JYU DISSERTATIONS 636

Jari Karppinen

Menopause, Physical Activity, and Whole-Body Metabolism with an Emphasis on Resting Energy Expenditure, Fat Oxidation, and Serum Metabolome

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF SPORT AND
HEALTH SCIENCES

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Esitetään Jyväskylän yliopiston liikuntatieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston päärakennuksen salissa C1
kesäkuun 16. päivänä 2023 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Sport and Health Sciences of the University of Jyväskylä,
in University Main Building, auditorium C1, on June 16, 2023, at 12 o'clock noon.

JYVÄSKYLÄN YLIOPISTO
UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2023

Editors
Anne Viljanen
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ISBN 978-951-39-9575-1 (PDF)
URN:ISBN:978-951-39-9575-1
ISSN 2489-9003

Permanent link to this publication: http://urn.fi/URN:ISBN:978-951-39-9575-1

ABSTRACT

Karppinen, Jari
Menopause, physical activity, and whole-body metabolism with an emphasis on resting energy expenditure, fat oxidation, and serum metabolome
Jyväskylä: University of Jyväskylä, 2023, 153 p.
(JYU Dissertations
ISSN 2489-9003; 636)
ISBN 978-951-39-9575-1 (PDF)

Menopause is thought to impair cardiometabolic health, while physical activity is believed to promote it. This PhD thesis investigated the associations between these two exposures and resting energy expenditure, fat oxidation at rest and during exercise, and changes in the serum metabolome. The thesis also examined the relationship between fat oxidation and blood glucose regulation abilities. The data came from four studies. The longitudinal ERMA study measured serum metabolites with nuclear magnetic resonance spectroscopy from 218 women who were in perimenopause and postmenopause stages (with a median follow-up of 14 months). During the follow-up period, 35 women began using menopausal hormone therapy. The other three studies were cross-sectional and measured resting energy expenditure and fat oxidation at rest and during exercise with indirect calorimetry. The EsmiRs study recruited 42 women (aged 52–58 years) representing different menopause stages. The resting energy expenditure data were supplemented with data from 17 women in the Calex study (aged 42–58 years). The FITFATTWIN study included 23 pairs of monozygotic male twins (aged 32–37 years). Cotwins of 10 pairs were discordant for physical activity over the past 3 years. EsmiRs and FITFATTWIN participants also underwent oral glucose tolerance testing. Based on the results, menopause was associated with increased apolipoprotein B-containing lipoprotein particle number and changes in system biomarkers indicative of overnutrition and declining metabolic health. The changes in metabolite levels were partially explained by shifts in the sex hormone levels. Menopausal hormone therapy improved the apolipoprotein B/A-I ratio. Menopause status or serum sex hormone levels were not associated with resting energy expenditure or fat oxidation. Physical activity discordant cotwins also did not differ in these measures. However, higher physical activity was associated with higher peak fat oxidation during exercise in middle-aged women. Neither resting nor peak fat oxidation indicated better glycemic control or insulin sensitivity. Therefore, menopause may increase cardiometabolic risk by altering the serum metabolome rather than by decreasing resting energy expenditure or fat oxidation capacity. Physical activity may improve fat oxidation capacity during exercise in middle-aged women but without necessarily increasing the ability to regulate blood glucose levels.

Keywords: menopause, physical activity, energy metabolism, metabolomics

TIIVISTELMÄ (ABSTRACT IN FINNISH)

Karppinen, Jari
Menopaussin ja fyysisen aktiivisuuden yhteydet lepoenergiankulutukseen, rasvan käytön tehoon ja muutoksiin seerumin metabolomissa
Jyväskylä: Jyväskylän yliopisto, 2023, 153 p.
(JYU Dissertations
ISSN 2489-9003; 636)
ISBN 978-951-39-9575-1 (PDF)

