4 | Progression of menopause and a healthier lifestyle are associated with adipose tissue accumulation in opposite ways

Linear mixed-effect models were constructed to study associations between menopausal progression and total, regional and mid-thigh fat variables, and to assess whether lifestyle habits (physical activity, diet quality score, and external hormone use) modulated these associations (Table 2). Self-reported leisure-time physical activity (SR-PA) was chosen as the physical activity measure instead of ACC-PA due to the larger number of valid measurements. Nonetheless, models using accelerometer data supported the following findings (data not shown). For all studied DXA fat variables, the progression of menopause was a significant predictor of adipose tissue accumulation ($p < 0.001$ for all). Higher diet quality score was associated with lower adiposity for all other variables ($p < 0.05$ for all) except for android-to-gynoid ratio. Higher physical activity was also associated with lower adiposity ($p < 0.01$ for all). Of the mid-thigh fat variables, time was a significant predictor of subcutaneous adipose tissue area ($p < 0.001$). Higher physical activity was associated with lower subcutaneous adipose tissue area and higher muscle density ($p < 0.05$ for both).

2.5 | Adipokines are associated with menopausal transition, and adiponectin and leptin are concomitantly associated with total and regional fat mass variables

To study the associations between adipokines and selected adiposity variables, linear mixed-effect models were constructed (Figure 3). Adiponectin levels were positively associated with menopause progression, even after adjusting the models for body adiposity ($p < 0.001$ for all). Of the FM variables, adiponectin was positively associated with gynoid FM ($p < 0.05$). Leptin was positively associated with menopause in the total, gynoid, and gluteofemoral FM-adjusted models ($p < 0.05$ for all). Combined estrogen and progestogen use and the total and regional FMs of android, gynoid, and gluteofemoral area were positively associated with leptin levels ($p < 0.01$ for all). Resistin concentration was negatively associated with menopause in all adiposity models ($p < 0.05$ for all) but was not associated with any FM variables.

Gluteofemoral fat mass (kg)			Android-to-gynoid ratio			Subcutaneous adipose tissue area (cm^2)			Muscle compartment adipose tissue area (cm^2)			Mean muscle density (HU)		
B	95%	CI	B	95%	CI	B	95%	CI	B	95%	CI	B	95%	CI
0.27***	0.22	0.32	**0.01*****	0.01	0.01	**1.24*****	0.77	1.72	0.15	−0.11	0.30	**−0.46****	−0.76	−0.17
0.27***	0.21	0.32	**0.01*****	0.01	0.01	**1.05*****	0.49	1.60	−0.14	−0.03	0.32	−0.23	−0.58	0.12
−0.07	−0.39	0.24	−0.01	−0.03	2×10^{-3}	−0.08	−2.37	2.22	-7×10^{-3}	−0.80	0.78	−0.86	−2.33	0.62
−0.17	−1.10	0.76	0.01	−0.03	0.05	-	-	-	-	-	-	-	-	-
−0.18	−0.47	0.12	1×10^{-4}	−0.01	0.01	0.37	−6.15	6.90	0.15	−2.17	2.47	−1.02	−5.61	3.58
−0.08**	−0.14	−0.02	-1×10^{-3}	-4×10^{-3}	1×10^{-3}	−0.19	−0.66	0.27	-2×10^{-3}	−0.15	0.15	0.05	−0.20	0.30
−0.07***	−0.10	−0.03	**-3×10^{-3}****	-4×10^{-3}	-1×10^{-3}	**−0.37***	−0.69	−0.04	−0.05	−0.16	0.06	**0.25***	0.04	0.46
-2×10^{-3}	−0.02	0.01	3×10^{-4}	-3×10^{-4}	1×10^{-3}	−0.18	−0.37	0.02	3×10^{-3}	−0.06	0.07	0.10	−0.03	0.22

2.6 | Fiber types differ in their lipid droplet content and oxidative and glycolytic capacities, but menopause is not associated with changes in these parameters

Muscle fiber oxidative capacity was investigated with succinate dehydrogenase and glycolytic capacity with α-glycerophosphate dehydrogenase staining. Lipid accumulation was quantified with lipid droplet staining. First, oxidative and glycolytic capacities were studied between fiber types and separately at the B, F1, and F2 time points (Figure 4a,b). Samples from 10 participants were analyzed at baseline, eight at F1, and seven at F2. None of the participants used HT. At all time points, type I fibers had higher oxidative capacity than type II fibers, whereas glycolytic capacity was higher in type II than in type I fibers ($p < 0.05$ for all). Secondly, single lipid droplet area and lipid accumulation index were studied separately among fiber types at all three time points (Figure 4c,d). At baseline, lipid droplet area was the largest in type I fibers ($p < 0.05$). No difference was observed at other time points. At all time points, lipid accumulation index was the largest in type I fibers ($p < 0.01$ for all).

Thirdly, to study the change in metabolic capacity and lipid accumulation during follow-ups, baseline results were used as a reference value and paired relative changes between timepoints were investigated ($n = 8$ for the short-term and $n = 7$ for the long-term follow-up) (Figure 4e-h). Only glycolytic capacity in type II fibers in the long-term follow-up increased significantly (1 vs 1.16, $p = 0.043$, Figure 4f). Overall tissue oxidative and glycolytic capacities were calculated using the information from fiber type distribution as the overall metabolic capacity of a tissue depends on the ratio of different fiber types. During the follow-ups, no significant changes were observed in the relative overall tissue oxidative or glycolytic capacity (Figure 4e,f), or in lipid droplet area or accumulation index (Figure 4g,h).

We also investigated associations between myofiber lipid and body adiposity variables at all measurement points (Figure 5). In type I fibers, lipid droplet area correlated positively with total FM, fat-%, android FM, gynoid FM, gluteofemoral FM, and leg FM in at least two time points (r_s =0.76–1.00, $p < 0.05$ for all). In type I fibers, lipid accumulation index correlated positively with all studied FM variables in at least two time points (r_s =0.66–0.93, $p < 0.05$ for all). In type IIA fibers, lipid accumulation index correlated positively with gynoid and leg FM at baseline and F2 (r_s =0.81–0.93, $p < 0.05$). For oxidative and glycolytic capacities, correlations were only found at single time points (results not shown). At F1, leg FM correlated positively with the glycolytic capacity of type II cells (r_s =0.76, $p = 0.037$) and total tissue glycolytic capacity (r_s =0.81, $p = 0.022$). At F2, oxidative capacity in type II cells and at the total tissue level correlated negatively with lipid accumulation index in type IIA cells (type II: r_s = −0.71, $p = 0.034$; overall tissue r_s = −0.82, $p = 0.034$).

3 | DISCUSSION

In this longitudinal study over the menopausal transition, changes were observed in multiple adiposity measures, from circulating adipokines to regional body fat. Our results show that menopause contributes to body fat accumulation and is uniquely associated with systemic leptin, adiponectin, and resistin levels. We observed fat

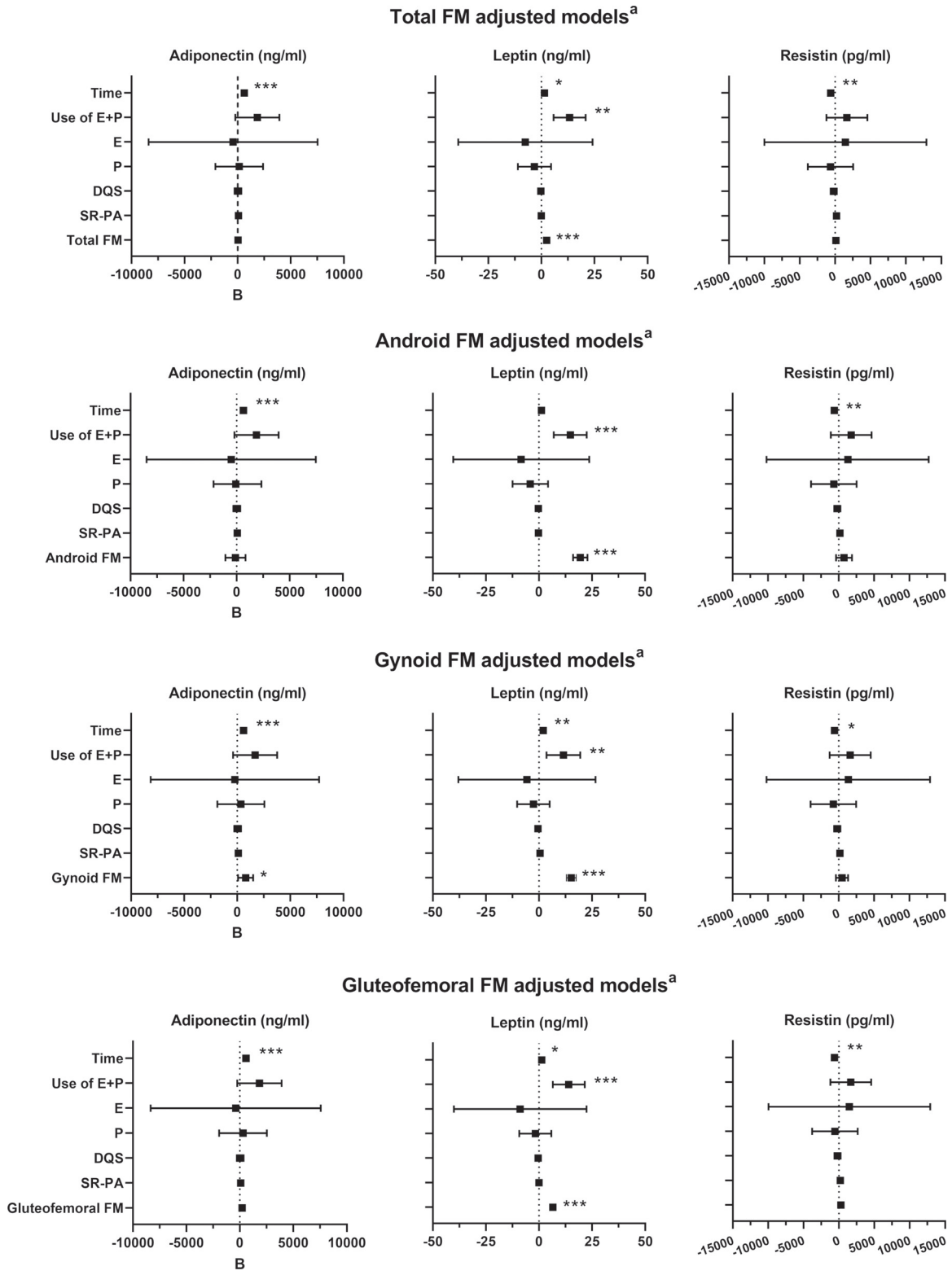

FIGURE 3 Results from the adjusted adipokine and adiposity variables linear mixed-effect models (total observations $n = 276$). Black squares represent the B coefficient of the model covariates, while lines represent the 95% confidence interval. Time, follow-up time in years; Hormone use, the reference group is non-users; E, estrogen; P, progestogen; DQS, diet quality score; SR-PA, self-reported physical activity (MET-hours/day); MET, metabolic equivalent of task; FM, fat mass (kg). All models were also adjusted for centered baseline age and education. $^{***}p < 0.001$ $^{**}p < 0.01$, $^{*}p < 0.05$

accumulation at multiple adipose depots from the total body to the limb level. The increase was detectable already during the short-term follow-up, which focused on the time between perimenopause and early postmenopause, and was more noticeable when the follow-up time was extended to cover the transition from premenopause to further postmenopause. The increase in android FM was relatively large compared with total body or other regional depots. Leptin and adiponectin levels increased during both follow-ups, while resistin levels decreased. At the cellular level, we observed muscle fiber type-specific oxidative and glycolytic capacities and lipid accumulation index, but no changes during menopausal transition except for an increment in type II fiber glycolytic capacity. Lipid droplet area and lipid accumulation index, especially in type I fibers, were positively correlated with total and regional body adiposity measurements during menopause.

During the reproductive years, women typically have higher total body FM than men, especially in the gluteofemoral region due to hormonal and enzymatic actions (Wells, 2007). Increasing evidence from longitudinal studies suggests that the loss of female sex hormones during menopause shifts the site of adipose tissue accumulation toward the waist and android area (Lee et al., 2009; Lovejoy et al., 2008). This observation is supported by our data—although adiposity increased in all body regions, android-to-gynoid ratio also increased, indicating higher fat accumulation in the central body regions. Furthermore, we observed higher relative fat accumulation in upper than in lower body regions. For instance, during the long-term follow-up, FM increased by 14% in the trunk and android areas compared with increments of 6% and 10% in the gynoid and gluteofemoral areas respectively. The link between menopause and total body fatness is still inconclusive. The main contributor to increased total body fat has been suggested to be either menopause (Greendale et al., 2019; Lovejoy et al., 2008) or aging (Ambikairajah et al., 2019). In a longitudinal study of women aged 40–66, Guo et al. observed a 0.41 kg mean annual increase in body fat and found that postmenopausal women had more total body fat than similar aged pre- or perimenopausal women (Guo et al., 1999). We observed a mean annual increase of 0.68 kg of body fat over a maximum period of four years around menopause. This, and similar results from other groups (Greendale et al., 2019; Lovejoy et al., 2008), suggests that the increase in adiposity is accelerated around menopause, and that the increase is pronounced in white women (Marlatt et al., 2020). Menopausal changes are part of normal female aging, thus differentiating them in an observational study is difficult, yet it seems that menopause could have an accelerating effect on adiposity.

Physical activity is a widely studied approach to manage body weight and adiposity in all age groups, including middle-aged women (Grindler & Santoro, 2015). In our study, we investigated associations between self-reported as well as device-measured physical activity level and body adiposity during menopausal transition. Similar to previous studies (Sternfeld et al., 2005), we also found that higher physical activity level was associated with lower body adiposity. Although associations between physical activity and body composition have been broadly studied in menopausal women, most studies are cross-sectional or conducted in postmenopausal women. To the best of our knowledge, only one study in addition to ours has reported longitudinal associations between voluntary physical activity level and changes in body adiposity during menopause (Sternfeld et al., 2004). Although the participants in our study were relatively active (approx. 50 min of MVPA per day), this was insufficient to prevent fat accumulation during menopause, as also reported by Sternfeld et al. (2004). We also found that diet quality was significantly associated with several adiposity variables. Although we did not directly assess energy intake, the diet quality score has been shown to negatively correlate with the intake of total and saturated fat and sucrose (Masip et al., 2019), which might imply an overall higher use of calorie-rich foods. Thus, these findings emphasize the importance of healthy dietary patterns for the management of body adiposity during middle age.

Adipokines play a vital role in tissue–tissue crosstalk and are broadly associated with different metabolic health parameters, such as total FM and insulin sensitivity. Our results suggest that regional differences in adipokine production, and especially gynoid area FM, have an important effect on adipokine levels (Figure 3). As menopause has previously been shown to be associated with declines in metabolic health parameters (Hyvärinen et al., 2021; Wang et al., 2018), and adiponectin as an indicator of good metabolic health (Recinella et al., 2020), the observed increase in adiponectin levels was unexpected and seems counterintuitive, but has also been reported by others (Lee et al., 2009). As the participants in our cohort had fairly healthy lifestyles, it is possible that they were more protected from metabolic deterioration than might have been expected based on the observed changes in body composition (Table 1). Longitudinal associations between leptin and menopause progression have previously been investigated in a few studies (Lee et al., 2009; Sowers et al., 2008), which suggested that body adiposity is the main contributor to leptin levels. We found that leptin levels were positively associated with several body adiposity variables, but also with the menopausal transition (Figure 3). We also observed that the use of estrogen- and progestogen-containing menopausal HT was associated with higher leptin levels, which contradicts our results on menopausal progression. E2 level has previously been shown to correlate with leptin levels in younger women (Geber et al., 2012), but not in women during menopause (Springer et al., 2014). As leptin is a manifold contributor to total body metabolism, more research is clearly needed to clarify its associations with female sex hormones. Resistin, the third adipokine we investigated, has been previously linked to increased BMI and body adiposity in some (Recinella et al., 2020; Zhang et al., 2015), but not all studies (Chu et al., 2006). In cross-sectional studies, resistin levels do not differ according to menopausal state (Gupta et al., 2008; Hong et al., 2007), while a longitudinal study by Sowers et al. found a decrease in resistin levels after menopause (Sowers et al., 2008). Here, we also observed a negative association with menopausal progression when the models were adjusted for FM variables. Mechanisms between E2 and resistin have been studied in animal models (Caja & Puerta, 2007), but the results remain inconclusive. As resistin is a potential candidate in preventing type 2 diabetes (Su et al., 2019), which is an emerging risk for postmenopausal women, more longitudinal studies are warranted.