Menopaussin ajatellaan heikentävän ja fyysisen aktiivisuuden edistävän aineenvaihduntaterveyttä. Tässä väitöskirjassa tutkittiin, ovatko menopaussi ja fyysinen aktiivisuus yhteydessä lepoenergiankulutukseen, rasvan käytön tehoon levossa ja liikkuessa tai muutoksiin seerumin metabolomissa. Lisäksi tarkasteltiin rasvan käytön tehon ja sokeriaineenvaihduntaterveyden välisiä yhteyksiä. Väitöskirjatutkimuksessa käytettiin neljän tutkimuksen aineistoa. ERMA-tutkimuksessa seurattiin 218 naista heidän ylittäessään menopaussin (seurannan mediaani 14 kk). Tutkimuksen aikana 35 tutkittavista aloitti hormonikorvaushoidon. Seerumin metabolomi määritettiin ydinmagneettisella resonanssispektroskopialla alku- ja loppumittausnäytteistä. Muissa tutkimuksissa mitattiin aineenvaihduntaa epäsuoralla kalorimetrialla levossa ja liikkuessa. EsmiRs-tutkimukseen rekrytoitiin 42 menopaussin suhteen eri vaiheissa olevaa naista (ikä 52–58 vuotta). Aineistoa täydennettiin Calex-tutkimuksen 17 naisen (ikä 42–58 vuotta) tuloksilla kun tutkittiin lepoenergiankulutusta. FITFATTWIN-tutkimukseen osallistui 23 identtistä mieskaksosparia (ikä 32–37 vuotta), joista 10 parin veljekset erosivat fyysiseltä aktiivisuudeltaan. EsmiRs- ja FITFATTWIN-tutkimukset sisälsivät myös sokerirasituskokeen. Menopaussi oli yhteydessä apoB:tä sisältävien lipoproteiinipartikkeleiden määrän kasvuun ja laajemmin aineenvaihduntaterveyden heikkenemisestä kertoviin biomarkkerimuutoksiin. Muutokset sukupuolihormonien pitoisuuksissa selittivät osan löydöksistä. Hormonikorvaushoito paransi apoB/A-I-suhdetta. Menopaussistatus ei ollut yhteydessä lepoenergiankulutukseen eikä rasvan käytön tehokkuuteen. Fyysiseltä aktiivisuudelta eroavat kaksosveljet eivät myöskään eronneet lepoenergiankulutuksen eivätkä rasvan käytön suhteen. Keski-ikäisten naisten aineistossa korkeampi fyysinen aktiivisuus oli yhteydessä korkeampaan rasvan käyttökykyyn liikkuessa. Korkeampi rasvan käytön teho levossa tai liikkuessa ei kertonut paremmasta sokerinsiedosta eikä insuliiniherkkyydestä. Menopaussi heikentää aineenvaihduntaterveyttä siis ennemmin muuttamalla seerumin metabolomia kuin alentamalla lepoenergiankulutusta tai rasvan käyttöä. Fyysinen aktiivisuus voi tehostaa rasvan käyttöä liikkuessa, mutta tällä ei välttämättä ole merkitystä sokeriaineenvaihduntaterveydelle.

Avainsanat: vaihdevuodet, liikunta, energia-aineenvaihdunta, metabolomiikka

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ACKNOWLEDGEMENTS

This PhD thesis marks the culmination of five years of work in the discipline of Sports and Exercise Medicine at the Faculty of Sport and Health Sciences, University of Jyväskylä. My passion for understanding human metabolism was ignited during my Master's studies, and this thesis embodies the knowledge I have acquired since then. I am grateful for all the experiences and people I have encountered on this journey, recognizing that this thesis is only the beginning of my exploration into the complexities of scientific research.

First and foremost, I would like to express my heartfelt appreciation to my supervisors, who have been instrumental in my development as a scientist. Associate Professor Eija Laakkonen has been a guiding force throughout my PhD journey, providing insightful feedback and encouraging me to pursue my interests. Her support has been crucial to my growth as a knowledgeable researcher. Eija, I could not have wished for a better mentor. I am also deeply grateful to Professor Emeritus Urho Kujala, who has served as a role model for me in understanding human function, scientific methodology, and interpretation of research findings. Lastly, I thank Professor Jari Laukkanen for his support and expertise. Our discussions during the data collection process will remain cherished memories, and I am grateful for all the knowledge you shared. I also want to acknowledge Professor Timo Lakka for serving as a steering group member for my dissertation.

I sincerely thank the official reviewers, Associate Professor Laurie Isacco and Professor Pirjo Nuutila, for their feedback and encouragement. I also want to thank Professor Edward Melanson for agreeing to be the opponent. It is truly an honor to have the opportunity to discuss my thesis with such a distinguished expert.

I express my gratitude to the Faculty of Sport and Health Sciences of the University of Jyväskylä for funding my research, allowing me to work full-time as a Doctoral Researcher and focus exclusively on my thesis. I also thank Professor Jari Parkkari, Senior Lecturers Katja Waller and Eero Haapala, fellow Doctoral Researcher Suvi Ravi, and other colleagues in Sports and Exercise Medicine for their guidance and help over the years. Additionally, I extend my appreciation to fellow researchers at the Gerontology Research Center for their valuable contributions to my development as a scientist. Although I have not been formally affiliated with the center, I have been fortunate to participate in conferences, meetings, and thoughtful discussions.

I am forever grateful to both past and present members of the Laakkonen lab, including Sira Karvinen, Matti Hyvärinen, Tero Sievänen, and others, for their support and encouragement. Special thanks go to my dear colleague, Hanna-Kaarina Juppi. Sharing the PhD journey with you has been a pleasure, and our friendship has made it even more exciting. I am thankful to my co-authors for their feedback and contributions to the studies included in this thesis. Their insights and suggestions have been invaluable in shaping this work. The lab personnel, Mervi Matero, Eeva-Maija Palonen, Anja Ahlgren, Hanne Tähti,

Bettina Hutz, and Jukka Hintikka, also deserve my heartfelt thanks for their contributions to data collection.

I would like to thank my parents, my sister Sari and brother Mika, and their families for their unwavering support throughout my life. Dad, I'm grateful for all the years I got to spend with you. Your work ethic was unparalleled. You taught me to never give up, and here I am. Mom, you have always been my greatest support. You believed in me even when I doubted myself. Without you, this thesis would never have been possible. Lastly, I would like to express my deepest gratitude to my partner, Timo. Thank you for your immeasurable support during this journey.

Lieto, 16.04.2023
Jari Karppinen

ORIGINAL PUBLICATIONS AND AUTHOR CONTRIBUTION

This PhD thesis is based on the below four original publications, which are referred to by their Roman numerals. The thesis also includes unpublished results.

I **Karppinen, J. E.**, Törmäkangas, T., Kujala, U. M., Sipilä, S., Laukkanen, J., Aukee, P., Kovanen, V. & Laakkonen, E. K. (2022). Menopause modulates the circulating metabolome: evidence from a prospective cohort study. *European Journal of Preventive Cardiology*, 29(10), 1448–1459. https://doi.org/10.1093/eurjpc/zwac060

II **Karppinen, J. E.**, Wiklund, P., Ihalainen, J. K., Juppi, H-K., Isola, V., Hyvärinen, M., Ahokas, E. K., Kujala, U. M., Laukkanen, J., Hulmi, J. J., Ahtiainen, J. P., Cheng, S. & Laakkonen, E. K. Age but not menopausal status is linked to lower resting energy expenditure. Submitted for publication.

III **Karppinen, J. E.**, Rottensteiner, M., Wiklund, P., Hämäläinen, K., Laakkonen, E. K., Kaprio, J., Kainulainen, H. & Kujala, U. M. (2019). Fat oxidation at rest and during exercise in male monozygotic twins. *European Journal of Applied Physiology*, 119(11–12), 2711–2722. https://doi.org/10.1007/s00421-019-04247-x

IV **Karppinen, J. E.**, Juppi, H-K, Hintikka, J., Wiklund, P., Haapala, E. A., Hyvärinen, M., Tammelin, T. H., Aukee, P., Kujala, U. M., Laukkanen, J. & Laakkonen, E. K. (2022). Associations of resting and peak fat oxidation with sex hormone profile and blood glucose control in middle-aged women. *Nutrition, Metabolism and Cardiovascular Diseases*, 32(9), 2157–2167. https://doi.org/10.1016/j.numecd.2022.06.001

I made a significant contribution to the overall design of the EsmiRs metabolism study, which provided data for *Studies II* and *IV*. I also developed the protocols for both resting and exercise metabolism testing and performed indirect calorimetry measurements. Additionally, I provided guidance for the Physique and NOREDS studies on measuring resting energy expenditure, resulting in the collection of data that was used in *Study II*. I processed most gas exchange data used in the current thesis into variables and planned the overall concept of each manuscript. I conducted the statistical analyses in *Studies II–IV*. In *Study I*, I created the diet quality score, performed preliminary statistical analyses, and assisted with the final analyses. I was responsible for interpreting the data and creating all figures. As the first author, I drafted all manuscripts and made revisions based on feedback from the coauthors and reviewers.

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ABBREVIATIONS

AEE	Activity energy expenditure
APO	Apolipoprotein
AUC	Area under the curve
BCAA	Branched-chained amino acid
BMI	Body mass index
CEE	Conjugated equine estrogens
CV	Coefficient of variation
CVD	Cardiovascular disease
DLW	Doubly labeled water
E2	Estradiol
ER	Estrogen receptor
FAT_{MAX}	Relative exercise intensity of peak fat oxidation
FFM	Fat-free mass
FM	Fat mass
FSH	Follicle-stimulating hormone
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model of insulin resistance
ICC	Intraclass correlation coefficient
IDL	Intermediate-density lipoprotein
LDL	Low-density lipoprotein
LM	Lean mass
MET	Metabolic equivalent of task
MHT	Menopausal hormone therapy
MUFA	Monounsaturated fatty acids
MZ	Monozygotic
NEFA	Nonesterified fatty acid
NEAT	Nonexercise activity thermogenesis
NMR	Nuclear magnetic resonance
OGTT	Oral glucose tolerance test
PFO	Peak fat oxidation
PUFA	Polyunsaturated fatty acid
QUICKI	Quantitative insulin sensitivity check index
REE	Resting energy expenditure
RER	Respiratory exchange ratio
RFO	Resting fat oxidation
RQ	Respiratory quotient
SEE	Sleeping energy expenditure
SFA	Saturated fatty acid
T2D	Type 2 diabetes
TEE	Total energy expenditure
VLDL	Very low-density lipoprotein
$\dot{V}CO_2$	Volume of carbon dioxide

$\dot{V}O_2$	Volume of oxygen
$\dot{V}O_{2MAX}$	Maximal oxygen uptake
$\dot{V}O_{2PEAK}$	Peak oxygen uptake
WHI	Women's Health Initiative study
W_{MAX}	Maximal work capacity

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ORIGINAL PAPERS

1 INTRODUCTION

The term metabolism originates from the Greek words *metabolē* or *metaballein*, meaning change or to change. Metabolism encompasses all the chemical reactions within living organisms, including both the breakdown and synthesis of molecules (Cammack et al., 2006). Hence, metabolism covers the cellular processes that allow us to be what we are and do what we do. The cost of these processes determines energy expenditure, which is met through the conversion of stored energy in the chemical bonds of mainly glucose and fatty acids into ATP, which is the energy currency of the body. The dependence on either substrate as an energy source is primarily influenced by their availability at a given time, yet it has been suggested that this choice may also reflect an individual's metabolic functioning and health status (Goodpaster & Sparks, 2017).

Two powerful stimuli that influence metabolism are endocrine hormones and physical activity. Endocrine hormones are signaling molecules secreted by specialized cells and that are transported in circulation to modulate the function of the target cells at another body location (Hall, 2016). The word hormone comes from the Greek word *hormōn*, which means to set into motion. Physical activity does the same but in a more concrete manner. According to the classic definition by Caspersen et al. (1985), physical activity is any bodily movement produced by skeletal muscles that result in energy expenditure. The endocrine system and physical activity are closely connected and mutually impact one another, the interrelated nature of physiology and behavior.