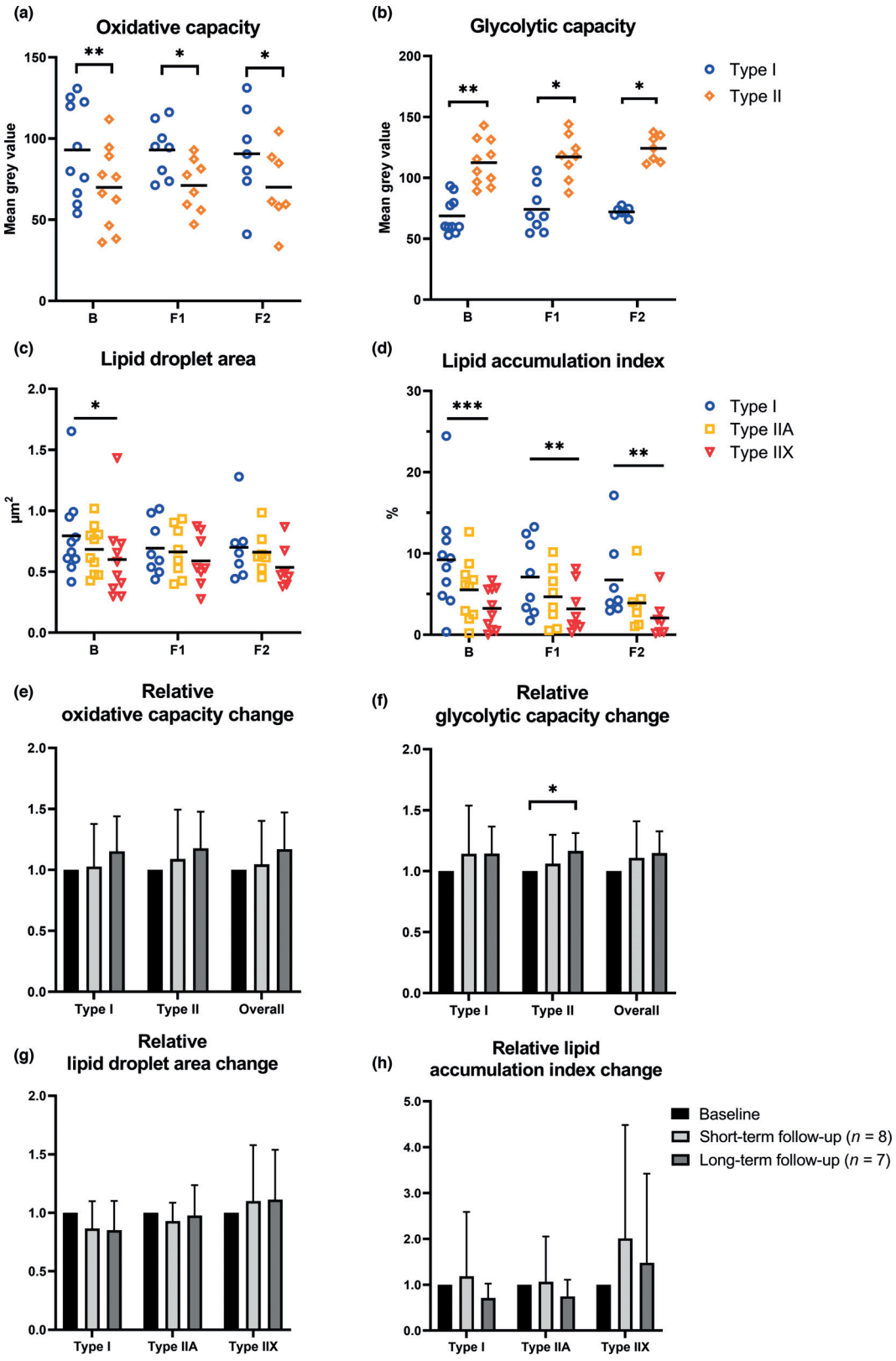
(a)
Oxidative capacity
Mean grey value
150
100
50
0
**
*
*
B
F1
F2
(b)
Glycolytic capacity
Mean grey value
200
150
100
50
0
**
*
*
B
F1
F2
Type I
Type II
(c)
Lipid droplet area
µm2
2.0
1.5
1.0
0.5
0.0
*
B
F1
F2
(d)
Lipid accumulation index
%
30
20
10
0
***
**
**
B
F1
F2
Type I
Type IIA
Type IIX
(e)
Relative
oxidative capacity change
2.0
1.5
1.0
0.5
0.0
Type I
Type II
Overall
(f)
Relative
glycolytic capacity change
2.0
1.5
1.0
0.5
0.0
*
Type I
Type II
Overall
(g)
Relative
lipid droplet area change
2.0
1.5
1.0
0.5
0.0
Type I
Type IIA
Type IIX
(h)
Relative lipid
accumulation index change
5.0
4.0
3.0
2.0
1.0
0.0
Type I
Type IIA
Type IIX
Baseline
Short-term follow-up (n = 8)
Long-term follow-up (n = 7)

FIGURE 4 Biopsy results (n = 7–10). Oxidative and glycolytic capacities, lipid droplet area, and lipid accumulation index in different fiber types at baseline (B), short-term follow-up point (F1), and long-term follow-up point (F2) (Figure 4a-d). In 4A-D, horizontal lines represent mean values. Figure 4e-h shows relative paired changes in oxidative and glycolytic capacity, lipid droplet area, and lipid accumulation index per fiber type, where baseline values were set as a reference value (=1) between short- and long-term follow-ups. Here, values are presented as mean ± *SD*. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

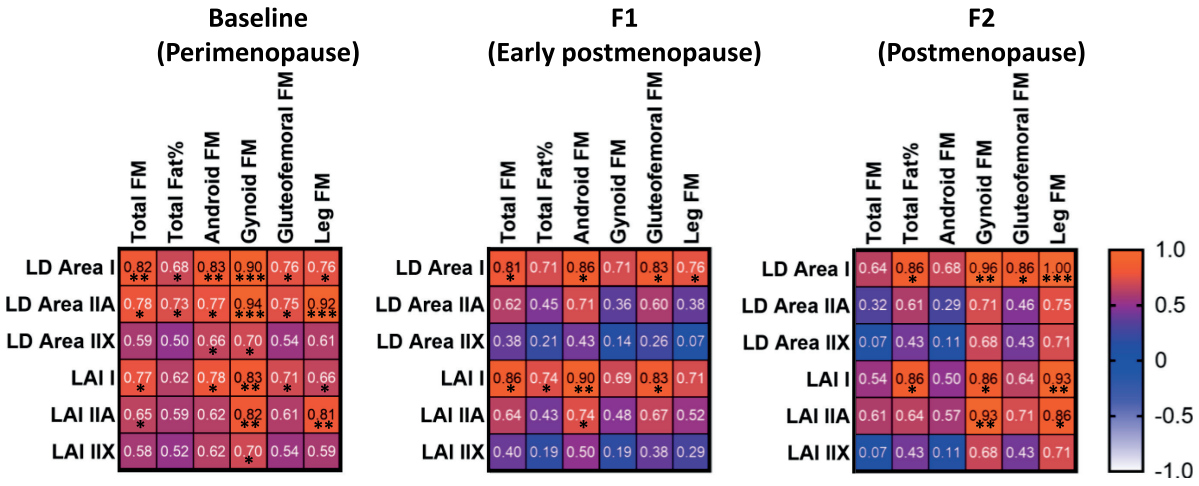

FIGURE 5 Correlation matrix of muscle fiber and body adiposity variables at three measurement points (n = 7–10). Correlation strength and direction are illustrated by correlation coefficient and background color. Red indicates a strong positive correlation and white indicates a strong negative correlation. F1, short-term follow-up point; F2, long-term follow-up point; FM, fat mass; LD, lipid droplet; LAI, lipid accumulation index. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

Cellular changes in skeletal muscle adiposity during menopause are understudied, and to the best of our knowledge, our study is the first to address this issue. Intracellular lipid droplets are dynamic in nature, but their concentration seems to correlate with total body adiposity (He et al., 2001). Similar to previous studies (He et al., 2001), we also found that lipid accumulation differs between the three muscle fiber types, but we did not detect a change in lipid droplet size or accumulation index during the follow-ups (Figure 4). As we did observe a decrease in muscle density in the long-term follow-up CT scans, it is possible that the accumulated low-density tissue is either located between the muscle fibers or is not adipose tissue, but connective tissue instead (Edmunds et al., 2018). We also observed strong positive correlations, especially between lower limb DXA and *m. vastus lateralis* biopsy adiposity variables, suggesting that the correlations between cell and tissue adiposity may be region-specific. Our results on the metabolic enzyme potential of skeletal muscle during menopause are also the first of their kind. Although we observed a small increase in glycolytic capacity of type II fibers, we cannot exclude the possible confounding effect of varying freezing times between the longitudinal samples. Nonetheless, the clear differences observed in oxidative and glycolytic capacities between different muscle fiber types might play a role in individual metabolic maintenance in middle age. As skeletal muscle is a metabolically important tissue with a high rate of energy consumption, more research in menopausal women is warranted.

The current study has several strengths and limitations. The strengths include careful characterization of menopausal status and a comprehensive set of adiposity measurements supplemented with measures of systemic adipokines and cellular muscle adiposity analysis. We used a modified version of the STRAW+10 criteria (Harlow et al., 2012), which is widely considered the gold standard for characterizing menopausal stages. Body composition was also measured using gold standard methods, DXA and CT. Limitations of the study include that due to exclusion criteria, participants represented a cohort of relatively healthy middle-aged Caucasian women, hindering their generalizability to other populations. Unfortunately, we were not able to separate visceral adipose fat from the other adipose depots of the central body. Thus, our analysis of android adiposity includes both subcutaneous and visceral depots. The small number of available muscle biopsy samples also prevents us from drawing stronger conclusions about cellular modifications.

We conclude that menopausal transition is associated with increasing regional and total body adiposity, particularly in central body regions. Physical activity alone does not mitigate increased adiposity, but higher physical activity and diet quality are associated with lower adiposity at baseline, and may therefore delay the accumulation of adipose tissue. Menopausal transition correlates with adipokine release, reflecting associations between female sex hormones and adipose tissue, yet the implications remain unclear. At the cellular level, skeletal muscle fiber characteristics are not affected by the menopausal transition. Information from the current

study can be used in health education of adult women to emphasize the importance of physical activity and a healthy diet in maintaining beneficial body composition.

4 | EXPERIMENTAL PROCEDURES

4.1 | Study design and participants

This study used longitudinal data from ERMA (Estrogenic Regulation of Muscle Apoptosis) and EsmiRs (The Estrogen, MicroRNAs, and the Risk of Metabolic Dysfunction) studies (Figure 1). In total, 1393 women were assigned to baseline menopausal groups based on their current FSH levels and menstrual bleeding diaries. Participants with conditions affecting normal ovarian function or the use of estrogen-containing medication were excluded. Assigned groups were premenopausal, early perimenopausal, late perimenopausal, and postmenopausal, based on the modified STRAW+10 guidelines (Kovanen et al., 2018). A subgroup of the perimenopausal women was invited to take part in the *ERMA longitudinal study*, in which women were followed individually to early postmenopause. During the follow-up, participants visited the laboratory every three to six months based on their previous FSH levels and bleeding diaries. When the participant was categorized as early postmenopausal, the baseline measurements were repeated. These data form the "*short-term follow-up*." After starting the follow-up, three participants were re-categorized as premenopausal, but as they reached early postmenopause during short-term follow-up, they were included in the current study.

Four years after the ERMA baseline, 206 women took part in the EsmiRs laboratory measurements, where the baseline measurements were repeated. Each participant's menopausal status was again determined based on FSH level, menstrual bleeding data, and additional information from the ERMA longitudinal study for those who participated in it. Participants categorized as postmenopausal were included in the "*long-term follow-up*."

Baseline ERMA data were collected during 2015–2016 and the ERMA follow-up measurements were performed between August 2015 and early 2019. EsmiRs data collection started in January 2019 and finished in March 2020.

The study followed the Declaration of Helsinki. All participants provided written informed consent, and the study was approved by the ethical committee of the Central Finland Health Care District (ERMA 8 U/2014 and EsmiRs 9 U/2018).

4.2 | Hormone measurements

Fasting serum samples were taken from the antecubital vein between 7 and 10 AM. At baseline, women with a regular menstrual cycle were asked to visit the laboratory between cycle days 1 to 5. Serum E2, FSH, sulfated dehydroepiandrosterone (DHEAS), and sex-hormone-binding globulin (SHBG) were measured with IMMULITE 2000 XPi (Siemens Healthcare Diagnostics, UK). Serum leptin, adiponectin, and resistin levels were measured with a Quansys Multiplex-kit (custom kits HCUM190820-ID and HA2M200303-ID, Quansys Biosciences, Utah, USA).

After baseline measurements, participants started the follow-ups. During the short-term follow-up, elevated FSH levels were verified with control blood samples. A participant was only categorized as early postmenopausal after two elevated FSH samples accompanied by at least six months without menstruation. The participant was then invited to the final short-term follow-up visit for physiological measurements, during which the E2 and FSH levels were again measured. For the final short-term follow-up analysis, the E2 and FSH values from the last regular follow-up visit and final follow-up visits were averaged to minimize the effect of daily fluctuation. Hormone levels from the participants who started using hormone therapy during the short follow-up were only collected from the final follow-up visit. For participants in the long-term follow-up, baseline hormone measurements were repeated during the EsmiRs laboratory visit. Serum leptin, adiponectin, and resistin levels were also measured at baseline.

4.3 | Fat mass measurements

Measurements were done after overnight fasting. Total, trunk, gynoid, android, and right leg FM and total fat-% were analyzed from the DXA scans (LUNAR Prodigy; GE Healthcare, Chicago, IL). Android-to-gynoid ratio was calculated by dividing android FM (kg) by gynoid FM (kg). Gluteofemoral area was outlined manually using the iliac crest line as the upper limit and the knee joint as the lower limit of a rectangle (Peppa et al., 2013). Corresponding FM and fat-% were calculated from images. DXA measurement of right leg FM was chosen to accompany the quantitative CT scans that were also taken from the right mid-thigh.

The right mid-thigh was scanned at the level of the muscle biopsy with CT (Siemens Somatom Emotion scanner, Siemens, Erlangen, Germany). Subcutaneous and muscle compartment (area inside muscle fascia) adipose tissue area was measured using appropriate thresholds in Python Software (version 3.6). From the cross-sectional image, the muscle portion including the femur was first separated using a U-net machine learning algorithm or manually if needed. Adipose tissue area was separated from muscle and bone tissues using Hounsfield Unit (HU) limits, and mean muscle density was calculated. Images were analyzed using ImageJ Software (v.1.52, NIH, USA) and Python.

4.4 | Muscle biopsies

Muscle biopsies were collected from the middle of *m. vastus lateralis* using suction-modified Bergström needle biopsies under local anesthesia. All visible connective and adipose tissues were removed, and

the sample was divided into three parts. Two parts were assigned to biochemical and molecular biology analyses and were snap-frozen in liquid nitrogen. The third part assigned for histology was embedded transversely on a cork with TissueTek and frozen in isopentane cooled in liquid nitrogen. All samples were stored at −150°C until analysis.

4.5 | Immunohistochemistry and lipid droplet staining

Transverse, 10-μm sections were cut with a cryostat at −21°C. Slides were air-dried and fixed in 4% paraformaldehyde, incubated with 100 mM glycine, and blocked in 5% goat serum (GS). Primary and secondary antibodies were added to 5% GS. Myosin heavy chain distribution was analyzed with antibodies against type I (A4.951, 2 μg/μl, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, USA) and type IIX (6H1, 5 μg/μl, DSHB). Anti-laminin (L9393, 1:250, Sigma-Aldrich, St. Louis, MO, USA) was used to detect cell borders. Primary antibodies were incubated at +4°C o/n, and the attachment was visualized with fluorescent secondary antibodies Alexa Fluor 405 goat anti-mouse (A31553, 1:500, Invitrogen, Oregon, USA), Alexa Fluor 647 goat anti-mouse (A21238, 1:500, Invitrogen), and Alexa Fluor 488 goat anti-rabbit (A11034, 1:500; Invitrogen). LD540 (0.1μg/ml, produced in the University of Jyväskylä Chemistry department) was incubated in sections for 20 min RT to stain neutral lipids. After rinsing with PBS, sections were mounted with Mowiol-Dabco and imaged with confocal microscopy (LSM 700, Axio Observer, Zeiss, Oberkochen, Germany). Laminin signal was enhanced, and broken cells were excluded in ImageJ with the Trainable Weka Segmentation plugin and later by human inspection. Cell segmentation and the measurement of lipid droplet size, number, and area fraction were achieved as previously reported (Fachada et al., 2022). Lipid accumulation index was calculated as the combined area of lipid droplets divided by mean fiber size (Goodpaster et al., 2000).

4.6 | Enzyme histology

Serial 12-μm transverse sections were cut at −21°C. Enzyme activities of succinate dehydrogenase (SDH) and α-glycerophosphate dehydrogenase (GPD) were measured with histological staining. SDH incubation medium consisted of 1 mg/ml NBT (N-6876, Sigma) and 27 mg/ml sodium succinate (S-2378, Sigma) in 0.2 M phosphate buffer, pH 7.4. Sections were incubated in prewarmed solution for 90 min at 37.2°C, washed with H_2O, and mounted with Mowiol. GPD medium consisted of 1.2 mM NBT, 2.3 mM menadione (47775, Sigma), and 9.3 mM alfa-glycerophosphate (G6501, Sigma) in 0.05 M Tris-Buffer, pH 7.4. Sections were incubated in prewarmed solution for 40 min at 37.2°C, washed with H_2O, and cleared with acetone. Lastly, sections were rinsed with H_2O and mounted with Mowiol. Samples were imaged with Olympus BX50 (10×/0.30) (Olympus, Tokyo, Japan). In ImageJ (v. 1.53c), images were transformed into 8-bit grayscale format. At least 55 cells per sample were manually cropped, and mean gray value was calculated, where 0 indicated black and 255 indicated white. To describe staining intensity more intuitively, mean cell gray values were subtracted from the maximum gray value of 255 and the resulting values were used for further analysis. Enzyme staining was paired with myosin fiber type staining in serial sections. 12-μm sections were air-dried and blocked in 10% GS. Primary and secondary antibodies were added to 10% GS. Fiber types were analyzed with antibodies against type I (A4.951, 2 μg/μl, DSHB) and type II (A4.74, 2 μg/μl, DSHB) fibers. Anti-laminin (L9393, 1:250, Sigma-Aldrich) was used to detect cell borders. Sections were incubated in primary antibodies at RT for 1 h, washed, and incubated with secondary antibodies Alexa Fluor 546 goat anti-mouse (A11003, 1:500, Invitrogen) and Alexa Fluor 488 goat anti-rabbit (A11034, 1:500, Invitrogen) at RT for 1 h. Sections were mounted with Mowiol-Dabco and imaged with confocal microscopy. Images were analyzed with ImageJ. Corresponding fibers were localized manually between histological and immunohistological images.

4.7 | Physical activity

Physical activity was evaluated with a structured questionnaire (Kujala et al., 1998) and hip-worn accelerometer as reported previously (Laakkonen et al., 2017). Briefly, the questionnaire included four questions about the frequency, intensity, and duration of leisure-time physical activity bouts and the average time spent in active commuting. Based on the answers, metabolic equivalent of a task (MET) hours per day for leisure-time physical activity was calculated. Objective physical activity was assessed with an accelerometer worn for seven consecutive days (ActiGraph GT3X+ or wGT3X+, Pensacola, FL, USA). The data analysis process has been reported previously (Hyvärinen et al., 2019). Briefly, the amount of moderate-to-vigorous physical activity (MVPA) was assessed using triaxial vector magnitude cutoff point of 2690 counts per minute and the daily averages were adjusted to 16 h of daily wear time (Laakkonen et al., 2017).

4.8 | Diet quality

Diet was assessed using food-frequency questionnaires and quantified using the diet quality score as done previously (Juppi et al., 2020). Shortly, diet quality score was calculated based on 11 components that are characteristic of a healthy diet, as described in the Nordic Nutrition Recommendations 2012. Regular intake of foods such as vegetables, fruits and berries, dark bread, low-fat dairy, fish and nuts, and seeds was considered beneficial. Moreover, limited intake of processed meats, processed grain products, sugar-sweetened beverages, fast food, and sweet or salty snacks was also favored. Each component was worth 1 point, and the maximum score available was 11 points. A higher score reflected a healthier diet.

4.9 | Health status and medications

Information about medical conditions and prescription medicine that may have affected adiposity was collected from the questionnaires throughout the follow-ups. Participants diagnosed with cancer during the study were excluded. The suitability of non-insulin-treated type 2 diabetics ($n = 2$) and new thyroid medication users ($n = 7$) for analysis was investigated, but since sensitivity analysis revealed no major difference in the results, they were included in the analysis.

The use of exogenous sex hormones was determined from questionnaires, resulting in a four-class variable: non-user, only estrogen-user, only progestogen-user, and combined estrogen and progestogen-user. Transdermal (patches, gels, and sprays), oral (tablets), and intra-uterine preparations were included, but local intravaginal estrogen therapy was not. The use of estrogen and progestogen preparations during menopause may affect blood FSH levels due to a negative feedback loop mechanism. We conducted sensitivity analyses (data not shown) between menopausal stages and external hormone use, and found that pre- and perimenopausal progestogen users had lower FSH levels compared with non-users. At postmenopause, estrogen use was associated with higher E2 levels and decreased FSH levels when compared to non-estrogen users. Yet, the difference was not consistent in all comparisons. At postmenopause, progestogen use was not associated with altered hormone levels.

4.10 | Background variables

Anthropometric measurements were done after overnight fasting. Body mass was measured with a digital scale and height with a stadiometer. Body mass index (BMI) was calculated as body mass divided by height squared (kg/m^2). Data about smoking (current/quitter/never smoked), alcohol consumption (portions per week), and level of education (primary, secondary, and tertiary) was determined with a questionnaire. Baseline missing information for education was $n = 14$ ($n = 6$ in premenopausal and $n = 8$ in perimenopausal group). These data were completed using information from the follow-up questionnaires, except for one completely missing questionnaire in the perimenopausal group.

4.11 | Statistical analysis

All variables were evaluated for normality and parametric tests were used whenever possible. Independent samples *t*-test, chi-squared test, and Mann–Whitney *U*-tests were used to compare baseline characteristics between pre- and perimenopausal groups. Paired *t*-test, Wilcoxon signed-rank test, and marginal homogeneity tests were used to test for differences in baseline characteristics, adipokines, and adiposity variables between baseline and follow-ups. Wilcoxon signed-rank test was used to test for differences in annual changes within participants whose body composition was measured at three time points. Friedman test was used to compare nonparametric lipid droplet size and lipid accumulation index between three fiber types. Wilcoxon signed-rank test was used to assess the difference in relative changes during follow-up in cell variables. Due to non-normal distributions, Spearman correlations were calculated and visualized with GraphPad Prism (v.9.1.1) to examine associations between cell and selected adipose tissue variables.

Linear mixed-effect models were created to examine associations between the adiposity measurements and covariates during the follow-ups. Since the models included information from both the short- and long-term follow-up and because menopausal status at baseline and four-year follow-up was not uniform, duration of follow-up in years was selected to represent time. To investigate associations between time and each of the adipose tissue mass variables, models were controlled for education, baseline mean-centered age, mean-centered physical activity, mean-centered diet quality score, the use of external hormones, and the interaction between time and physical activity (fixed effects). The models were constructed using unstructured longitudinal correlation matrix, and intercept and time were used as random effects. Longitudinal associations between adipokines and adiposity variables were investigated with similar linear mixed-effect models, but without the interaction between time and physical activity. The possible effect of smoking was tested, but because we did not see associations in any of the variables, it was left out of the final models. All variables and covariates were evaluated for outliers. A value was considered to be an outlier if it was above 3rd quartile +3*interquartile range or 1st quartile −3*interquartile range. Based on the extreme values in body adiposity ($n = 3$), SR-PA ($n = 5$), ACC-PA ($n = 3$), and adipokines ($n = 3$), participants were removed from the analyses. Multicollinearity between covariates was assessed with variance inflation factor analyses. The model assumptions were tested using Q–Q plots of residuals. Statistical data analysis was carried out using IBM SPSS Statistics Software version 26 (Chicago, IL, USA), and a p-value < 0.05 was considered statistically significant. Due to the observational nature of the study with predetermined associations of interest, the results are presented without multiple comparison corrections. With the large number of statistical comparisons in the study, this may increase the risk of type I error.

5 | PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

For graphical abstract, BioRender's Academic License has been granted.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

VK, EKL, and SS designed the ERMA study and UK, and PA contributed to the planning. EKL designed the EsmiRs study, and VK and SS substantially contributed to the planning. UMK and PA also supported planning of the study. HKJ was responsible for the majority of the laboratory measurements and analysis including adipokine analysis, tissue staining, microscopy imaging, DXA, and CT analyses, while EKL supervised them. HKJ performed statistical analysis with the guidance of MH. SS performed the CT scanning and supervised the analyses. VF was responsible for preparing a script for confocal microscopy image analysis and performed the analysis. NC prepared and analyzed CT scans. PA and UMK offered clinical knowledge and guidance to data analysis. JEK prepared the diet quality score. HS was the clinician responsible for performing muscle biopsies. VK and EKL provided funding for the study. SK assisted with laboratory assays. HKJ prepared the first version of the manuscript. SS, VF, MH, NC, PA, JEK, HS, UMK, VK, SK, and EKL have participated in the interpretation of the results and critically commented on the manuscript during the writing process. All the authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the project leader (eija.k.laakkonen@jyu.fi). The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

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II

METABOLIC HEALTH, MENOPAUSE, AND PHYSICAL ACTIVITY—A 4-YEAR FOLLOW-UP STUDY

by

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Epidemiology and Population Health

Metabolic health, menopause, and physical activity—a 4-year follow-up study

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BACKGROUND: In women, metabolic health deteriorates after menopause, and the role of physical activity (PA) in mitigating the change is not completely understood. This study investigates the changes in indicators of metabolic health around menopause and evaluates whether PA modulates these changes.

METHODS: Longitudinal data of 298 women aged 48–55 years at baseline participating in the ERMA and EsmiRs studies was used. Mean follow-up time was 3.8 (SD 0.1) years. Studied indicators of metabolic health were total and android fat mass, waist circumference, waist-to-hip ratio (WHR), systolic (SBP) and diastolic (DBP) blood pressure, blood glucose, triglycerides, serum total cholesterol, and high- (HDL-C) and low-density (LDL-C) lipoprotein cholesterol. PA was assessed by accelerometers and questionnaires. The participants were categorized into three menopausal groups: PRE-PRE (pre- or perimenopausal at both timepoints, $n = 56$), PRE-POST (pre- or perimenopausal at baseline, postmenopausal at follow-up, $n = 149$), and POST-POST (postmenopausal at both timepoints, $n = 93$). Analyses were carried out using linear and Poisson mixed-effect models.

RESULTS: At baseline, PA associated directly with HDL-C and inversely with LDL-C and all body adiposity variables. An increase was observed in total ($B = 1.72$, 95% CI [0.16, 3.28]) and android fat mass (0.26, [0.06, 0.46]), SBP (9.37, [3.34, 15.39]), and in all blood-based biomarkers in the PRE-POST group during the follow-up. The increase tended to be smaller in the PRE-PRE and POST-POST groups compared to the PRE-POST group, except for SBP. The change in PA associated inversely with the change in SBP (−2.40, [−4.34, −0.46]) and directly with the change in WHR (0.72, [0.05, 1.38]).

CONCLUSIONS: In middle-aged women, menopause may accelerate the changes in multiple indicators of metabolic health. PA associates with healthier blood lipid profile and body composition in middle-aged women but does not seem to modulate the changes in most of the studied metabolic health indicators during the menopausal transition.

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INTRODUCTION

Metabolic health is an umbrella term for factors that combine several aspects of cellular, cardiovascular, and cardiorespiratory health and well-being. Body adiposity, anthropometrics, blood pressure, and blood-based biomarkers, such as serum lipids and blood glucose, can be clinically used to evaluate metabolic health. One established method is to use the diagnostic criteria of metabolic syndrome (MetS) [1], a multifaceted disorder predisposing individuals to severe health concerns, such as atherosclerotic heart disease [2] and type II diabetes [3]. Although there is a significant genetic component in the individual variance of metabolic health and emergence of MetS risk factors [4], unhealthy lifestyle habits, such as physical inactivity, are proposed to be a major contributor.

The effect of menopause on metabolic health and the development of MetS has been an increasing area of interest, as nowadays women in Western countries are expected to live in the postmenopausal state for more than one third of their lives [5–7]. Menopausal transition and the accompanying changes in the hormonal milieu (e.g., decrease in the systemic estradiol (E2) levels) have been associated with unfavorable changes in several indicators of metabolic health [8, 9]. For instance, increased blood glucose [10], accumulation of abdominal adiposity [11] as well as unhealthy changes in serum lipids [12] have been reported during menopausal transition. Additionally, menopause-related increase in inflammation marker levels [13] and decrease in muscle mass [14] have an additive negative impact on metabolic health. Therefore, it is not surprising that in women the incidence of MetS and cardiovascular disease increases after menopause [8, 15].

Physical activity (PA) has been widely proposed to improve the metabolic risk factor profile and cardiovascular health. Literature suggests that regular PA decreases total and visceral fat mass,

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improves insulin sensitivity, prevents dyslipidemia, and decreases systolic (SBP) and diastolic (DBP) blood pressure [16–18]. Thus, it can be used for the prevention and treatment of MetS. However, the associations between PA and changes in indicators of metabolic health around menopause are understudied as only few longitudinal studies have been conducted using device-measured PA [12, 19]. Moreover, these studies included only women transitioning from pre- or perimenopause to postmenopause and therefore could not address the contemporaneous aging-related changes.

The objective of this study was to investigate the changes around menopause in serum lipids and glucose, blood pressure, and body adiposity as indicators of metabolic health. Additionally, the aim was to evaluate whether PA modulates these changes using unique longitudinal data from the study of middle-aged women with different menopausal status.

MATERIALS AND METHODS

Study design and population

This study utilized the data from the observational Estrogenic Regulation of Muscle Apoptosis (ERMA) and Estrogen, MicroRNAs and the Risk of Metabolic Dysfunction (EsmiRs) studies. The participant selection for the ERMA study has been described in detail elsewhere [20]. Briefly, out of the 6 878 randomly selected women aged 47–55 years living in Central Finland, 1393 consented and met the inclusion criteria for the baseline measurements (Fig. 1). Exclusion criteria included conditions and the use of medications affecting ovarian function and systemic hormone or inflammatory status, such as bilateral oophorectomy, pregnancy, lactating, severe obesity (self-reported body mass index (BMI) ≥ 35 kg/m^2), or the use of estrogen-containing medications and continuous cortisone or inflammatory drug treatment [20].

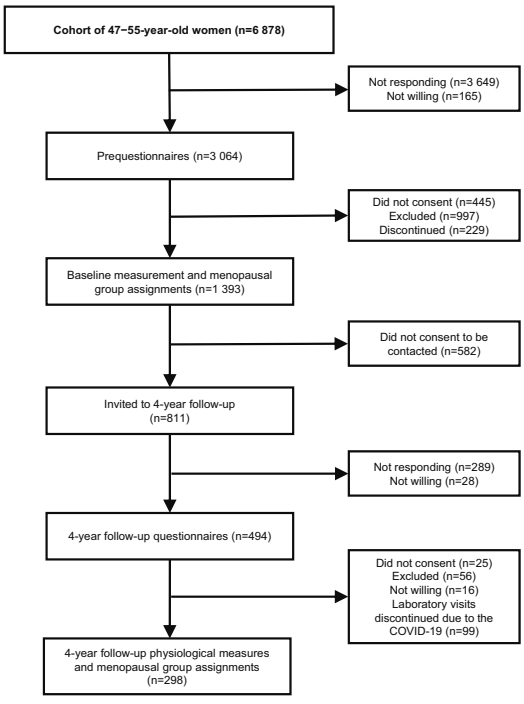

Fig. 1 Flow chart of the study. The flow chart describes the participant enrollment and selection procedure of the ERMA and EsmiRs studies with detailed information about the exclusions and discontinuations during each phase of the study.

The 4-year follow-up measurements were carried out in the EsmiRs study. Out of the 811 participants measured in the ERMA baseline who consented to be contacted, 494 were willing to participate in the EsmiRs questionnaire. Of these participants, 56 were excluded, 25 did not consent, and 16 were not willing to continue to physiological measurements. The participants were excluded due to having more than 7 years from menopause based on the self-reports ($n = 46$), diabetes requiring regular insulin therapy ($n = 2$), severe cardiovascular dysfunction ($n = 2$), or diagnosed with cancer during the follow-up ($n = 6$). Furthermore, 99 participants could not be measured because of the COVID-19 lockdown. Consequently, the final study sample included 298 white women (Fig. 1). To estimate potential selection bias, sensitivity analyses comparing the included sample to the rest of the measured participants ($n = 1095$) at the ERMA baseline for all outcome variables and accelerometer-measured PA were conducted.

The recruiting for the ERMA study was conducted in 2014. The baseline measurements were initiated at the beginning of 2015, and they lasted until the end of 2016. The recruiting for the EsmiRs study started in November 2018 and laboratory measurements were initiated in January 2019. They were discontinued on March 16, 2020 due to the COVID-19 pandemic. The study was performed in accordance with the Declaration of Helsinki. All participants provided written informed consent, and the study was approved by the ethical committee of the Central Finland Health Care District (ERMA 8U/2014 and EsmiRs 9U/2018).

Menopausal status assignments

Blood sampling after overnight fasting was performed in a supine position from the antecubital vein during days 1–5 of menstrual cycle if the cycle was predictable. Serum was separated from whole blood and stored at −80 °C before analysis. Serum concentrations of E2 and follicle-stimulating hormone (FSH) were determined using IMMULITE® 2000 XPi (Siemens Healthineers, Erlangen, Germany) according to the manufacturer's instructions.

Participants were categorized as pre-, peri-, or postmenopausal in both measurements based on the FSH concentrations and self-reported menstrual bleeding diaries using the adapted Stages of Reproductive Aging Workshop (STRAW + 10) guidelines [20]. The participants were divided into three groups based on how their menopausal status changed during the study. PRE-POST group ($n = 149$) consisted of women who experienced menopause during the follow-up period. That is, they were categorized as pre- or perimenopausal in the baseline and postmenopausal in the follow-up measurement. Furthermore, women that were pre- or perimenopausal (PRE-PRE, $n = 56$) or postmenopausal (POST-POST, $n = 93$) in both measurements were designated to their respective groups.

Indicators of metabolic health

Blood pressure and anthropometrics were measured after overnight fasting. SBP and DBP was measured twice in a sitting position after 10 min rest using Omron M6 Comfort (Omron Healthcare, Kioto, Japan) with a standard size cuff and the mean values of the measurements were used. Waist circumference was measured in light underwear midway between the superior iliac spine and the lower rib margin, and hip circumference at the level of the greater trochanters [21]. Body mass and height were measured with standard procedures and BMI was computed by dividing the body mass with squared body height. Total body fat mass and percentage, android fat mass, and fat free mass were assessed with dual-energy X-ray absorptiometry (DXA; LUNAR, GE Healthcare, Chicago, IL, USA).

Serum samples collected during menopausal status assignment were also used for outcome variable analysis. Serum glucose, high- (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol, and triglycerides were measured with KONELAB 20 XTi analyzer (Thermo Fischer Scientific, Vantaa, Finland).

The updated ATP III criteria for MetS risk factors was used [1]. The defining levels for risk factors were ≥88 cm for waist circumference, ≥130/≥85 mmHg for blood pressure, ≥1.69 mmol/l for serum triglycerides, ≥5.6 mmol/l for blood glucose, and <1.29 mmol/l for HDL-C.

Physical activity

Accelerometry-measured PA was assessed in both timepoints by triaxial ActiGraph GT3X and wGT3X accelerometers (ActiGraph LLC, Pensacola, FL, USA) with an accompanied diary. Participants were instructed to wear the accelerometers for seven consecutive days on their right hip during

waking hours, except during water-based activities. The data were collected at 60 Hz and the Euclidian norm of the resultant acceleration was computed for each timepoint. Consequently, the mean amplitude deviations (MAD) were computed for non-overlapping 5 s epochs, and the mean MAD value for 1 min epochs were determined based on the 5 s MAD values [22]. The accelerometer-measured MAD (ACC-MAD) reflects the directly measured acceleration and captures the volume of the activity in the entire intensity profile [23] and has been validated against oxygen consumption [24]. Non-wear time was identified as any epoch of at least 60 min with 1 min MAD continuously less than 0.001 g (g denotes the gravitational acceleration on Earth).[1] A minimum of 3 days with a wear time of 10 h or more was regarded as a valid measurement.[2] Finally, the ACC-MAD was determined for wear time for each measurement. For supplementary information, we defined activity with intensity higher or equal to 0.091 g as moderate-to-vigorous physical activity (MVPA) [24]. The ACC-MAD was strongly associated ($r = 0.88$ and $r = 0.79$) with the amount of MVPA and ActiGraph counts [25], respectively.

Additionally, PA was assessed by a self-reported questionnaire (SR-PA) [26]. Briefly, the questionnaire included four questions about the average frequency, intensity, and duration of leisure time PA bouts as well as the average duration of the commuting activity. Based on the responses, the metabolic equivalent (MET) hours per day for leisure time PA was calculated.

Covariates

The use of medications and lifestyle habits were assessed by a structured questionnaire at baseline and follow-up measurements. Responses were used to assess alcohol consumption in portions per week and current smoking status (nonsmoker/smoker). Participants also reported their use of regular prescription medications that were categorized using the Anatomical Therapeutic Chemical (ATC) classification [27]. The use of medications was assessed (non-user/user) separately in preparations affecting blood pressure (ATC C02–05 and C07–09), serum lipids (ATC C10), and thyroid function (ATC H03).