Menopause is a normal physiological process most women encounter, usually during their midlife. Because menopause occurs when women are generally active in work and have various caregiving roles within their families (Davis & Baber, 2022), menopause touches not only the lives of women but also those close to them. The exact reason for menopause is unknown, and the phenomenon is extremely rare in the animal kingdom, with only the females of humans and four whale species experiencing menopause and having long postreproductive life spans (Dalton, 2022). The grandmother hypothesis posits that, in these species, females gain evolutionary advantages from menopause because it redirects them to allocate more energy resources toward increasing the

longevity of their grand-offspring (Hawkes, 2003). For example, killer whale calves have a lower life expectancy if their grandmother dies (Nattrass et al., 2019). Menopause leads to changes in sex hormone levels, which influences the functioning of cells that have receptors for these hormones, even if these cells are not directly involved in reproduction. The effects of these cellular changes are, however, not fully understood. Research has suggested that these adaptations may increase the risk of cardiometabolic diseases in women, at least in industrialized nations (El Khoudary et al., 2020; Lambrinoudaki et al., 2022). Therefore, it is crucial to further investigate the effects of menopause on metabolism to better understand and mitigate the associated health risks.

In contrast to menopause, regular physical activity causes cellular adaptations that promote cardiometabolic health (Pedersen & Saltin, 2015). Evolutionarily, the human body is designed to be active throughout life because physical activity used to be a necessity for survival (Cordain et al., 1998). However, this is no longer the case in our current environment. Today, physical inactivity is a global burden and is estimated to be, based on observational data, responsible for 7.2% and 7.6% of all-cause and cardiovascular disease (CVD) mortality, respectively (Katzmarzyk et al., 2022). The benefits of physical activity are diverse and mediated through numerous metabolic pathways. An emerging model has proposed that physical activity may protect health by redirecting energy resources away from the metabolic processes that contribute to cardiometabolic diseases (Pontzer, 2018), although more evidence is required to solidify this theory.

Metabolism cannot be characterized using a single method. Therefore, the research on metabolism employs multiple techniques to gain a more comprehensive understanding of the research question at hand. The present thesis has incorporated both traditional and modern methods. Gas exchange measurements have been used to study energy expenditure and substrate use since the nineteenth century (Kenny et al., 2017). Despite the rapid progression of biotechnology, the indirect calorimetry technique has held its place as one of the most widely used methods in metabolism research. In comparison, metabolomics is a relatively new method, with its first mention appearing in the PubMed database in 1993. Metabolomics offers another way to study whole-body metabolism by measuring a large number of small molecules less than 1500 Da in size, called metabolites, which are used or produced by cellular processes, hence providing insight into the biological status of the individual (X. Liu & Locasale, 2017). The use of metabolomics has grown rapidly, with a sixfold increase in publications in the PubMed database from 2011 to 2021. Reflecting this substantial and rapid progression, some metabolomics platforms have already ventured into clinical and commercial markets (Tikkanen et al., 2021), highlighting the potential of this field to contribute to medical advancements

In the present dissertation, I present my research findings on the complex relationships between menopause, physical activity, and metabolism at the whole-body level. Additionally, I reflect on what I have learned while studying the interconnections between fat oxidation and blood glucose regulation abilities.

2 LITERATURE REVIEW

2.1 Measures of whole-body metabolism

2.1.1 Resting and total energy expenditure

Adolf Magnus-Levy conceived of the concept of basal metabolic rate to define a variable that could be used to study intraindividual and interindividual variation in elementary processes consuming energy while activity is limited to a minimum (Magnus-Levy, 1947). Basal metabolic rate and resting energy expenditure (REE) characterize the same phenomenon and are often used interchangeably. As explained by Heymsfield et al. (2021), the term basal metabolic rate is reserved for situations when the measurements are performed in strict conditions similar to the pioneering research (e.g., Harris & Benedict, 1918), where, for example, muscular repose was automatically confirmed.

In the general population, approximately 60–70% of total daily energy expenditure (TEE) is covered by REE. The energy expended on movement, which is known as activity energy expenditure (AEE), accounts for 20–30% of TEE, and the energy spent on processing and storing energy and nutrients from food, which is known as the thermic effect of food, makes up the remaining 10%. Among the three components, AEE varies the most between individuals (Warren et al., 2010), while the thermic effect of food is the most consistent (Calcagno et al., 2019). The TEE components are closely interconnected, despite often being portrayed as separate. For example, a rise in AEE can lead to an increase in food intake, slightly elevating the thermic effect of food (Calcagno et al., 2019). The relationship between AEE and REE is even more complex and will be explored further in section 2.3.2.

2.1.1.1 Assessment of energy expenditure

The first law of thermodynamics states that energy cannot be created or destroyed, only converted into another form. Therefore, when the body uses chemical energy, it eventually transforms this energy into heat that dissipates

into the surrounding environment. Direct calorimetry, which measures this heat dissipation, is the gold standard for assessing energy expenditure at the whole-body level. However, the method is challenging to use, especially when the participants are physically active (Kenny et al., 2017). The body uses almost all the oxygen it takes in to synthesize ATP, producing CO_2 (Heymsfield et al., 2021). Studies that have simultaneously measured heat dissipation with the oxygen and CO_2 breathed in and out found that gas exchange analysis can also closely estimate energy expenditure (Kenny et al., 2017). Nowadays, indirect calorimetry is the most commonly used method for measuring energy expenditure at the whole-body level.