Based on self-reports, participants were classified as being either non-user, only estrogen, only progestogen or combined estrogen and progestogen users. Exogenous sex hormone preparations for contraceptive and hormone replacement therapy use, such as pills, intra-uterine device, patches, and transdermal gels but not intravaginal local estrogen therapy were included. Diet quality score (DQS) was computed based on a food-frequency questionnaire as reported previously [14]. Shortly, DQS consisted of 11 elements characteristic to a healthy diet by the Nordic Nutrition Recommendations 2012. A higher intake of whole-grains, vegetables, fruits and berries, low-fat dairy, fish, and nuts and seeds, and a lower intake of processed grains, processed meats, sugary beverages, fast foods, and sweet or salty snacks were regarded as beneficial. Each component accounted for was worth of 1 point, and the maximum score available was therefore 11 points. A higher DQS score was regarded to reflect a healthier diet. The DQS was partly adapted from Masip et al. [28].

Missing data

The percentage of missing values across the variables separately for each timepoint varied from 0 to 21%. The number of valid measurements for 298 participants in each variable is presented in Table 1. The total number of missing data values was 741 out of 13,708 (5%). Missing data occurred due to invalid or missing measurements as well as unclear or incomplete questionnaire responses. Missing data were assumed to occur at random and multiple imputation was used to create and analyse 50 multiply imputed data sets. Multiple imputation was carried out in R [29] using the "mice" package [30]. All variables measured at the same timepoint and the target variable measurement from the other timepoint were used for imputation of each variable. The number of iterations was set to 50 and passive imputation was used for the derived waist-to-hip ratio (WHR) variable. The model parameters were estimated in each imputed dataset separately and pooled using Rubin's rules [31]. For comparison, we also performed the complete case analysis and there was no notable difference in the results that would have led to different conclusions.

Statistical analysis

The main analyses were carried out using linear and Poisson mixed-effect models with random intercept [32]. For each outcome variable, the fixed effects were time (0 = baseline, 1 = follow-up), menopausal group, ACC-MAD, and interactions between time and group as well as time and ACC-MAD. The interactions were included in the models to study how the change in PA associate with the change in outcome variables during the follow-up. Furthermore, the covariates included as fixed effects were mean centered age at baseline and the use of hormonal preparations. Residual plots, Q–Q plots, and correlation analysis were used for testing the model assumptions. The analyses were carried out in R using the "nlme" [33] and "lme4" [34] packages.

Based on the literature, we identified candidate covariates related to lifestyle habits and the use of medications that may be associated with the outcome variables. Their distributions in the study population are presented in detail in Supplementary Table 1. However, to our consideration, lifestyle habits and the use of antihypertensives, lipid-modifying agents or thyroid therapy do not significantly affect the progression of menopausal transition, and the use may even be caused by the menopause-induced changes in the outcome variables. Thus, only the use of sex hormone therapy was controlled for confounding. Nonetheless, we also performed the analysis including the relevant variables and their interaction with time as covariates, but it did not have a notable effect on the results. Furthermore, we conducted sensitivity analyses for blood lipids and blood pressure by excluding the participants who used lipid-modifying agents and antihypertensives, respectively.

RESULTS

Characteristics of the study population

The average follow-up-time was 3.8 years in all groups (Table 1). At baseline, the participants were slightly overweight with mean BMI of 25.3 ± SD 3.7 and had slightly elevated SBP (132.0 ± 3.7), DBP (84.1 ± 9.2), total cholesterol (5.23 ± 0.91), and LDL-C (3.05 ± 0.80). Other outcome variable means were within the normal range [35, 36]. Participants in the PRE-PRE group were the youngest and had the lowest FSH and highest E2 levels at baseline. Respectively, the participants in the POST-POST group were the oldest and had the highest FSH and lowest E2 levels. The most notable changes in E2 and FSH levels occurred in the PRE-POST group during the follow-up. The percentage of the participants with three or more MetS risk factors was 16% at baseline and at follow-up. The sensitivity analyses using unpaired *T*-test indicated the study sample to have slightly lower blood glucose (5.15 ± 0.45 and 5.28 ± 0.63, t (1387) = 3.319, $p = 0.001$) and higher ACC-MAD (30.2 ± 10.0 and 28.8 ± 8.8, t (782) = −2.044, $p = 0.041$) compared to participants that did not participate in the follow-up. No differences were observed for other outcome variables (data not shown).

Blood-based biomarkers

The PRE-POST group had lower total cholesterol and HDL-C compared to the POST-POST group (Table 2). In the full sample, ACC-MAD was directly associated with HDL-C ($B = 0.06$, 95% CI [0.01, 0.11]) and inversely with LDL-C ($B = -0.11$, 95% CI [−0.21, −0.01]). The levels of all blood-based biomarkers increased during the follow-up in the PRE-POST group and the increase tended to be smaller in the PRE-PRE and, especially, in the POST-POST group. The change in ACC-MAD was not associated with the change in any of the outcome variables measured from blood. The use of progestogen was associated with lower HDL-C, while the combined progestogen and estrogen use was associated with a lower blood glucose. The results did not differ notably when using SR-PA as a PA measure (Supplementary Table 2) or excluding participants using lipid-modifying agents (Supplementary Table 3).

[1]The threshold of 0.001 g was determined based on the correspondence with the self-reported wear time in this population ($r = 0.70$). Self-reported wear time was not used in the analysis due to the invalid and missing entries in the diaries.

[2]Seven valid days were recorded in 89% (462/528) of the measurements.

Table 1. Characteristics of the study population in full sample and separately for each group.

	Full sample			PRE-POST			PRE-PRE			POST-POST		
	BL	**FU**	***Change*[a]**	**BL**	**FU**	***Change*[a]**	**BL**	**FU**	***Change*[a]**	**BL**	**FU**	***Change*[a]**
Age and blood-based biomarkers [*n*]	298	298	*298*	149	149	*149*	56	56	*56*	93	93	*93*
Age [year]	51.3 ± 1.8	55.1 ± 1.8	*3.8 ± 0.1*	51.3 ± 1.7	55.2 ± 1.7	*3.8 ± 0.2*	50.0 ± 1.4	53.8 ± 1.4	*3.8 ± 0.1*	52.1 ± 1.8	55.9 ± 1.8	*3.8 ± 0.1*
Estradiol [nmol/l]	0.38 ± 0.53	0.26 ± 0.28	*−0.12 ± 0.62*	0.47 ± 0.49	0.20 ± 0.21	*−0.27 ± 0.54*	0.52 ± 0.98	0.58 ± 0.39	*0.06 ± 1.09*	0.15 ± 0.10	0.17 ± 0.13	*0.02 ± 0.10*
Follicle-stimulating hormone [IU/l]	39.9 ± 37.1	69.5 ± 37.5	*29.5 ± 40.5*	24.2 ± 21.8	80.3 31.9	*56.0 ± 36.3*	11.7 ± 16.6	18.9 ± 12.3	*7.1 ± 21.5*	82.0 ± 29.1	82.6 ± 29.6	*0.6 ± 24.7*
Total cholesterol [mmol/l]	5.23 ± 0.91	5.67 ± 1.00	*0.43 ± 0.88*	5.14 ± 0.90	5.75 ± 1.02	*0.61 ± 0.75*	5.07 ± 0.78	5.41 ± 0.99	*0.34 ± 0.92*	5.50 ± 0.95	5.69 ± 0.94	*0.20 ± 0.98*
HDL-C [mmol/l]	1.72 ± 0.47	1.91 ± 0.50	*0.19 ± 0.39*	1.68 ± 0.42	1.93 ± 0.48	*0.25 ± 0.39*	1.61 ± 0.38	1.78 ± 0.41	*0.17 ± 0.29*	1.86 ± 0.55	1.97 ± 0.56	*0.11 ± 0.42*
LDL-C [mmol/l]	3.05 ± 0.80	3.41 ± 0.88	*0.37 ± 0.76*	2.98 ± 0.75	3.49 ± 0.91	*0.51 ± 0.67*	2.97 ± 0.75	3.27 ± 0.86	*0.30 ± 0.80*	3.20 ± 0.89	3.37 ± 0.85	*0.17 ± 0.82*
Glucose [mmol/l]	5.15 ± 0.45	5.16 ± 0.62	*0.02 ± 0.55*	5.12 ± 0.43	5.22 ± 0.70	*0.10 ± 0.64*	5.18 ± 0.42	5.20 ± 0.45	*0.03 ± 0.36*	5.18 ± 0.48	5.05 ± 0.55	*−0.13 ± 0.45*
Triglycerides [mmol/l]	1.08 ± 0.61	1.27 ± 0.73	*0.19 ± 0.53*	1.06 ± 0.53	1.31 ± 0.70	*0.25 ± 0.52*	1.03 ± 0.49	1.12±0.54	*0.10 ± 0.41*	1.13 ± 0.76	1.29 ± 0.88	*0.16 ± 0.60*
Blood pressure and anthropometrics [*n*]	249	298	*249*	139	149	*139*	46	56	*46*	64	93	*64*
Systolic blood pressure [mmHg]	132.0 ± 16.3	133.2 ± 18.3	*2.0 ± 13.4*	132.2 ± 17.4	133.6 ± 18.0	*2.0 ± 13.4*	132.0 ± 15.8	133.6 ± 19.1	*2.2 ± 13.6*	131.4 ± 14.6	132.2 ± 18.4	*1.8 ± 13.4*
Diastolic blood pressure [mmHg]	84.1 ± 9.2	81.9 ± 10.0	*−2.1 ± 6.5*	83.9 ± 9.7	82.2 ± 10.4	*−1.5 ± 7.1*	84.3 ± 8.6	81.5 ± 9.3	*−3.1 ± 5.9*	84.3 ± 8.8	81.7 ± 10.0	*−2.8 ± 5.3*
Waist circumference [cm]	82.9 ± 9.7	83.7 ± 10.4	*1.2 ± 4.2*	83.0 ± 10.3	83.8 ± 11.1	*1.3 ± 3.9*	83.2 ± 8.8	84.6 ± 9.6	*0.9 ± 4.8*	82.3 ± 9.0	83.1 ± 9.8	*1.1 ± 4.4*
Waist-to-hip ratio × 100	82.5 ± 6.4	84.2 ± 5.5	*1.7 ± 3.7*	82.4 ± 6.7	83.8 ± 5.4	*1.4 ± 4.1*	82.8 ± 6.0	85.1 ± 5.8	*2.1 ± 3.7*	82.7 ± 6.0	84.4 ± 5.4	*2.2 ± 2.5*
Weight [kg]	69.5 ± 10.8	70.9 ± 11.5	1.8 ± 3.9	69.8 ± 11.0	71.7 ± 12.3	2.3 ± 3.6	70.0 ± 10.3	72.0 ± 10.4	1.5 ± 3.3	68.3 ± 10.8	69.1 ± 10.6	1.0 ± 4.5
Body mass index [kg/m^2]	25.3 ± 3.7	25.8 ± 4.1	0.7 ± 1.4	25.5 ± 3.9	26.2 ± 4.4	0.9 ± 1.4	25.1 ± 3.1	25.9 ± 3.3	0.5 ± 1.2	24.9 ± 3.6	25.3 ± 3.8	0.4 ± 1.6
Body mass index [kg/m^2][b]												
<18.5	0 (1)	1 (3)		0 (0)	1 (1)		0 (0)	0 (0)		2 (1)	2 (2)	
18.5–24.9	53 (131)	44 (131)		51 (71)	44 (66)		52 (24)	38 (21)		56 (36)	47 (44)	
25–29.9	36 (90)	39 (117)		36 (50)	36 (54)		39 (18)	48 (27)		34 (22)	39 (36)	
≥30	11 (27)	16 (47)		13 (18	19 (28)		9 (4)	14 (8)		8 (5)	12 (11)	
Body composition [*n*]	244	292	*240*	137	145	*134*	44	55	*44*	63	92	*62*
Total fat mass [kg]	24.2 ± 8.4	25.9 ± 9.1	*2.0 ± 3.3*	24.7 ± 8.9	26.7 ± 9.8	*2.6 ± 2.8*	23.5 ± 7.3	25.1 ± 7.9	*1.2 ± 3.3*	23.7 ± 7.9	25.1 ± 8.4	*1.3 ± 4.0*
Android fat mass [kg]	2.14 ± 0.91	2.39 ± 1.01	*0.27 ± 0.42*	2.18 ± 0.96	2.47 ± 1.09	*0.35 ± 0.38*	2.06 ± 0.81	2.26 ± 0.87	*0.15 ± 0.35*	2.11 ± 0.88	2.34 ± 0.96	*0.17 ± 0.50*
Total fat percentage [%]	34.0 ± 7.4	35.7 ± 7.6	2.0 ± 2.8	34.3 ± 8.0	36.4 ± 8.0	2.5 ± 2.3	32.9 ± 5.9	34.2 ± 6.6	1.1 ± 3.0	34.1 ± 7.0	35.5 ± 7.4	1.4 ± 3.3
Fat free mass [kg]	45.2 ± 4.3	44.8 ± 4.4	−0.4 ± 1.5	45.2 ± 4.3	44.6 ± 4.5	−0.5 ± 1.6	46.6 ± 4.5	46.8 ± 4.0	0.1 ± 1.3	44.2 ± 4.0	44.0 ± 4.2	−0.5 ± 1.5
	249	298		139	149		46	56		64	93	

Table 1 continued

	Full sample			PRE-POST			PRE-PRE			POST-POST		
	BL	FU	*Change*[a]	BL	FU	*Change*[a]	BL	FU	*Change*[a]	BL	FU	*Change*[a]
Metabolic syndrome risk factors[b] [*n*]												
0	31 (77)	29 (87)		31 (43)	28 (42)		28 (13)	29 (16)		33 (21)	31 (29)	
1	32 (79)	35 (103)		30 (42)	35 (52)		37 (17)	39 (22)		31 (20)	31 (29)	
2	22 (54)	21 (61)		24 (33)	20 (29)		15 (7)	18 (10)		22 (14)	24 (22)	
3	10 (25)	8 (25)		10 (14)	8 (12)		9 (4)	11 (6)		11 (7)	8 (7)	
4	4 (11)	5 (15)		4 (6)	6 (9)		7 (3)	4 (2)		3 (2)	4 (4)	
5	1 (3)	2 (7)		1 (1)	3 (5)		4 (2)	0 (0)		0 (0)	2 (2)	
Accelerometer-measured PA [*n*]	235	283	*222*	134	141	*126*	43	55	*43*	58	87	*53*
ACC-MAD [mg]	30.2 ± 10.0	28.3±8.6	*−1.9 ± 7.2*	29.7 ± 11.1	28.0 ± 8.3	*−1.8 ± 7.8*	31.7 ± 7.8	29.1 ± 8.8	*−3.3 ± 5.8*	30.3 ± 8.6	28.4 ± 9.0	*−0.9 ± 6.7*
Use of hormonal preparations[b] [*n*]	298	298		149	149		56	56		93	93	
Non-user	62 (186)	60 (180)		62 (101)	66 (99)		39 (22)	34 (19)		68 (63)	67 (62)	
Progestogen	38 (112)	19 (56)		32 (48)	15 (23)		61 (34)	41 (23)		32 (30)	11 (10)	
Estrogen	0 (0)	3 (10)		0 (0)	3 (4)		0 (0)	4 (2)		0 (0)	4 (4)	
Progestogen + Estrogen	0 (0)	18 (52)		0 (0)	15 (23)		0 (0)	21 (12)		0 (0)	18 (17)	
Lifestyle habits [*n*]	276	298	*276*	144	149	*144*	53	56	*53*	79	93	*79*
Alcohol consumption [portions/wk]	3.73 ± 3.92	3.24 ± 3.43	*−0.53 ± 2.63*	3.93 ± 3.32	3.68 ± 3.69	*−0.26 ± 2.32*	3.00 ± 2.43	2.54 ± 1.94	*−0.58 ± 1.91*	3.86 ± 5.43	2.98 ± 3.62	*−0.99 ± 3.45*
Diet quality score	5.87 ± 2.45	5.85 ± 2.26	*−0.02 ± 1.90*	5.84 ± 2.52	5.70 ± 2.33	*−0.04 ± 1.90*	5.77 ± 2.28	6.20 ± 2.34	*0.38 ± 1.91*	5.99 ± 2.46	5.88 ± 2.08	*−0.27 ± 1.89*
Smoking[b]												
Non-smoker	95 (262)	94 (280)		95 (136)	94 (140)		96 (51)	96 (54)		95 (75)	92 (86)	95 (262)
Smoker	5 (13)	6 (18)		5 (7)	6 (9)		4 (2)	4 (2)		5 (4)	8 (7)	5 (13)

Data are mean ± SD unless otherwise specified.

PRE-POST participants who were pre- or perimenopausal at baseline and postmenopausal at follow-up, *PRE-PRE* participants who were pre- or perimenopausal in both measurements, *POST-POST* participants who were postmenopausal already at baseline, *BL* baseline measurement, *FU* follow-up measurement, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *PA* physical activity, *ACC-MAD* accelerometer-measured mean amplitude deviation, *mg* milligravity (0.00981 m/s^2).

[a]For participants with baseline and follow-up measurement.

[b]Data are % (*n*).

Table 2. Pooled fixed effect estimates for blood-based biomarkers ($n = 298$).

	Total cholesterol [mmol/l]		HDL-C [mmol/l]		LDL-C [mmol/l]		Glucose [mmol/l]		Triglycerides [mmol/l]	
	B	**95% CI**	***B***	**95% CI**	***B***	**95% CI**	***B***	**95% CI**	***B***	**95% CI**
Intercept (PRE-POST)	5.48***	[5.12, 5.83]	1.54***	[1.38, 1.72]	3.33***	[3.02, 3.62]	5.17***	[4.97, 5.38]	1.24***	[0.99, 1.48]
Main effects										
Group										
PRE-POST (ref.)	–		–		–		–		–	
PRE-PRE	0.03	[−0.27, 0.33]	−0.05	[−0.20, 0.10]	0.05	[−0.22, 0.32]	0.04	[−0.13, 0.22]	0.02	[−0.19, 0.24]
POST-POST	0.33*	[0.09, 0.59]	0.18**	[0.05, 0.31]	0.21	[−0.02, 0.43]	0.06	[−0.08, 0.20]	0.05	[−0.13, 0.22]
ACC-MAD [10 mg]	−0.10	[−0.21, 0,01]	0.06*	[0.01, 0.11]	−0.11*	[−0.21, −0.01]	−0.02	[−0.08, 0.04]	−0.06	[−0.13, 0.01]
Age at baseline [year]	0.03	[−0.03, 0.09]	−0.00	[−0.03, 0.03]	0.02	[−0.03, 0.07]	−0.00	[−0.03, 0.03]	0.03	[−0.01, 0.07]
Use of hormonal preparations										
Non-user (ref.)	–		–		–		–		–	
Progestogen	−0.14	[−0.32, 0.04]	−0.11**	[−0.20, −0.03]	−0.05	[−0.21, 0.10]	0.06	[−0.05, 0.16]	−0.03	[−0.15, 0.09]
Estrogen	−0.19	[−0.68, 0.30]	0.16	[−0.07, 0.38]	−0.16	[−0.59, 0.27]	0.12	[−0.18, 0.42]	−0.13	[−0.45, 0.18]
Progestogen + Estrogen	−0.17	[−0.42, 0.07]	−0.08	[−0.19, 0.03]	−0.15	[−0.37, 0.06]	−0.19*	[−0.33, −0.04]	−0.02	[−0.17, 0.14]
Time (PRE-POST)	0.45*	[0.07, 0.84]	0.35***	[0.18, 0.52]	0.40*	[0.06, 0.74]	0.32**	[0.08, 0.55]	0.28*	[0.03, 0.52]
Interactions										
Time × Group										
Time × PRE-POST (ref.)	–		–		–		–		–	
Time × PRE-PRE	−0.28*	[−0.54, −0.01]	−0.07	[−0.18, 0.05]	−0.21	[−0.44, 0.02]	−0.06	[−0.22, 0.11]	−0.15	[−0.32, 0.01]
Time × POST-POST	−0.42***	[−0.65, −0.20]	−0.15*	[−0.24, −0.05]	−0.34**	[−0.53, −0.14]	−0.22*	[−0.36, −0.08]	−0.09	[−0.23, 0.05]
Time × ACC-MAD	0.05	[−0.07, 0.18]	−0.04	[−0.09, 0.02]	0.04	[−0.07, 0.15]	−0.07	[−0.14, 0.01]	−0.01	[−0.09, 0.07]

HDL-C high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *CI* Confidence interval, *PRE-POST* participants who were pre- or perimenopausal at baseline and postmenopausal at follow-up (reference group), *PRE-PRE* participants who were pre- or perimenopausal in both measurements, *POST-POST* participants who were postmenopausal already at baseline, *ACC-MAD* accelerometer-measured mean amplitude deviation, *mg* milligravity (0.00981 m/s^2), *Time* from baseline to follow-up.
*$p \leq 0.05$; **$p \leq 0.01$; ***$p < 0.001$.

Table 3. Pooled fixed effect estimates for body composition and anthropometrics ($n = 298$).

	Fat mass [kg]		Android fat mass [kg]		Waist circumference [cm]		Waist-to-hip ratio × 100	
	B	**95% CI**	***B***	**95% CI**	***B***	**95% CI**	***B***	**95% CI**
Intercept (PRE-POST)	26.66***	[24.59, 28.73]	2.46***	[2.19, 2.73]	85.41***	[82.81, 88.00]	84.95***	[82.85, 87.04]
Main effects								
Group								
PRE-POST (ref.)	–		–		–		–	
PRE-PRE	0.46	[−2.33, 3.26]	0.04	[−0.27, 0.35]	1.96	[−1.30, 5.22]	1.10	[−0.92, 3.12]
POST-POST	−0.74	[−3.05, 1.57]	−0.04	[−0.30, 0.21]	−0.75	[−3.48, 1.97]	−0.05	[−1.78, 1.68]
ACC-MAD [10 mg]	−0.77**	[−1.27, −0.26]	−0.11**	[−0.18, −0.03]	−0.92**	[−1.60, −0.24]	−0.89**	[−1.51, −0.27]
Age at baseline [year]	0.40	[−0.18, 0.98]	0.04	[−0.02, 0.11]	0.61	[−0.07, 1.29]	0.32	[−0.08, 0.72]
Use of hormonal preparations								
Non-user (ref.)	–		–		–		–	
Progestogen	−0.61	[−1.55, 0.34]	−0.06	[−0.18, 0.05]	−0.28	[−1.48, 0.92]	−0.12	[−1.19, 0.94]
Estrogen	1.02	[−1.08, 3.13]	0.09	[−0.17, 0.36]	1.14	[−1.58, 3.86]	0.19	[−2.37, 2.75]
Progestogen + Estrogen	−0.60	[−1.81, 0.62]	−0.14	[−0.29, 0.01]	−0.47	[−1.94, 0.99]	0.58	[−0.76, 1.92]
Time (PRE-POST)	1.72*	[0.16, 3.28]	0.26**	[0.06, 0.46]	0.44	[−1.64, 2.51]	−0.75	[−2.77, 1.26]
Interactions								
Time × Group								
Time × PRE-POST (ref.)	–		–		–		–	
Time × PRE-PRE	−1.33*	[−2.41, −0.26]	−0.15*	[−0.29, −0.02]	−0.19	[−1.61, 1.22]	0.61	[−0.81, 2.03]
Time × POST-POST	−1.20*	[−2.12, −0.28]	−0.12	[−0.24, 0.00]	−0.47	[−1.76, 0.81]	0.36	[−0.92, 1.64]
Time × ACC-MAD	0.24	[−0.28, 0.75]	0.03	[−0.04, 0.09]	0.23	[−0.46, 0.91]	0.72*	[0.05, 1.38]

CI Confidence interval, *PRE-POST* participants who were pre- or perimenopausal at baseline and postmenopausal at follow-up (reference group), *PRE-PRE* participants who were pre- or perimenopausal in both measurements, *POST-POST* participants who were postmenopausal already at baseline, *ACC-MAD* accelerometer-measured mean amplitude deviation, *mg* milligravity (0.00981 m/s^2), *Time* from baseline to follow-up.
*$p \le 0.05$; **$p \le 0.01$; ***$p < 0.001$.

Body composition and anthropometrics

ACC-MAD was inversely associated with total fat mass ($B = -0.77$, 95% CI [−1.27, −0.26]) and android fat mass ($B = -0.12$, 95% CI [−0.18, −0.03]), waist circumference ($B = -0.92$, 95% CI [−1.60, −0.24]), and WHR ($B = -0.89$, 95% CI [−1.51, −0.27]) in the full sample (Table 3). Total ($B = 1.72$, 95% CI [0.16, 3.28]) and android fat mass ($B = 0.26$, 95% CI [0.06, 0.46]) increased during the follow-up in the PRE-POST group and the change was smaller in the PRE-PRE and POST-POST groups compared to the PRE-POST group. The change in ACC-MAD was directly associated with the change in WHR ($B = 0.72$, 95% CI [0.05, 1.38]). Combined progestogen and estrogen use was associated with lower android fat mass when compared to non-hormone users. The results were relatively similar when using SR-PA, however, SR-PA was not associated with the change in WHR (Supplementary Table 4).

Blood pressure

ACC-MAD was not associated with SBP and DBP in the full sample (Table 4). SBP increased during the follow-up in the PRE-POST group ($B = 9.37$, 95% CI [3.34, 15.39]) and the change did not differ between the groups. Additionally, the change in ACC-MAD was inversely associated with the change in SBP ($B = -2.40$, 95% CI [−4.34, −0.46]), but this association was not observed with SR-PA (Supplementary Table 5). The combined progestogen and estrogen use was associated with lower SBP and DBP. The results did not differ notably when excluding participants using antihypertensives (Supplementary Table 6).

Number of MetS risk factors

In the Poisson mixed-effect models (Table 5), age at baseline was directly associated with the number of MetS risk factors at baseline (exp(B) = 1.07, 95% CI [1.00, 1.14]) in the full sample. The number of risk factors at baseline and the change in the number of risk factors during the follow-up did not differ between the groups. Furthermore, ACC-MAD was not associated with the number of risk factors at baseline nor with the change in the number. The results did not differ notably when using SR-PA (Supplementary Table 7) or excluding participants using lipid-modifying agents or antihypertensives (Supplementary Table 8).

DISCUSSION

In this longitudinal study of middle-aged women, an increase in several indicators of metabolic health, ranging from blood-based biomarkers and SBP to body adiposity, were observed during the follow-up. The increase was greater during menopausal transition, and the rate of change decelerated after menopause, especially in blood-based biomarkers. Higher PA was associated with favorable levels in metabolic health indicators; however, the change in PA did not associate with the rate of change during the follow-up in most of the studied metabolic health indicators. Nonetheless, associations of higher PA with a greater increase in WHR and a smaller increase in SBP were observed. PA was not associated with the number of MetS risk factors.

We observed a significant increase in total cholesterol, HDL-C, LDL-C, triglycerides, and blood glucose in women going through menopause during the follow-up. Several other longitudinal studies have also reported an increase in serum total cholesterol, LDL-C, and triglycerides during the menopausal transition [37–40]. However, the literature on the associations of menopause and HDL-C is more inconsistent. Previous studies have reported HDL-C to increase [12, 19, 39, 41], peak right before menopause [40], as

Table 4. Pooled fixed effect estimates for blood pressure ($n = 298$).

	Systolic blood pressure [mmHg]		Diastolic blood pressure [mmHg]	
	B	**95% CI**	***B***	**95% CI**
Intercept (PRE-POST)	130.91***	[125.01, 136.80]	84.90***	[81.83, 87.96]
Main effects				
Group				
PRE-POST (ref.)	–		–	
PRE-PRE	1.31	[−4.41, 7.03]	1.10	[−2.07, 4.27]
POST-POST	−1.97	[−6.79, 2.85]	0.01	[−2.66, 2.68]
ACC-MAD [10 mg]	0.28	[−1.42, 2.00]	−0.36	[−1.24, 0.51]
Age at baseline [year]	1.10	[−0.03, 2.23]	0.42	[−0.22, 1.06]
Use of hormonal preparations				
Non-user (ref.)	–		–	
Progestogen	0.46	[−2.66, 3.59]	−0.18	[−1.80, 1.43]
Estrogen	1.76	[−6.24, 9.76]	−0.95	[−4.91, 3.01]
Progestogen + Estrogen	−5.55**	[−9.61, −1.49]	−4.33***	[−6.36, −2.30]
Time (PRE-POST)	9.37**	[3.34, 15.39]	−0.16	[−3.11, 2.79]
Interactions				
Time × Group				
Time × PRE-POST (ref.)	–		–	
Time × PRE-PRE	0.62	[−3.67, 4.91]	−0.81	[−2.88, 1.26]
Time × POST-POST	−0.03	[−3.80, 3.73]	−0.73	[−2.57, 1.12]
Time × ACC-MAD	−2.40*	[−4.34, −0.46]	−0.28	[−1.24, 0.68]

CI Confidence interval, *PRE-POST* participants who were pre- or perimenopausal at baseline and postmenopausal at follow-up (reference group), *PRE-PRE* participants who were pre- or perimenopausal in both measurements, *POST-POST* participants who were postmenopausal already at baseline, *ACC-MAD* accelerometer-measured mean amplitude deviation, *mg* milligravity (0.00981 m/s^2), *Time* from baseline to follow-up.
*$p \leq 0.05$; **$p \leq 0.01$, ***$p < 0.001$.

well as continuously decline during menopausal transition [42]. In addition to increase in HDL-C in the PRE-POST group, higher baseline HDL-C levels and lower increase rate in the postmenopausal group were also observed. These conflicting results suggest that the change in HDL-C during menopausal transition is a complicated process related to, e.g., aging and genetic background. As HDL-C and its antiatherogenic functionality have a major role in promoting cardiovascular health, it is obvious that more detailed longitudinal studies are needed to clarify this process.

Previous findings on associations of menopausal transition and blood glucose are also contradictory. Some longitudinal studies have reported a decrease [15, 19] during the menopausal transition, but in cross-sectional design postmenopausal women have been reported to have higher blood glucose compared to pre- and perimenopausal women [10, 43]. We observed an increase in fasting blood glucose in women going through menopause and the increase was attenuated in the POST-POST group. Our findings indicate that in addition to aging, the increase in blood glucose may be explained by the decreasing E2 levels during menopausal transition, since E2 is known to enhance insulin sensitivity and glucose disposal in women [44].

The observed increase in total and android fat masses in this study are consistent with previous literature [45–47]. The decrease in female sex hormone levels during menopausal transition is proposed to lead to increased accumulation of adipose tissue especially in the waist and visceral area [11, 48], yet the association of menopause to total adipose tissue accumulation is somewhat debated [9]. Although android fat mass increased during the follow-up, we did not observe a change in waist circumference. Similar results have also been reported by others [19, 49]. This indicates a change in the ratio between android lean and fat masses during the follow-up. A comparable change in muscle-to-fat ratio is also observed in total body level during the menopausal transition [11, 47]. Furthermore, we observed an increase in SBP that did not differ between the groups. This finding is supported by the previous review by Taddei [50] that suggested the changes in SBP to be more dependent on age than menopausal status in middle-aged women.

Regular PA is a well-established contributor to a healthier blood lipid profile and body composition also in menopausal women [12, 51]. With both accelerometry-measured and self-reported measures, higher PA was associated with lower levels in blood-based biomarkers and body composition variables but, surprisingly [52], not in blood pressure. When exploring the combined effect of PA and follow-up time, increased PA was associated with an accelerated increase in WHR. This result suggests accelerated decrease in hip circumference in more active women, since the change in PA was not associated with the change in waist circumference. While estradiol levels are associated with both gluteal adipose [53] and muscle mass [14, 54], we suspect that the pronounced decrease in more physically active women is caused especially by the loss of muscle mass due to the potentially higher muscle mass on their gluteal area at baseline. However, in the current study, we were not able to accurately identify the lost tissue type at the hip area. We also observed higher PA to be associated with a smaller increase in SBP during the follow-up. As discussed earlier, the observed changes in SBP may have been related to aging rather than menopausal transition [50], but our results indicate that regular PA may be efficient for controlling SBP in menopausal women similar to other populations [55, 56].

Table 5. Pooled fixed effect estimates for number of metabolic syndrome risk factors ($n = 298$).

	Number of metabolic syndrome risk factors	
	exp (B)	**95% CI**
Intercept (PRE-POST)	1.41	[0.91, 2.16]
Main effects		
Group		
PRE-POST (ref.)	–	
PRE-PRE	1.31	[0.93, 1.84]
POST-POST	0.95	[0.71, 1.28]
ACC-MAD [10 mg]	0.91	[0.79, 1.04]
Age at baseline [year]	1.07*	[1.00, 1.14]
Use of hormonal preparations		
Non-user (ref.)	–	
Progestogen	0.98	[0.78, 1.23]
Estrogen	0.85	[0.41, 1.74]
Progestogen + Estrogen	0.71	[0.50, 1.02]
Time (PRE-POST)	1.59	[0.93, 2.73]
Interactions		
Time × Group		
Time × PRE-POST (ref.)	–	
Time × PRE-PRE	0.77	[0.53, 1.14]
Time × POST-POST	0.93	[0.67, 1.30]
Time × ACC-MAD	0.88	[0.73, 1.06]

CI Confidence interval, *PRE-POST* participants who were pre- or perimenopausal at baseline and postmenopausal at follow-up (reference group), *PRE-PRE* participants who were pre- or perimenopausal in both measurements, *POST-POST* participants who were postmenopausal already at baseline, *ACC-MAD* accelerometer-measured mean amplitude deviation, *mg* milligravity (0.00981 m/s^2), *Time* from baseline to follow-up.
*$p \le 0.05$; **$p \le 0.01$; ***$p < 0.001$.

Although PA was associated with individual indicators of metabolic health, no associations with PA and the number of MetS risk factors or change in the number were observed. This may be caused by the strict cutoff points used in the clinical identification of MetS that does not capture the change unless the cutoff point is reached. Our findings are somewhat contradictory to a recent longitudinal study [57] in which higher PA was associated with lower incidence and better recovery from MetS in middle-aged women. Nonetheless, also in our study, the number of MetS factors tended to be smaller and the increase in the number was slightly lower in more active participants. Thus, PA might be beneficial for preventing the unwanted changes in individual MetS risk factors, but more studies on the associations of PA and number of MetS risk factors during menopause are required.

An interesting additional finding of the study was the observed associations of external hormone use with multiple indicators of metabolic health, highlighted by the distinctive association between the combined use of estrogen and progestogen and lower SBP. The use of hormone replacement therapy has been previously shown to reduce abdominal fat, blood glucose, LDL-to-HDL ratio and blood pressure [58], similar to our results. The individual effects of progestogen use on body composition and metabolic health are less studied, but estrogen is recognized to associate directly with gynoid adipose tissue volume [48, 59], better insulin sensitivity [44], and beneficial effects on vasodilatation and LDL-C concentration [60]. Although our results from exogenous hormone use are mostly in agreement with previous results, the results need to be interpreted with caution, since we did not consider the dosage, the duration of use, or form of the exogenous hormones.

One of the limitations was that the measurements were repeated only once. The homogenous sample of white, middle-aged women with exclusion of women with severe obesity and different medical disorders may limit the generalizability of the results for more heterogeneous populations including participants with disabling conditions. Furthermore, based on the sensitivity analysis, dropouts during the study have caused healthy selection bias particularly towards slightly better glucose control and higher PA which also limits the generalizability of the results. However, this is unlikely to have caused overestimation of the observed unhealthy menopause-related changes in outcome variables. The strengths of the study included the use of accelerometers for PA and DXA for body composition measurements. Additionally, the study design in which women of similar age but different menopausal status were followed for the same amount of time allowed to study the menopause-related changes in outcome variables while taking into account the simultaneous aging.

In conclusion, the results indicate that undesirable changes in blood lipids, body adiposity, and blood pressure occur in middle-aged women, and the rate of change accelerates near menopause, especially in blood lipids. Although habitual PA associated with a healthier blood lipid profile and lower body adiposity in middle-aged women in this study, it did not significantly modulate the menopause-related changes in most of the studied metabolic health indicators. However, higher PA may attenuate the increase in SBP and associate with an accelerated increase in WHR. These results indicate that significant increases in PA around menopause may be needed to counteract the menopause-related changes in blood-based biomarkers and body adiposity. Nonetheless, our findings could encourage professionals working with menopausal women to highlight the importance of PA in the early prevention of hypertension and cardiovascular disease. Further longitudinal studies on the role of PA on the metabolic health during the menopausal transition are needed.

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AUTHOR CONTRIBUTIONS

MH and HKJ prepared the original draft and share the first authorship of the paper. MH was the major contributor in statistical analyses, while ST offered guidance. MH analyzed the raw accelerometer data and HKJ processed the DXA data. JEK was responsible for forming the diet analysis used. VK and EKL obtained funding for the project. ST, JEK, SK, THT, VK, PA, UMK, TR, SS, and EKL all gave their professional effort in the writing process. All authors read and approved the final paper.

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COMPETING INTERESTS

The authors declare no competing interests.