To assess REE, gas exchange is usually measured using open-circuit respirometers, such as metabolic carts. A valid REE assessment requires four conditions to be met (Henry, 2005). First, the participants must be awake, lying supine and rested, and with no physical activity before the assessment. A commonly used protocol is a 30-minute resting period followed by a 30-minute measurement period (Fullmer et al., 2015). Second, the test should be conducted after fasting for at least 10 hours to control the thermic effect of food. Third, thermoneutral conditions (22–26°C) are needed. REE increases in response to cold stress (Claessens-van Ooijen et al., 2006; Kashiwazaki et al., 1990), while the effect of raising room temperature is unclear (Henderson & Halsey, 2022). Finally, the participants should be relaxed and familiar with the equipment because anxiety can distort the measurements. REE is typically measured in the early morning to meet these requirements, representing a snapshot of basal metabolism close to its nadir. However, it is important to acknowledge that REE follows a circadian rhythm and increases later in the day (van Moorsel et al., 2016; Zitting et al., 2018). REE estimates from different devices can vary by over 200 kcal/d, with a measurement error in popular metabolic carts ranging from 2–14% (Alcantara et al., 2022). Therefore, the same cart should be used throughout the study, and calibration procedures should be followed to ensure the validity and reliability of measurements. Overall, REE measurements in standardized conditions are repeatable, with the mean coefficient of variation (CV) for intraindividual variability of REE over 6 months being 5–6% (Bader et al., 2005).

In a laboratory setting, TEE can be measured using a whole-room indirect calorimeter. The sleeping energy expenditure (SEE) is also often measured in this scenario, which is roughly equal to REE minus the effects of arousal (Heymsfield et al., 2021). However, it should be noted that human behavior can vary between laboratory and real-life conditions (Kumahara et al., 2010). The most accurate method to assess free-living TEE is the doubly labeled water (DLW) method (Strath et al., 2013). In the DLW method, the participants are dosed with deuterium and ^{18}oxygen-enriched water (Speakman & Hambly, 2016). The isotopes merge in the body water pool, and their disappearance can be measured from bodily fluids, typically from urine. Deuterium (^{2}H) disappears from the body as water (H_2O) and ^{18}oxygen also as CO_2. Therefore, CO_2 production can be estimated by the elimination rate difference between the isotopes. TEE can then be calculated when the average diet macronutrient distribution is known or esti-

mated because macronutrient consumption influences the ratio of oxygen consumption and CO_2 production (discussed in section 2.1.2.1).

2.1.1.2 Main determinants of resting energy expenditure

Tissue mass. REE shows notable variation between individuals, primarily because of differences in the fat-free mass (FFM) (M. J. Müller et al., 2004; Pontzer et al., 2021). In adult populations with typical FFM (40–80 kg), FFM has a linear relationship with REE and accounts for about 60–85% of its variance, depending on sample homogeneity (M. J. Müller et al., 2018). The two-compartment model, which considers both FFM and fat mass (FM), slightly improves REE estimation (Heymsfield et al., 2021). However, FM contributes less to REE variance because of its lower mass-specific metabolic rate (FFM 20 kcal/d, FM 4.5 kcal/d) (Elia, 1992). FFM is composed of organs and tissues with varying metabolic rates, with proportions differing between individuals, hence leading to an error in REE estimation when using the two-compartment model. Measuring FFM constituent volumes with magnetic resonance imaging can explain nearly 90% of REE variability (Heymsfield et al., 2021). However, this proportion may be understated as volume estimation assumes constant tissue quality, which, in real-life scenarios, varies between individuals and alters the mass-specific metabolic rate of organs and tissues (M. J. Müller et al., 2018). Tissue-specific metabolic rates can also vary because of differences in cellular metabolism. Most studies using REE as an outcome aim to understand how the energy expenditure by all body cells differs between individuals or within individuals over time. The metabolic rate of cells is shaped by intrinsic factors such as mitochondrial characteristics and their stimulation level, for example, by the nervous and endocrine systems (M. J. Müller et al., 2018). The below sections will examine those factors that may affect REE at the cellular level.

Age. REE changes during the human lifespan, as shown by Pontzer et al. (2021). A newborn's adjusted REE (for FFM and FM) is similar to that of adults, but it increases to a peak in the first year before then declining to adult levels by age 18. TEE shows a similar pattern. In old age, REE decreases because of slowing cellular metabolism (Z. Wang et al., 2010), but the exact timing is unclear. Pontzer et al. (2021) found that REE decline starts after 45 years, while TEE declines only after 63 years. This discrepancy has led these researchers to argue that metabolic slowing may not manifest until about 60 years of age, which was argued because the limited sample size of middle-aged participants with REE measurements created some uncertainty about the estimate. In contrast, Geisler et al. (2016b) found that, when adjusted for FFM constituents, REE started declining already in the 30s. In the end, estimating the exact age of decline is challenging because of limitations in current body composition assessment methods that cannot account for age-associated changes in tissue quality (Fragala et al., 2015; Giorgio et al., 2010) or FFM composition (Bosy-Westphal et al., 2003) when using a two-compartment model. Age does improve REE explanation and prediction at the population level (M. J. Müller et al., 2004), likely because of partially mitigating these limitations.