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III

ROLE OF MENOPAUSAL TRANSITION AND PHYSICAL ACTIVITY IN LOSS OF LEAN AND MUSCLE MASS: A FOLLOW-UP STUDY IN MIDDLE-AGED FINNISH WOMEN

by

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Role of Menopausal Transition and Physical Activity in Loss of Lean and Muscle Mass: A Follow-Up Study in Middle-Aged Finnish Women

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Abstract: In midlife, women experience hormonal changes due to menopausal transition. A decrease especially in estradiol has been hypothesized to cause loss of muscle mass. This study investigated the effect of menopausal transition on changes in lean and muscle mass, from the total body to the muscle fiber level, among 47–55-year-old women. Data were used from the Estrogenic Regulation of Muscle Apoptosis (ERMA) study, where 234 women were followed from perimenopause to early postmenopause. Hormone levels (estradiol and follicle stimulating hormone), total and regional body composition (dual-energy X-ray absorptiometry (DXA) and computed tomography (CT) scans), physical activity level (self-reported and accelerometer-measured) and muscle fiber properties (muscle biopsy) were assessed at baseline and at early postmenopause. Significant decreases were seen in lean body mass (LBM), lean body mass index (LBMI), appendicular lean mass (ALM), appendicular lean mass index (ALMI), leg lean mass and thigh muscle cross-sectional area (CSA). Menopausal status was a significant predictor for all tested muscle mass variables, while physical activity was an additional significant contributor for LBM, ALM, ALMI, leg lean mass and relative muscle CSA. Menopausal transition was associated with loss of muscle mass at multiple anatomical levels, while physical activity was beneficial for the maintenance of skeletal muscle mass.

Keywords: menopause; female aging; skeletal muscle; sarcopenia; estradiol; physical activity

1. Introduction

Skeletal muscle is responsible for movements under voluntary control, but it also has an important role in metabolism [1,2]. During aging, muscle mass decreases due to an imbalance in muscle protein turnover and cell atrophy [3]. In women, aging-related hormonal changes accelerate especially during menopause, which women face in middle age [4,5]. Estradiol, the main female sex steroid hormone lost due to menopause, has been proposed to be among the molecular regulators of female skeletal

muscle properties [6]. Menopausal transition starts when the hypothalamus begins to show signs of aging and the release of gonadotropin-releasing hormone becomes unsynchronized [7]. This leads to an imbalance between the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH), and when combined with simultaneous ovarian aging, estradiol levels decrease [7]. When menopause occurs, i.e., when the menstrual cycle ceases completely, systemic FSH levels are set high and estradiol levels low. These hormonal changes, particularly the loss of systemic estradiol from the ovaries, have been suggested to have an impact on whole body and skeletal muscle composition, whereby more adipose tissue is accumulated and total muscle mass decreases [8–10]. To date, the majority of human studies regarding menopause and muscle mass have been based on cross-sectional studies (e.g., [11,12]), and to our knowledge, the number of longitudinal studies [8,13,14] is limited.

Total muscle mass is dependent on the number and size of muscle cells. Human skeletal muscle consists of three major muscle fiber types, which differ in functional and metabolic properties. This distinction is based on the content of the dominant myosin heavy chain (MHC) type, the main determinant of muscle cell contractile properties. Type I myofibers (MHC-I) or "slow fibers" are myofibers with high mitochondrial and capillary density, and they provide power for long-term contractions, e.g., during postural maintenance and endurance sports. Type IIA (MHC-IIA) and type IIX (MHC-IIX) fibers or "fast fibers" are more glycolytic in nature, and contribute to short-duration high-intensity activities [15]. While the proportion of different fiber types in muscles is mostly attributable to genetics, it seems that age, level and type of physical activity, inactivity and body weight also have an effect [16,17]. Only a few studies have focused specifically on the role of estradiol in fiber type distribution [18–20], so the role of the main female sex hormone in this process remains largely unknown.

The aim of this longitudinal study was to investigate how menopause affects lean and skeletal muscle mass in women. We were also interested in the possible association between physical activity level and skeletal muscle tissue properties during the menopausal transition. To gain a comprehensive insight into the menopausal transition, we used whole body, limb and cellular variables to estimate lean body mass (LBM), appendicular lean mass (ALM), thigh muscle cross-sectional area and *m. vastus lateralis* muscle fiber composition before and after menopause, as presented in Figure 1.

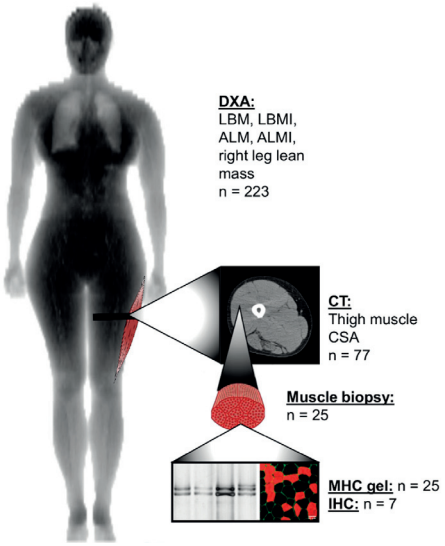

Figure 1. Anatomical levels of lean and muscle mass measurements and the number of the same participants measured at baseline and final follow-up time-points. ALM, appendicular lean mass; ALMI, appendicular lean mass index; CSA, cross-sectional area; CT, computed tomography; DXA, dual-energy X-ray absorptiometry; IHC, immunohistochemistry; LBM, lean body mass; LBMI, lean body mass index; MHC, myosin heavy chain.

2. Materials and Methods

2.1. Study Design and Participants

This study utilized data and samples from the Estrogenic Regulation of Muscle Apoptosis (ERMA) study [21]. The flow chart of the study is presented in Figure 2. Postal invitations to participate were sent to 6878 randomly selected 47–55-year-old women from the Finnish National Registry who were living in the city of Jyväskylä or neighboring regions. Of these 6878, 3064 returned the pre-questionnaire. After applying the exclusion criteria of self-reported body mass index (BMI) over 35 kg/m^2, use of estrogen-containing contraceptives or other medications and conditions affecting ovarian function, altered systemic inflammatory status and health concerns or conditions jeopardizing ordinary physical function, 1627 participants were invited to the laboratory for further assessment. Of these, 1393 came to the first blood sampling session and were assigned to menopausal groups (premenopausal, early perimenopausal, late perimenopausal and postmenopausal), based on their FSH levels and menstrual bleeding reported in a diary. The ERMA follow-up study (core-ERMA) invited 381 women assigned to the early or late perimenopausal groups to participate in a follow-up study over the menopausal transition (Figure 2). These core-ERMA participants were followed from perimenopause to early postmenopause (mean follow-up time 15.3 ± 8.6 months), with regular laboratory visits every 3–6 months. Follow-up visits were continued until FSH level was above 30 IU/mL and no menstrual bleeding was reported during the last five to six months. Within a few days of the detection of high FSH concomitant with lack of menstruation, the participant was asked to come to the laboratory for a control visit to re-test the FSH levels and re-check the bleeding diary. If in the control visit FSH levels were still high and there was still no menstrual bleeding, the participant was considered to be early postmenopausal and she was asked to come to the laboratory for final follow-up measurements. During the follow-up period, 234 women became postmenopausal and the final follow-up measurements repeating the baseline measurement protocol were performed. The number of participants who took part in the lean mass measurements used in the current study is presented in Figure 1. Eleven women did not participate in the dual-energy X-ray absorptiometry (DXA) measurements, therefore the final number of participants for total body composition measurements was 223. For the quantitative computed tomography (CT) scans and biopsies, all participants who at baseline did not use progestogen-containing contraceptives and had not had a hysterectomy were invited for measurements. The only exclusion criterion for DXA and CT was a previous history of cancer. Exclusion criteria for muscle biopsy were the use of blood thinning medications or hemophilia. During the follow-up, 37 women started using hormone replacement therapy (HT). The average follow-up time for these participants was 16.6 (6.4–35.6) months. At baseline, 21 participants in the HT-group were early perimenopausal and 16 were late perimenopausal. The most commonly used product was tablet form estradiol hemihydrate combined with dydrogesterone (Femoston, n = 15) and estradiol valeriate-containing tablets (Progynova, n = 7). Because HT use masks the progression of menopausal transition, HT users were invited to the final follow-up measurements approximately six months after their HT use was informed to the laboratory. This time period was considered to be sufficient to allow HT to have exerted its phenotypic effects. The duration of HT use varied from 2 to 337 days. During final follow-up measurements, one participant informed us that she had started using HT just two days prior to the laboratory visit. As she was already regarded to be postmenopausal, the final follow-up measurements were done as planned, but she was classified as an HT user. In the statistical analysis, the duration of HT use was controlled for all participants. All participants provided written informed consent prior to inclusion. The study was approved by the ethical committee of the Central Finland Health Care District (Dnro 8U/2014).

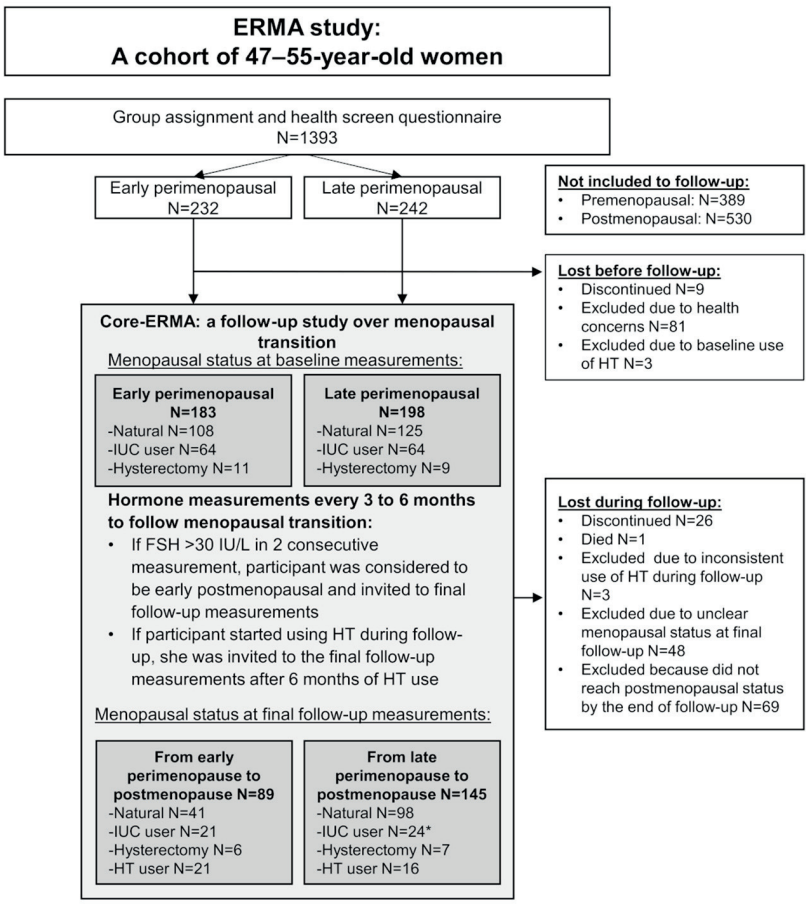

Figure 2. Flow chart of the longitudinal Estrogenic Regulation of Muscle Apoptosis (ERMA) study. FSH, follicle stimulating hormone; HT, hormone replacement therapy; IUC, intra-uterine hormonal contraceptive. * One former IUC user did not inform us if her IUC-status had changed during follow-up, so she was still counted as an IUC user at final follow-up measurements. IUC-group also includes women using progestogen pills.

2.2. Hormone Measurements

Fasting serum samples were taken from an antecubital vein between 7–10 a.m. At baseline, women with a menstrual cycle were asked to come to the laboratory between cycle days 1 to 5. Serum estradiol (E_2), follicle stimulating hormone (FSH), sulfated dehydroepiandrosterone (DHEAS) and sex hormone binding globulin (SHBG) levels were measured with IMMULITE 2000 XPi (Siemens Healthcare Diagnostics, UK).

After baseline measurements, participants started the follow-up. During follow-up, elevated FSH levels were checked with FSH control blood samples and after two similarly elevated levels combined with a lack of menstruation for at least 6 months, the participant was considered postmenopausal. The participant was then invited to the final follow-up visit for physiological measurements, during which the E_2 and FSH levels were again measured. For the final analysis, the E_2 and FSH values of the most recent follow-up visit and final follow-up visits were averaged to minimize the effect of daily fluctuations. Hormone levels for participants who started using HT were only obtained from the final follow-up visit.

2.3. Lean and Muscle Mass Measurements

Lean and muscle mass measurements were done after overnight fasting. Total LBM, appendicular lean mass (summed lean mass of arms and legs, ALM) and lean mass of the right leg were analyzed from DXA scans (LUNAR Prodigy; GE Healthcare, Chicago, IL). DXA measurement of right leg lean mass was chosen to accompany the quantitative computed tomography scans (CT) that were taken from the right thigh. LBM index (LBMI) was calculated by dividing LBM (kg) by height squared (m^2). ALM index (ALMI) was calculated similarly with ALM and height squared. An ALMI cut off-limit of 5.67 kg/m^2 [22] was used to detect sarcopenia. The right mid-thigh was scanned at the level of the muscle biopsy with CT (Siemens Somatom Emotion scanner, Siemens, Erlangen, Germany) (Figure 1). Total thigh cross-sectional area (CSA) and absolute and relative muscle areas were measured using appropriate thresholds in Python Software (version 3.6). From the cross-sectional image, the muscle portion including the femur was first separated using a machine learning algorithm called U-net [23] or manually if needed. Muscle cross-sectional area was separated from adipose tissue and bone by using Hounsfield unit (HU) limits for muscle. Relative muscle area was calculated by dividing absolute muscle area by total CSA. All images were analyzed using ImageJ Software (v.1.52, NIH) and Python.

2.4. Muscle Biopsies

Muscle biopsies were collected from the subpopulation of women at baseline and final follow-up who did not use progestogen at baseline and did not start using HT during the follow-up, i.e., those who went through the menopausal transition naturally. Biopsies were taken from the middle portion of the *m. vastus lateralis* by percutaneous needle biopsy under local anesthesia. All visible connective and adipose tissue was removed, and the sample was quickly divided into three parts. Two parts were assigned to biochemical and molecular biology analyses and were snap frozen in liquid nitrogen. The third part was embedded transversely on a cork with TissueTek and frozen in isopentane cooled in liquid nitrogen. All samples were stored at −150 °C until analysis.

2.5. Myosin Heavy Chain Isoform Separation with SDS-PAGE

Muscle samples assigned for protein analysis (weight ~ 4–12 mg) were homogenized in 1:100 myosin extraction buffer (0.1 M KCL, 0.1 M KH_2PO_4, 0.05 M $K_2HPO_4{\cdot}3H_2O$, 0.01 M EDTA, 0.02 M NaPPi, BME, Pepstatin A, Halt Proteinase and Phosphatase Inhibitor (ThermoFisher Scientific, Waltham, MA, USA)) with TissueLyser II (Qiagen, Germany). Homogenization was extended with 24-h shaking at +4 °C, followed by centrifugation for 10 min at 10,000× *g* at +4 °C (Eppendorf 5424, FA-45-24-11, Hamburg, Germany). Then, 20 µL of obtained supernatant was mixed with working Laemmli sample buffer and glycerol. Samples were heated for 4 min at +100 °C and then frozen to −20 °C. 200–300 ng of total protein was loaded into the SDS-PAGE gel system, consisting of 3% stacking gel and 6.7% separating gel with 30% glycerol. Electrophoresis was run in Bio-Rad Protean II Xi Cell for 42–44 h at 70–90 V at +4 °C. After the run, gels were fixed for one hour (40% ethanol, 10% acetic acid and 50% H_2O) and washed with water. A sensitizer (0.02% sodiumthiosulphate in water) was applied to gels for 1 min. Gels were washed and incubated in cold 0.1% silvernitrate solution with formaldehyde. After staining, the gels were again washed and developed with 3% sodiumcarbonate solution, until the staining was visible. Developing was terminated in 5% acetic acid solution. Gels were imaged with ChemiDoc MP (v.2.2.0.08, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and images analyzed with Image Lab (v.6.0.1, Bio-Rad Laboratories, Inc.).

2.6. Myofiber Type Distribution and Size Measurement

Serial transverse sections of 10 µm in thickness were cut on a cryostat at −20 °C and attached to glass slides. Slides were air dried and fixed in 4% paraformaldehyde in PBS (pH 7.4). Samples were blocked with 5% goat serum and primary antibodies were added in 1% goat serum. Myosin heavy chain distribution was analyzed with mouse antibodies against type I or type II fibers (A4.74

(1:35) for type II and A4.951 (1:40) for type I, Developmental Studies Hybridoma Bank, University of Iowa, IA, USA). Rabbit antibody for laminin (L9393, 1:250, Sigma-Aldrich, St. Louis, MO, USA) was used to mark cell borders. Primary antibodies were incubated at +4 °C overnight and the next morning, the attachment of primary antibodies was visualized with fluorescent secondary antibodies (Alexa Fluor 546 for goat anti-mouse (A11003, 1:500) and Alexa Fluor 488 for goat anti-rabbit (A11008, 1:500), Invitrogen, Carlsbad, CA, USA). Sections were mounted with Prolong Gold with Dapi (P36931, Invitrogen) and imaged with confocal microscopy (LSM 700, Axio Observer, Zeiss, Oberkochen, Germany). Images were analyzed manually with ImageJ software (v.1.52, NIH).

2.7. Physical Activity

The intensity and volume of physical activity were evaluated using a structured physical activity questionnaire [24] and ActiGraph hip-worn accelerometers (GT3X+ or wGT3X+, Pensacola, FL, USA), as reported earlier [25]. The physical activity questionnaire was used to calculate metabolic equivalent (MET) index as the product of intensity*duration*frequency of activity to give a score of MET-hours per day [24]. The data analysis process for accelerometer measurements has been reported previously [26]. Briefly, the amount of time spent at different physical activity intensities was evaluated using triaxial vector magnitude cutoff points for light, moderate and vigorous physical activity intensities: 450, 2690 and 6166 counts per minute, respectively [25,27]. Moderate-to-vigorous physical activity (MVPA) was defined by computing the sum of moderate and vigorous physical activity. The participants wore the accelerometers for seven consecutive days during waking hours, leading to some variation in accelerometer wearing times between participants. Therefore, MVPA was normalized to 16-hour wearing time per day [28]. Longitudinal accelerometer data were obtained from 173 participants, because not all participants were willing to wear accelerometers, some devices were lost in return transit and some data were lost due to technical errors.

2.8. Dietary Analysis

Diet quality score (DQS) was calculated based on a food-frequency questionnaire, which the participants completed at baseline and final follow-up measurements. The food-frequency questionnaire included 45 typical Finnish food items and 6 answer options. The DQS consisted of 11 elements characteristic of a healthy diet according to the Nordic Nutrition Recommendations 2012 (http://dx.doi.org/10.6027/Nord2014-002). The regular consumption of vegetables, fruits, and berries, dark or crispbread, low-fat dairy and fish, as well as nuts and seeds, was classified as beneficial. In contrast, a healthy diet was considered to only rarely include refined baked products, processed meats, sugary beverages, fast food, and sweet or salty snacks. Each component was worth 1 point, and the maximum score available was 11 points. A higher DQS score was considered to reflect a healthier diet. The DQS was partly adapted from [29]. Reconstruction of the original DQS was necessary, as our food-frequency questionnaire included some different food items and different wording of answers.

2.9. Background Variables

Anthropometrics were measured after overnight fasting. Body mass was measured with a digital scale and height with a stadiometer. Body mass index (BMI) was calculated as body mass divided by height squared (kg/m^2). Level of education was determined with a questionnaire and categorized as primary, secondary and tertiary. Data about smoking and alcohol consumption were collected with a structured questionnaire.

2.10. Statistical Analysis

Descriptive characteristics are reported as means and standard deviations (SD). All variables were evaluated for normality and parametric tests were used whenever possible. Independent samples *t*-test, chi-squared test and Mann–Whitney *U*-tests were used to compare baseline characteristics between early and late perimenopausal groups. Generalized estimating equations (GEE) tests of model effects

was also used to study the possible differences between the menopausal groups in the longitudinal set-up. For longitudinal analyses, differences in lean and muscle mass variables between early and late perimenopausal groups, between baseline progestogen users and non-users, and between participants who did and did not start to use HT, were tested. Because no differences were found between the groups, they were combined for analysis. Paired *t*-test and Wilcoxon rank test were used to test for differences in lean and muscle mass variables between baseline and final follow-up. Pearson and Spearman correlations were calculated to examine associations between changes in physical activity level and lean mass measures, and variables from muscle biopsies. GEE-modelling was performed to examine more detailed associations between the change in lean and muscle mass measurements and covariates during the follow-up. To investigate associations between menopausal status and each of the lean and muscle mass variables, models were controlled for baseline progestogen use, differences in the duration of HT use, and follow-up time. Education was also tested as a possible predictor, but it failed to reach significance for all variables, so it was not included as a covariate. To further investigate whether physical activity and age were also significant predictors of some or all of the muscle mass variables, they were included in the model step by step. Statistical data analysis was carried out using IBM SPSS Statistics Software version 24 (Chicago, IL, USA), and a P-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the Population at Baseline

Age, demographics and systemic hormone levels are shown in Table 1. As expected, early perimenopausal women were younger than late perimenopausal women ($P = 0.013$). The early perimenopausal group had higher serum E_2 levels than the late perimenopausal group ($P < 0.001$). Serum FSH levels were higher in the late perimenopausal group ($P < 0.001$). DHEAS and SHBG did not differ between the groups. Education level, smoking habits and alcohol consumption were similar in both menopausal groups. At baseline, more than half of the participants had natural bleeding status, meaning that they did not have conditions that could confound the detection of menstrual cycle, such as the use of intrauterine or other hormonal contraception, or hysterectomy. Notably, 94% of subjects in the early and 89% in the late perimenopausal group fulfilled the national recommendations of moderate-to-vigorous physical activity (at least 150 min of MVPA per week, ≈21 min per day) and can therefore be considered to be active. No differences were observed between the groups in accelerometer-measured or self-reported physical activity.

Table 2 shows that the early and late perimenopausal groups did not differ in anthropometry or any of the lean and muscle mass variables at baseline. Approximately 50% of the participants were normal weight according to BMI, and LBM was on average more than 50% of the total body mass. LBMI was similar in both groups. ALMI was similar in both groups, and of the whole perimenopausal group, 4.5% (four participants in the early and six in the late perimenopausal group) were sarcopenic at baseline based on the previously reported cut-off values [22]. Right leg lean mass was similar in the early and late perimenopausal groups. No differences were observed in relative or absolute muscle area between the groups at baseline.

Table 1. Characteristics of the perimenopausal ERMA baseline population (n = 234).

	Early Perimenopausal (n = 89) [#]	Late Perimenopausal (n = 145) [¤]	*P*
Age, y	51.2 ± 2.0	51.8 ± 1.8	**0.013** [a]
E_2, nmol/L	0.46 ± 0.34	0.26 ± 0.18	**<0.001** [b]
FSH, IU/L	18.3 ± 5.0	46.9 ± 20.6	**<0.001** [b]
DHEAS, µmol/L	2.64 ± 1.42	2.91 ± 1.36	0.135 [b]
SHBG, nmol/L	58.5 ± 24.3	53.1 ± 22.3	0.140 [b]
Education level			
Primary	1.1%	3.4%	
Secondary	59.1%	47.6%	0.261 [b]
Tertiary	39.8%	49.0%	
Smoking			
Never	74.2%	66.9%	
Quitter	23.6%	24.1%	0.159 [b]
Current smoker	2.2%	9.0%	
Alcohol use, units/week	4.5 ± 4.5	3.9 ± 3.2	0.528 [b]
Bleeding status			
Natural	56.2%	66.9%	
IUC	34.8%	29%	0.151 [c]
Hysterectomy	9.0%	4.1%	
Physical activity			
MVPA, min/day [X]	53.6 ± 22.8	52.0 ± 32.9	0.195 [b]
MET-hours/day [XX]	4.6 ± 4.2	4.3 ± 3.8	0.743 [b]

Values are given as mean ± SD or as percentage. DHEAS, sulfated dehydroepiandrosterone; E_2, estradiol; FSH, follicle stimulating hormone; IUC, intra-uterine contraception/progestogen use; MET, metabolic equivalent; MVPA, moderate-to-vigorous physical activity; SHBG, sex hormone binding globulin.[#] In the early perimenopausal group, there are missing data regarding education, alcohol use and MET-h/day (n = 1) and regarding MVPA (n = 21). [¤] In the late perimenopausal group, there are missing data regarding DHEAS and SHBG (n = 1) and MVPA (n = 21). [a] Independent samples *t*-test, [b] Mann–Whitney *U*-test, [c] Chi-squared test, [X] accelerometer-measured, [XX] self-reported. Significant results ($P \leq 0.050$) are shown in bold.

Table 2. Anthropometry and lean and muscle mass variables of perimenopausal women in the ERMA baseline population (n = 234).

	Early Perimenopausal (n = 89) [#]	Late Perimenopausal (n = 145) [¤]	*P*
Body mass, kg	69.2 ± 11.9	70.2 ± 10.8	0.534 [a]
Body height, cm	165.2 ± 5.6	165.0 ± 5.8	0.771 [a]
BMI, kg/m^2	25.4 ± 4.2	25.8 ± 3.8	
Underweight (<18.5)	0%	0%	
Normal weight (18.5–24.99)	57.3%	47.6%	0.287 [b]
Overweight (25.0–29.99)	28.1%	36.6%	
Obese (>30)	14.6%	15.9%	
DXA-measurements			
LBM, kg	42.3 ± 4.8	41.4 ± 4.1	0.141 [a]
LBMI, kg/m^2	15.4 ± 1.4	15.2 ± 1.2	0.204 [a]
ALM, kg	18.2 ± 2.4	17.9 ± 2.1	0.416 [a]
ALMI, kg/m^2	6.6 ± 0.7	6.6 ± 0.6	0.553 [a]
Right leg lean mass, kg	6.8 ± 0.9	6.8 ± 0.8	0.494 [a]
Computed tomography	(n = 24)	(n = 53)	
Absolute muscle area, cm^2	166.1 ± 8.1	167.3 ± 10.3	0.636 [a]
Relative muscle area, %	69.3 ± 4.2	69.8 ± 6.1 *	0.722 [a]

Values are given as mean ± SD. ALM, appendicular lean mass; ALMI, appendicular lean mass index; BMI, body mass index; LBM, lean body mass; LBMI, lean body mass index. [#] In the early perimenopausal group there are missing data regarding DXA-measurements (n = 5). [¤] In the late perimenopausal group, there are missing data regarding DXA-measurements (n = 6). * Because of a technical failure in one CT scan, relative muscle area could not be calculated for n = 1 participant. [a] Independent samples *t*-test, [b] Mann–Whitney *U*-test.

3.2. Changes in Characteristics and Lean and Muscle Mass Variables during the Follow-Up

Because there were no differences in lean and muscle mass variables between the early and late perimenopausal groups at baseline, the groups were combined for the longitudinal analysis. Table 3 presents the characteristics and lean and muscle mass results from the whole group at baseline and final follow-up measurements. The duration from baseline to final follow-up was on average 465 (133–1323) days. Both total body mass and BMI significantly increased during the transition ($P < 0.001$ for both). Due to the pulsatile nature of estradiol and FSH during menopausal transition, the differences in these variables were analyzed with non-parametric tests. A significant change was observed for both hormones during the follow-up ($P < 0.001$), even when the HT-users were included. Significant decreases were seen in LBM ($P = 0.019$), LBMI ($P = 0.018$), ALM ($P < 0.001$), ALMI ($P < 0.001$), right leg lean mass ($P = 0.002$) and absolute ($P < 0.001$) and relative muscle CSA ($P < 0.001$), during the menopausal transition. No change was observed in DQS between baseline and final follow-up.

Table 3. Characteristics and lean and muscle mass at baseline and final follow-up (n = 234).

	Baseline (Perimenopausal) n = 234	**Final Follow-Up (Postmenopausal) n = 234**	**Difference %**	***P***
Age, y	51.6 ± 1.9	53.0 ± 1.9	**+2.7**	**<0.001** [a]
Body mass, kg	69.8 ± 11.2	70.4 ± 11.6	**+0.9**	**<0.001** [b]
BMI, kg/m^2	25.6 ± 4.0	25.8 ± 4.1	**+0.8**	**<0.001** [b]
E_2, nmol/L	0.34 ± 0.27	0.24 ± 0.19	**−30**	**<0.001** [b]
FSH, IU/L	36.0 ± 21.6	66.9 ± 28.1	**+86**	**<0.001** [b]
DQS, points	5.7 ± 2.3	5.5 ± 2.2		0.207 [a]
Physical activity				
MVPA, min/day [X] (n = 173)	51.8 ± 29.3	49.7 ± 23.6		0.567 [b]
MET-hours/day [XX] (n = 231)	4.5 ± 3.9	4.7 ± 3.6		0.057 [b]
DXA-measurements				
LBM, kg (n = 223)	41.7 ± 4.4	41.5 ± 4.4	**−0.5**	**0.019** [a]
LBMI, kg/m^2 (n = 223)	15.3 ± 1.3	15.2 ± 1.3	**−0.7**	**0.018** [a]
ALM, kg (n = 223)	18.0 ± 2.2	17.8 ± 2.2	**−1.1**	**<0.001** [a]
ALMI, kg/m^2 (n = 223)	6.6 ± 0.6	6.5 ± 0.6	**−1.1**	**<0.001** [a]
Right leg lean mass, kg (n = 223)	6.8 ± 0.9	6.7 ± 0.8	**−1.5**	**0.002** [a]
Computed tomography				
Absolute muscle area, cm^2 (n = 77)	166.9 ± 9.6	165.3 ± 10.1	**−1.0**	**<0.001** [a]
Relative muscle area (%) (n = 76) *	69.6 ± 5.6	68.9 ± 6.0	**−1.0**	**<0.001** [a]

Values are given as mean ± SD. ALM, appendicular lean mass; ALMI, appendicular lean mass index; BMI, body mass index; DQS, diet quality score; E_2, estradiol; FSH, follicle stimulating hormone; LBM, lean body mass; LBMI, lean body mass index; MET, metabolic equivalent; MVPA, moderate-to-vigorous physical activity. * n = 76: because of a technical failure in one CT scan, relative muscle area could not be calculated. [a] paired t-test, [b] Wilcoxon Signed rank test, [X] accelerometer-measured, [XX] self-reported. Significant results ($P \leq 0.050$) are shown in bold.

Progesterone has been suggested to affect female muscle function [30,31]. Therefore, to increase the robustness of our analysis, we separately investigated changes in lean and muscle mass measures among women who used progestogen-containing contraceptives at baseline (Table S1) and women who did not (Table S2). A similar analysis was also performed for non-HT users (Table S3) and those who started using HT during follow-up (Table S4). At baseline, progestogen users and non-users did not differ from each other regarding estradiol and FSH levels, physical activity or lean mass measurements. Only age statistically differed at baseline ($P = 0.004$), whereby non-progestogen users were 0.8 years older than users. Of the 73 participants who used progestogen-based medication at baseline, 44 reported still using only progestogen containing medication (IUC or tablets) at final follow-up, and 15 reported using estradiol + progestogen containing hormone replacement therapy at final follow-up. Thirteen participants reported not using any hormone contraception or hormone replacement therapy at final follow-up, one of whom had undergone hysterectomy. One participant did not answer this question at final follow-up, but she was regarded as a current progestogen-user at final follow-up measurements. During follow-up, no significant changes were seen in any of the lean or muscle mass variables in the baseline-progestogen users. A decrease was observed in estradiol

(−37%, $P = 0.002$) and an increase in FSH (+82%, $P < 0.001$). Non-progestogen users at baseline had very similar results to those presented in Table 3 for the whole follow-up group.

In the HT using subgroup, due to HT-use, the serum level of estradiol was higher at final follow-up when compared to baseline, but just failed to reach significance. FSH levels were also higher at final follow-up ($P = 0.047$). A significant decrease was only observed in absolute muscle area ($P = 0.021$), while LBM, LBMI, ALM, ALMI, right leg lean mass and relative muscle area remained unchanged during the transition. The lean and muscle mass results obtained from the sub-group of non-HT users did not differ from the results presented in Table 3, except for self-reported physical activity, which was significantly higher at final follow-up in the non-HT users (4.3 ± 4.0 vs. 4.6 ± 3.7 MET-hours/day, $P = 0.043$).

3.3. Longitudinal Associations Between Menopausal Status, Covariates and Lean and Muscle Mass Variables

A correlation analysis was performed between the change in physical activity measures and important lean mass measures. Variables were formed by subtracting the baseline value from the final follow-up value (Δvariable). The change in the level of accelerometer-measured physical activity (ΔMVPA) was weakly, yet significantly positively, associated with ΔLBM (r = 0.182, $P = 0.027$), ΔLBMI (r = 0.182, $P = 0.027$), ΔALM (r = 0.235, $P = 0.004$), ΔALMI (r = 0.238, $P = 0.004$) and Δright leg lean mass (r = 0.241, $P = 0.003$).

Longitudinal associations between menopausal status and different lean and muscle mass variables during the menopausal transition were also tested with GEE-model (Table 4). As GEE tests of model analysis did not reveal any categorical differences between groups discordant for HT or progestogen use, the whole perimenopausal group was combined and possible hormone use was added to the model. Calculated self-reported MET-hours/day values were used as a measure of physical activity, because data were available from a higher number of participants than for MVPA. However, the same analyses were also performed using accelerometer-measured MVPA and the results, which were very similar to those for self-reported MET-hours/day, are presented in Table S5. For LBM and ALMI menopausal status, baseline use of progestogen and physical activity level measured in MET-hours/day were significant predictors (for all $P \leq 0.050$). For LBMI, menopausal status, baseline use of progestogen and age were significant predictors ($P \leq 0.037$ for all). For ALM, right leg lean mass and relative muscle area menopausal status and physical activity remained significant in the adjusted model ($P \leq 0.011$ for all). For absolute muscle area, only menopausal status remained significant ($P < 0.001$) when the same explanatory models were used.

Table 4. GEE-model with self-reported MET-hours per day as a measure of physical activity.

	Model 1		Adjusted Model	
	B	**P**	**B**	**P**
LBM				
Menopausal status	−0.193	**0.026**	−0.203	**0.019**
Use of HT	0.000	0.833	0.000	0.836
Use of progestogen	1.193	0.057	1.263	**0.050**
Follow-up time	0.001	0.503	0.001	0.420
MET-hours/day	-	-	0.053	**0.036**
Age	-	-	0.109	0.506
LBMI				
Menopausal status	−0.069	**0.029**	−0.073	**0.020**
Use of HT	0.000	0.696	0.000	0.753
Use of progestogen	0.290	0.110	0.386	**0.037**
Follow-up time	0.000	0.426	0.000	0.183
MET-hours/day	-	-	0.017	0.054
Age	-	-	0.122	**0.009**

Table 4. *Cont.*

	Model 1		Adjusted Model	
	B	**P**	**B**	**P**
ALM				
Menopausal status	−0.231	**<0.001**	−0.238	**<0.001**
Use of HT	0.001	0.362	0.001	0.354
Use of progestogen	0.555	0.061	0.557	0.069
Follow-up time	0.001	0.304	0.001	0.278
MET-hours/day	-	-	0.038	**0.009**
Age	-	-	0.025	0.766
ALMI				
Menopausal status	−0.085	**<0.001**	−0.088	**<0.001**
Use of HT	0.000	0.386	0.000	0.326
Use of progestogen	0.143	0.083	0.175	**0.039**
Follow-up time	0.000	0.200	0.000	0.082
MET-hours/day	-	-	0.014	**0.005**
Age	-	-	0.046	0.055
Right leg lean mass				
Menopausal status	−0.088	**0.001**	−0.091	**0.001**
Use of HT	0.000	0.398	0.000	0.395
Use of progestogen	0.214	0.054	0.209	0.066
Follow-up time	0.000	0.224	0.000	0.217
MET-hours/day	-	-	0.017	**0.011**
Age	-	-	0.005	0.870
Absolute muscle area *				
Menopausal status	−1.597	**<0.001**	−1.586	**0.001**
Use of HT	−0.001	0.811	−0.001	0.797
Follow-up time	−0.001	0.833	0.000	0.938
MET-hours/day	-	-	0.047	0.801
Age	-	-	0.236	0.748
Relative muscle area *				
Menopausal status	−0.007	**<0.001**	−0.007	**<0.001**
Use of HT	-1.5×10^{-5}	0.488	-6.7×10^{-6}	0.721
Follow-up time	-7.5×10^{-6}	0.716	-6.9×10^{-6}	0.747
MET-hours/day	-	-	0.002	**0.011**
Age	-	-	−0.002	0.667

Model 1: adjusted for menopausal status, HT use in days, baseline use of progestogen and follow-up time in days. Adjusted model: adjusted for menopausal status, HT use in days, baseline use of progestogen, follow-up time in days, MET-hours/day and age at baseline. * Absolute and relative muscle areas were not adjusted for baseline progestogen use, as all participants were non-users at baseline. ALM, appendicular lean mass; ALMI, appendicular lean mass index; HT, hormone replacement therapy; LBM, lean body mass; LBMI, lean body mass index; MET, metabolic equivalent. Significant results ($P \leq 0.050$) are shown in bold.