Sex. Males have a higher mean absolute REE (M. J. Müller et al., 2018) and greater REE variability than females when controlling for FFM, FM, and age (Halsey et al., 2022). Notably, Pontzer et al. (2021) did not find sex differences in adjusted REE across the life course. The effect of sex on REE disappears when FFM is substituted by its components, highlighting the similarity in the impact of sex and age on REE in explanatory and predictive models and contributing to resolving the limitations of body composition assessments (Heymsfield et al., 2021).

Menstrual cycle. Levels of various hormones fluctuate during the menstrual cycle, with the most characteristic changes seen in the production of estradiol (E2) and progesterone by the ovaries. The present thesis focuses primarily on changes in metabolism during menopause, but it is important to briefly address the menstrual cycle as well. Typically, the menstrual cycle lasts around 28 days and is divided into the follicular and luteal phases, with each lasting approximately 14 days. However, hormone levels can also vary within these phases. Measuring women at different phases of the cycle can make it difficult to compare study results. Hence, it is now recommended to divide the menstrual cycle into four distinct phases to clearly capture specific hormonal profiles (Elliott-Sale et al., 2021). Phase 1 (early follicular) covers the first 5 days of the cycle, a time that is characterized by menstrual bleeding and low levels of E2 and progesterone. E2 levels then rise and reach their peak in Phase 2 (late follicular), which occurs 14–16 hours before ovulation, while progesterone levels remain low. Phase 3 (ovulatory), lasting 24–36 hours after a positive ovulation test, has moderate E2 levels and low progesterone levels. In Phase 4 (luteal), roughly 7 days after ovulation, progesterone levels are at their peak, and E2 levels are high but lower than in Phase 2. In reality, however, the menstrual cycle varies between and within individuals, making phase identification challenging.

The impact of the menstrual cycle on REE remains debatable. A meta-analysis of 26 studies by Benton et al. (2020) found that REE may be slightly higher in the luteal phase than in the follicular phase (standardized mean difference = 0.33; 95% CI 0.17–0.49). However, the total number of participants in the included studies was relatively small (n = 318), and the studies had a high risk of bias, reducing the certainty of the findings. Additionally, the effect size was smaller when restricting the analysis to studies with more than 10 participants or those published after 2000. Moreover, the studies performed measurements at different menstrual cycle phases. For example, follicular phase measurements were performed 2–12 days after the start of menstruation, reflecting time points with varying E2 levels.

Genotype. The heritability of REE adjusted for age, sex, and FFM has been estimated as being 30–40% (Bosy-Westphal et al., 2008; Bouchard et al., 1993), which is lower compared with many other physiological traits. The lower heritability can be attributed to a combination of factors, such as difficulties in accurately measuring REE and body composition. Additionally, REE may be more vulnerable to environmental factors affecting the metabolic state of an individual.

Energy balance and weight fluctuations. Perhaps the most studied stimuli in the REE literature are altered energy balance and body composition. Even short periods of negative energy balance cause a decline in REE, and extended periods of energy restriction lead to a larger REE decline than expected, which is solely based on tissue mass losses (M. J. Müller et al., 2021). Additionally, even if energy balance is restored, FFM- and FM-adjusted REE may not return to pre–weight loss levels (Fothergill et al., 2016). On the other hand, the energy surplus will elevate adjusted REE (M. J. Müller et al., 2021), and an increase in skeletal muscle mass or adiposity, especially when obese, may result in systemic adaptations that increase REE more than what is estimated based on tissue-specific metabolic rates alone (Heymsfield et al., 2022; Hwaung et al., 2019).

2.1.2 Fat oxidation

Resting fat oxidation (RFO) is the rate of fatty acid use that is measured under conditions similar to those used for assessing REE. On the other hand, the protocols used to measure fat oxidation during exercise vary. When comparing the research on exercise fat oxidation, it is crucial to take into account testing intensity and duration. The testing intensity is commonly expressed as a percentage of maximal oxygen uptake ($\dot{V}O_{2MAX}$) or peak oxygen uptake ($\dot{V}O_{2PEAK}$). At low-intensity exercise (25% of $\dot{V}O_{2MAX}$), the primary fuel source are nonesterified fatty acids (NEFAs) derived from circulation, with exercise duration having a minimal impact (Romijn et al., 1993). As the intensity of exercise increases to moderate levels (65% of $\dot{V}O_{2MAX}$), the absolute rate of fat oxidation also increases, and the use of fatty acids from intramyocellular lipids reaches that of circulating NEFA. Moreover, the amount of muscle glycogen used to fuel the exercise also increases. During moderate-intensity, prolonged exercise, the dependence on fat oxidation rises over time as glycogen storage gradually decreases, with the use of intramyocellular lipids following a somewhat inverted U-shaped curve. The increase lasts for the first 30 minutes, before declining around the 60-minute mark. In high-intensity exercise (85% $\dot{V}O_{2MAX}$), glycogen becomes the primary substrate source. The mode of exercise also has an impact on fat oxidation by engaging more muscle mass, which leads to higher absolute fat oxidation rates during running compared with cycling (Achten et al., 2003).