3.4. Changes at The Cellular Level

A subpopulation of participants gave biopsies at baseline and final follow-up (n = 25). They all underwent menopause naturally, as they did not use progestogen-containing contraception, nor did they start HT use during follow-up. Mean time between biopsy samples was 385 days (115–999 days). More specific information about the characteristics of these women is provided in Table S6. Changes in lean and muscle mass variables during the follow-up did not differ between participants in the biopsied and non-biopsied groups (Table S6).

Muscle biopsy samples assigned for protein analysis were used for myosin protein extraction. Relative myosin heavy chain proportion was analyzed with SDS-PAGE and silver-staining (Figure 3). Table 5 presents the proportion of different myosin isoforms, as percentages at baseline and final follow-up.

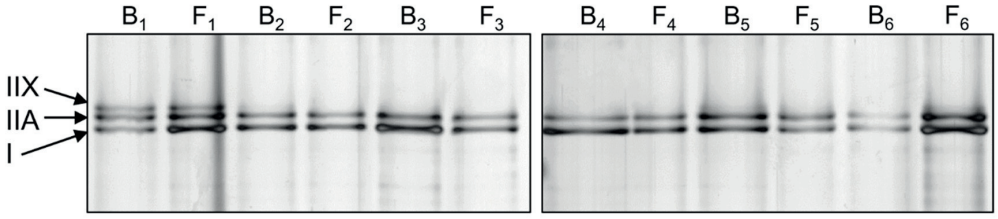

Figure 3. Representative silver-stained myosin heavy chain (MHC) SDS-PAGE results from six participants (1–6, B, baseline; F, final follow-up).

Table 5. Relative proportions of different myosin isoforms in SDS-PAGE at perimenopause and early postmenopause.

Myosin Isoform	Baseline n = 25	Final Follow-Up n = 25	*P*
Type I (%)	50.6 ± 11.2	52.9 ± 8.5	0.619
Type IIA (%)	40.6 ± 9.6	41.4 ± 6.0	0.563
Type IIX (%)	8.8 ± 10.8	5.7 ± 9.8	0.116

Values are presented as mean ± SD. Wilcoxon Rank test was used for statistical analysis.

No statistically significant differences were seen in the myosin isoform proportions when advancing through menopause. At baseline, type I myosin proportion was positively associated with the level of MVPA (r = 0.424, $P = 0.035$). At final follow-up, the proportion of type IIA myosin was negatively associated with ALM (r = −0.465, $P = 0.019$) and absolute muscle area (r = −0.521, $P = 0.013$). The proportion of type IIX myosin was positively associated with ALMI (r = 0.434, $P = 0.030$) and LBMI (r = 0.409, $P = 0.042$) at final follow-up.

Biopsies collected for immunohistological staining were used to analyze mean cross-sectional area and proportions of type I and II fibers. Figure 4 and Table 6 show the results of these analyses.

Counted cell number per participant varied from 940 to 6300 cells. The size of individual muscle fibers was unchanged between baseline and final follow-up. On average, type II fibers were smaller than type I fibers at baseline and final follow-up (for both $P < 0.001$). No changes were seen in the fiber type ratios from baseline to final follow-up.

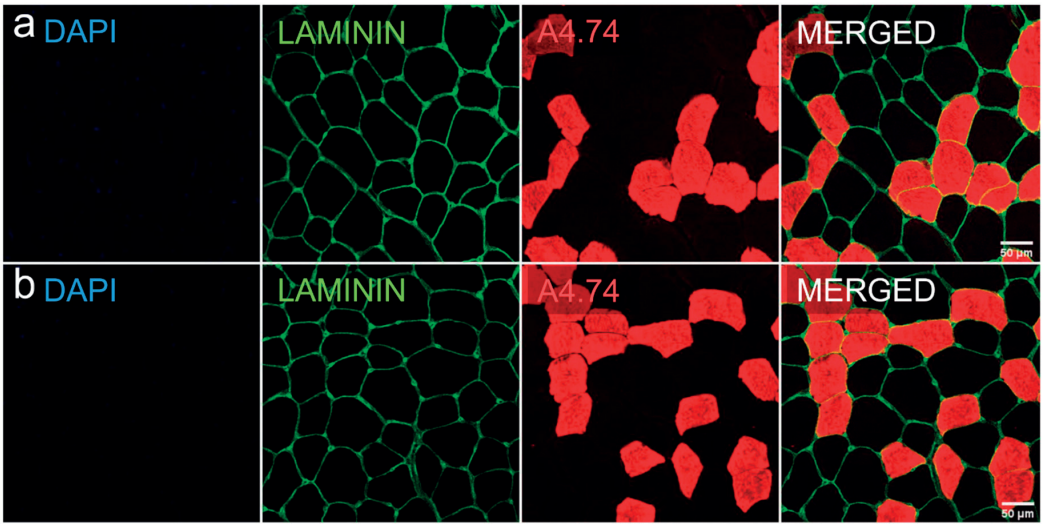

Figure 4. Representative cross-sectional area and fast myosin-based fiber typing of a muscle biopsy with Dapi (blue), laminin (green) and A4.74 (red, type II)-staining. Row (**a**) baseline, row (**b**) final follow-up.

Table 6. Mean cross-sectional area and proportions of type I and II fibers measured with immunohistological stainings.

	Baseline n = 7	Final Follow-Up n = 7	*P*
Slow (type I) cells, μm^2	3526 ± 1334	3525 ± 1618	0.735 [a]
Percentage	53.3 ± 10.9	51.1 ± 14.4	0.398 [a]
Fast (type II) cells, μm^2	2098 ± 948	2399 ± 1218	0.128 [a]
Percentage	46.7 ± 10.9	48.9 ± 14.4	0.398 [a]
P-value for difference between cell types	**<0.001** [b]	**<0.001** [b]	

Values are presented as mean ± SD. [a] Wilcoxon rank test, [b] Mann–Whitney *U*-test. Significant results ($P \leq 0.050$) are shown in bold.

At baseline, a positive correlation was observed between type II fiber size and age (r = 0.786, $P = 0.036$). A higher proportion of slow fibers was also associated with higher single leg lean mass (r = 0.857, $P = 0.014$), appendicular lean mass (r = 0.964, $P < 0.001$) and body mass (r = 0.893, $P = 0.007$). At final follow-up, larger type II fiber size correlated with higher ALMI (r = 0.786, $P = 0.036$). Positive correlations were also found at postmenopause between type I fiber proportion and total lean mass (r = 0.893, $P = 0.007$) and body mass (r = 0.929, $P = 0.003$). No significant correlations were found between the amount of physical activity and muscle fiber size or fiber type proportion.

4. Discussion

This study showed significant declines in several lean and muscle mass parameters in a longitudinal study design spanning the menopausal transition. We were able to show decreases in total lean mass, appendicular lean mass and thigh muscle cross-sectional area at a range of anatomical levels that, to our knowledge, has not been studied and showed before. This study examined the amount of skeletal muscle in relation to menopausal status, as well as possible associations between physical activity level and skeletal muscle tissue during menopausal transition. The results highlight the importance of menopause-related hormonal changes in the loss of lean and muscle mass that appears to be independent of the effects of aging. Our results also highlight the moderate yet significant importance of physical activity in maintaining lean mass during middle age.

4.1. Menopausal Transition Decreases Lean and Muscle Mass

Studies of the association between menopausal status and muscle mass have mostly used cross-sectional study designs and compared different menopausal groups [11,12,32,33]. Together with a limited number of longitudinal studies [8,13,14], they have found menopause to be associated with a decrease in skeletal muscle mass. Our results are in line with these previous studies, and here we also show changes in skeletal muscle at more anatomical levels and by using a shorter timespan over the menopausal transition than has been used before. We found that the decrease in lean mass occurs between peri- and early postmenopause, which was reflected in total body and appendicular measures. The results obtained by DXA and CT imaging collectively indicated a 0.5%–1.5% reduction in muscle mass due to menopausal transition, regardless of whether muscle mass was assessed at the anatomical level of the whole body, the limbs or the thigh.

In this study, participating women were not allowed to use any estradiol-containing medication at baseline, but we did not exclude those who began using HT during the follow-up. HT use was controlled in the main analysis and the inclusion of HT users did not affect the results of this study. However, when examining HT users and non-users separately, we did not find a similar, significant decrease in lean mass measures during the transition as with the non-HT-using subpopulation. This is in line with previous literature presenting data about the protective role of HT in muscle loss [34,35]. Our study was not designed to investigate the effects of HT, so our results in this respect need to be interpreted with caution. In our study, the participants were heterogenous in their HT supplementation

methods, dosages and usage times, as we did not control their hormone use. Furthermore, the number of HT-participants was relatively low (n = 37), which may have left our study underpowered to detect the effects of HT use. Since recent literature, as reviewed by Javed et al. [36], also presents conflicting results about the protective role of HT on muscle mass even after long-term use, clearly more studies are needed to resolve this issue.

The systemic levels of another abundant female sex hormone- progesterone- also decrease during menopause, but the role of progesterone in lean and muscle mass maintenance is less studied. Progesterone has been shown to increase protein synthesis in postmenopausal women [31], and beneficial neuronal properties of progesterone are well established, especially when combined with estradiol [30,37]. Here, we included women who used progestogen contraception at baseline and the use was controlled as "yes" or "no" in the main analysis. CT scans and muscle biopsies were taken from women who did not use progestogen at baseline. In our analysis, in the GEE-model progestogen use remained significant for multiple lean mass variables, and we did not observe a similar decrease in lean mass variables during the menopausal transition for the progestogen-using subgroup as for the non-users. Unfortunately, we did not have information about the exact duration of progestogen use for all participants, so assessing the specific role of progesterone in lean mass maintenance is difficult. That being said, progesterone might have beneficial effects on muscle mass maintenance during menopause, possibly due to increased muscle protein synthesis.

4.2. Associations of Menopausal Transition at the Single Cell Level

Aging has been shown to decrease muscle fiber area and there is evidence, albeit conflicting, of a shift in the muscle fiber type ratio towards a slower or more hybrid phenotype [38–40]. Most studies have reported that aging causes more visible changes in the size of type II fibers, while the size of slow type I fibers stays relatively unchanged in both sexes [41–44]. The role of aging in muscle tissue myosin expression has been previously studied in women in one cross-sectional study that compared 30- and 68-year old women, and the results also suggested a shift toward a slower phenotype in the older group [40]. Studies of the associations between menopause and skeletal muscle fiber size, type and myosin isoform expression are scarce. A few studies have examined the role of estrogen in animals, but the results are conflicting [19,45,46]. According to animal studies, a loss of ovarian function leads to a decline in myosin function in mature female mice [47], suggesting that myosin isoform availability may be functionally relevant. In humans, only a few studies have examined the response of skeletal muscle fibers to HT and revealed no difference between users and non-users [18,48]. To our knowledge, prior to the present study, no longitudinal studies have examined the myosin isoform distribution and muscle fiber size of perimenopausal and early postmenopausal women without HT use.

During the menopausal transition, we did not observe a change in the cross-sectional area of either type I or type II fibers, but the two fiber types did differ from each other in size both at baseline and at final follow-up. The cross-sectional area of type I fibers was larger at both time points and remained rather unchanged, similarly to what has been reported cross-sectionally in a similar age group with differing hormonal statuses [18]. The aging-related reduction in the CSA of type II fibers that has been found in earlier studies [39,49,50] was not phenocopied in our study. This suggests that muscle fiber size is not influenced by menopause, although it is also possible that changes in cell size were not detected, due to the short follow-up duration or the limited number of participants. Participants in this study had about a 50/50 ratio of type I and II fibers, which is more evenly distributed than has been previously observed in women in the same age range [18]. Widrick et al. reported that 49–57-year-old postmenopausal women had a fiber ratio of 38/62, with no difference between HT users and non-users. In our longitudinal study, it may be that type II fibers were lost early in the menopausal transition and thus that reductions were no longer detectable during the transition from perimenopause to postmenopause. However, we consider it more likely that changes in fiber type ratio occur later during aging than immediately concomitant to menopause. A higher proportion of type II fibers in muscles might have more drastic effects on muscle mass, if type II fibers are more affected by

aging. Studies of the relationship between fiber types and muscle mass have in fact reported a positive correlation between type II fiber proportion and skeletal muscle mass, in both men and women [39,40]. These results differ from our results, most likely because our study population was smaller and only included women.

4.3. Physical Activity Helps to Maintain Lean and Muscle Mass during Menopause

Here, we showed that while menopausal transition was a strong predictor of decreased lean and muscle mass, physical activity was positively associated with lean mass. Previous studies have shown that staying physically active during aging could potentially slow down the changes in skeletal muscle tissue caused by aging [51]. Physical activity has also been shown to be associated with female reproductive factors and menopausal symptoms [25], and together with estradiol, it seems to preserve favorable skeletal muscle properties [11,35,52]. We measured physical activity in two different ways; self-reports via a questionnaire, which measures mostly commute and leisure time activity, and 7-days of hip-worn accelerometer data, which included all wear-time activity, especially activities including steps. The results regarding the role of physical activity were similar with both measurements, which highlights the reliability of the results.

The combined effects of muscle fiber size/type and physical activity have not been widely studied in women, but it seems that older women are as capable of responding to both endurance and resistance exercise as males of the same age [43,53,54]. Subjects in our study remained physically relatively active and healthy during the transition, which might have positively affected their muscle fiber maintenance, and could therefore explain why no obvious decrease was seen in fiber size. No differences in physical activity levels were observed between the participants whose biopsies were used for fiber cross-sectional area analysis and non-biopsied subjects. In a recent study, the use of estradiol and progestogen-containing oral contraceptives combined with training was associated with larger type I CSA [20]. Moreover, after the training period, the fiber type shift from IIX to IIA was larger in women who used oral contraceptives, which, in contrast to our study, points to the role of estradiol in muscle cell modifications. We only analyzed muscle biopsies from women who went through natural menopause, without exposure to female hormones via oral contraceptives or HT, so they experienced fluctuating estradiol levels during the transition. We did not find associations between the level of physical activity and single muscle fiber size.

4.4. Strengths and Limitations

One of the limitations of this study is the relatively short follow-up time, especially after menopause. Although we carefully monitored the menopausal transition with sequential hormone measurements and menstrual bleeding diaries, we might have classified some women as still being perimenopausal at baseline or as postmenopausal too early, due to the relatively short follow-up time and typical fluctuations in hormone levels during the transition. Furthermore, although the study was intended to be observational, some of the participants might have increased their physical activity level during the follow-up, due to participation in this study and increased self-consciousness, which might have affected the outcomes when comparing only two timepoints. Whether or not this was the case, we were still able to see a clear decrease in lean mass at multiple body levels, which emphasizes the essential role of menopausal transition in the decline of lean and muscle mass.

This study also has several strengths. One of the main strengths is the longitudinal design, which was conducted based on a personalized timetable. As well as being a limitation, the relatively short follow-up time may be considered as a considerable strength, as we were able to show significant changes already on this time scale, emphasizing the role of menopause over purely aging-related effects. The number of participants remained relatively high throughout the study, even though the follow-up period was not long enough for all of the original core-ERMA participants to reach early postmenopause before the end of the study. It may also be considered as a strength that we utilized several parameters from different anatomical levels to examine possible changes in muscle mass.

We used DXA and CT imaging, which are highly accurate and repeatable and thus recommended methods for lean and muscle mass assessment at the whole body and limb levels. We also took muscle biopsies to examine potential cellular level changes. We had a reasonable number of repeated samples (n = 25) for myosin isoform expression analysis, but we were only able to do immunohistological staining for seven peri- and early postmenopausal women. Therefore, we cannot totally exclude the possibility that small sample size may have affected our results. When interpreting the results, it is also important to note that both muscle fiber CSA and fiber/myosin isoform proportions are known to be affected by the site of biopsy [41,44], and this might also have affected our results. Although we were obviously not able to obtain the follow-up biopsy from the exact same location as at baseline, an effort was made to take it from the closest proximity (~1 cm apart). Furthermore, multiple sections and all intact cells in them were counted to gain more data from the immunohistological samples. In addition, we took care that the CT scans were taken from the same location as the biopsies, to maximize the representativeness of these two completely different measurements.

5. Conclusions

This longitudinal study showed a significant decline in total body lean mass, appendicular lean mass and absolute and relative muscle cross-sectional area during the menopausal transition, suggesting an important role of female sex hormones in loss of muscle mass in women. Menopausal transition seems to have a role in loss of muscle mass that is independent of aging. Physical activity was associated with the maintenance of muscle mass during middle age, suggesting that women should stay physically active in order to reduce the risk of muscle mass loss-related symptoms, such as sarcopenia. Because muscle tissue is important not only for locomotion, but also for thermoregulation and whole-body metabolism, the menopause-related reductions in muscle mass demonstrated here may represent the onset of widespread negative effects on women's health.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/5/1588/s1, Table S1: Characteristics of the participants who used progestogen-based contraception at baseline, Table S2: Characteristics of the participants who did not use progestogen-based contraception at baseline, Table S3: Characteristics of the participants who went through natural menopausal transition (non-HT users), Table S4: Characteristics of the participants who started using HT during follow-up, Table S5: GEE-model with accelerometer-measured moderate-to-vigorous physical activity as a measure of physical activity (MVPA, min/day), Table S6: Characteristics of biopsied participants.

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Conflicts of Interest: The authors declare no conflict of interest.

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IV

SKELETAL MUSCLE MRNA TRANSCRIPTOME IS AFFECTED BY THE MENOPAUSAL TRANSITION

by

Juppi, H.-K., Korhonen, T.-M., Sievänen, T., Kovanen, V., Kujala, U. M., Aukee, P., Tammelin, T. H., Cronin, N. J., Sipilä, S., Karvinen, S., & Laakkonen, E. K. 2023

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