Assessing the capacity for fat oxidation during exercise has traditionally been done by testing participants at one or a few specific submaximal relative exercise intensities, such as 50% of $\dot{V}O_{2MAX}$. However, the relative exercise intensity at which fat oxidation peaks can vary substantially between individuals (Chrzanowski-Smith, Edinburgh, Thomas, et al., 2021; G. Fletcher et al., 2017; M. C. Venables et al., 2005). In 2002, Achten et al. developed the FAT_{MAX} protocol to address this issue; this protocol assesses fat oxidation at multiple exercise intensities during a single testing session. The variables obtained from the test include the highest rate of fat oxidation achieved, which is known as maximal or peak fat oxidation (PFO), and the relative exercise intensity at which PFO occurs as a percentage of $\dot{V}O_{2MAX}$, which is known as FAT_{MAX}.

2.1.2.1 Assessment of fat oxidation

The law of conservation of mass states that mass, like energy, cannot be created or destroyed but can only change form. Hence, the atoms of energy substrates do not disappear during energy extraction from chemical bonds; they transform into metabolic water and CO_2 instead. Glucose and fatty acids are the primary energy substrates in human metabolism. As outlined by Jeukendrup and Wallis (2005), a key difference between the substrates is that glucose contains more oxygen per carbon; therefore, breaking down a mole of glucose requires less oxygen than a mole of fatty acid. This structural difference leads to variations in the ratio of oxygen consumption and CO_2 production when cellular respiration primarily relies on either glucose or fatty acids. This ratio is represented by the respiratory quotient (RQ) at the tissue level. A RQ of around 0.7 indicates that ATP production relies on fatty acids, while a RQ of 1.0 signifies glucose use. However, the interpretation of RQ across its range is not straightforward because other molecules, such as amino acids and ketones, which have different RQs, can also be used in ATP production.

Whole-body RQ cannot be measured directly. However, the ratio of oxygen uptake to CO_2 excretion can be assessed at the respiratory level using indirect calorimetry, which is known as the respiratory exchange ratio (RER). This distinction is crucial because RER can only be considered equal to RQ when RQ remains constant for long enough for RER to match it (Jeukendrup & Wallis, 2005). Additionally, stoichiometric equations have been developed to estimate the rates of fat and carbohydrate oxidation using respirometry data. These estimates can be corrected for amino acid oxidation if urinary nitrogen excretion is also measured (Frayn, 1983; Péronnet & Massicotte, 1991).

The assessment of fat oxidation during exercise with indirect calorimetry requires additional considerations, as also reviewed by Jeukendrup and Wallis (2005). First, indirect calorimetry is a viable tool in substrate use assessment only at exercise intensities below approximately 75% of $\dot{V}O_{2MAX}$ because, at higher intensities, bicarbonate buffering of hydrogen ions causes nonmetabolic CO_2 production and distorts the fat oxidation assessment, making it appear as if fat oxidation eventually has dropped to zero, even though it has not. Second, each submaximal exercise test stage should be long enough for RER to match RQ. This is usually not an issue in traditional single-stage protocols, but it can be a problem in PFO testing using the FAT_{MAX} protocol. The original protocol (Achten et al., 2002), which was developed in young and trained cyclists, used three-minute exercise stages, but this may not be long enough for participants with low cardiorespiratory fitness to reach a steady state (Dandanell et al., 2017). However, increasing the stage duration to 4 minutes can solve the issue (Chrzanowski-Smith et al., 2018). An important methodological limitation of PFO assessment is that it has a substantial day-to-day intraindividual variation (Chrzanowski-Smith et al., 2018, 2020), with the typical error being ± 0.06 g/min.

2.1.2.2 Main determinants of resting fat oxidation

A common assumption in metabolism research has been that fasting RER or RFO characterizes the fat oxidation capacity and metabolic phenotype of an individual. As a result, many studies have tried to explain the differences in FM between groups or changes in FM over time by looking at fasting substrate use (Péronnet & Haman, 2019). However, these traditionally held assumptions have been questioned (Galgani & Fernández-Verdejo, 2021).

The determinants of resting substrate use, as measured after an overnight fast, are primarily energy balance (Goris & Westerterp, 2000) and the distribution of macronutrients in the diet when energy balance is maintained (Miles-Chan et al., 2015). Therefore, RFO increases when energy intake does not meet expenditure and when the proportion of fats in the diet is high. Additionally, RFO does not truly reflect maximal fat oxidation capacity. Even in people with low cardiorespiratory fitness, mitochondrial oxidative capacity is not the limiting factor of substrates used at rest (Holloszy, 2009). For these reasons, resting RER and RFO are perhaps better suited for assessing nutritional status (Miles-Chan et al., 2015), especially energy balance (Goris & Westerterp, 2000), than the metabolic functioning and health of an individual.

2.1.2.3 Main determinants of peak fat oxidation

It is unclear which step in the fat oxidation pathway is the rate-limiting factor for PFO. However, both the ability to mobilize and transport fatty acids from adipose tissue and intramyocellular lipid stores and the ability of the mitochondrial capacity to oxidize fatty acids likely play a role (Purdom et al., 2018). At the skeletal muscle level, high PFO is associated with a higher proportion of type I fibers, capillary density, the content of multiple cytosolic and mitochondrial proteins in the fat oxidation pathway, and total mitochondrial capacity (Chrzanowski-Smith, Edinburgh, Smith, et al., 2021; Dandanell et al., 2018; Shaw et al., 2020). However, circulating NEFA availability may still be a key factor in regulating PFO. Frandsen et al. (2019) showed that, in male athletes who overnight fasted, PFO does not represent the actual maximal fat oxidation rate: three subsequent tests performed at 4-hour intervals resulted in increasing levels of PFO. Circulating NEFA levels rose similarly in each ensuing trial, and NEFA concentration strongly explained PFO variation ($R^2 = 0.73$). Additionally, increasing NEFA availability by activating lipoprotein lipase activity with heparin infusion was found to increase fat oxidation during exercise in athletes (Hawley, 2002) and individuals with type 2 diabetes (T2D) (Akanji et al., 1993). Overall, these factors are associated with several temporal and phenotypic characteristics that are discussed next.

Diet and body composition. PFO is higher in the overnight fasted state compared with the postabsorptive state (Frandsen et al., 2019). However, diet on preceding days is not a major determinant of PFO, here in contrast to RFO (Chrzanowski-Smith, Edinburgh, Thomas, et al., 2021; G. Fletcher et al., 2017). Because absolute PFO is closely related to the number of oxidative cells, particularly in the skeletal muscle, studies should adjust PFO for FFM or lean mass (LM) in statistical analyses. Despite this, FM or body fat percentage does

not appear to be a significant determinant of PFO, even though it may be tempting to assume that lower fat oxidation capacity during exercise could lead to obesity. A systematic review by Arad et al. (2020) that compiled 24 exercise studies comparing fat oxidation between individuals whit overweight or obesity and individuals with normal weight found no systematic differences in exercise fat oxidation between the two groups. In some studies, participants with more FM even exhibited higher fat oxidation rates.

Cardiorespiratory fitness. When adjusted for FFM, cardiorespiratory fitness is the primary PFO determinant (Chrzanowski-Smith, Edinburgh, Thomas, et al., 2021; G. Fletcher et al., 2017; M. C. Venables et al., 2005). This conclusion is supported by evidence from case-control studies, where PFO has been found to be higher in endurance athletes than nonathletes (Dandanell et al., 2018; Shaw et al., 2020) and in participants with higher fitness levels compared with those with lower levels (Frandsen, Hansen, et al., 2021; Stisen et al., 2006). The strong association between PFO and $\dot{V}O_{2MAX}$ is expected because the variables are closely related. For example, better skeletal muscle oxidative machinery leads to higher arterio-venous oxygen difference, which leads to higher $\dot{V}O_{2MAX}$ (Bassett & Howley, 2000). Additionally, high cardiac output, which is the primary determinant of $\dot{V}O_{2MAX}$, may contribute to higher PFO by increasing oxygen delivery.

Genotype. The heritability of $\dot{V}O_{2MAX}$ is about 50% (Bouchard et al., 1998), which suggests that PFO may also have a significant genetic component. However, this has not yet been directly studied. The heritability estimates of RER at low-to-moderate exercise intensities have been similar to those for $\dot{V}O_{2MAX}$ (Bouchard et al., 1989). Therefore, it is likely that genotype is a major contributor to the interindividual variation of PFO and the association between $\dot{V}O_{2MAX}$ and PFO. Genetic pleiotropy may also be a factor in the relationship between physical activity and PFO, which is discussed in section 2.3.3.

Sex. The research has consistently shown that females have higher PFO than males (Chrzanowski-Smith, Edinburgh, Thomas, et al., 2021; G. Fletcher et al., 2017; M. C. Venables et al., 2005). The sex difference is also observed when nonathlete participants are tested using traditional single-stage protocols, but it may disappear in highly trained athlete populations (Cano et al., 2022). The exact reason for the sex difference is unknown, but it is presumed to be rooted in E2 concentration differences based mainly on evidence from rodent and cell models and E2 supplementation trials in men (Oosthuyse et al., 2022). According to Oosthuyse et al. (2022), E2 signaling can enhance fat oxidation in skeletal muscle by multiple mechanisms. E2 activates peroxisome proliferator-activated receptors α and δ, directly or via AMP-activated kinase, increasing the expression of beta-oxidation enzymes and pyruvate dehydrogenase kinase 4, which promote fat oxidation in the mitochondria. To meet this demand, the expression of long-chain fatty acid transport proteins in the plasma membrane, cytosol, and mitochondrial membranes are also increased. Additionally, E2 increases the expression of lipoprotein lipase to support triglyceride hydrolysis and cytosolic proteins involved in intracellular triglyceride storage. Furthermore, E2 improves adipose tissue lipolysis by heightening its sensitivity to norepinephrine and

downregulating adipose tissue lipoprotein lipase activity and those proteins involved in triglyceride storage. Chrzanowski-Smith, Edinburgh, Smith, et al. (2021) tried to explain the sex difference in PFO by quantifying adipose tissue and skeletal muscle protein contents without success. Ultimately, the sex differences originated from multiple biological levels, so finding a specific explanation for the phenotypic outcomes such as PFO may be challenging.

Menstrual cycle. The literature on the influence of the menstrual cycle on fat oxidation during exercise has been inconsistent. Some studies have demonstrated that fat oxidation is enhanced and that glycogen sparing occurs during the luteal phase, when E2 levels are high, compared with the early follicular phase (Boisseau & Isacco, 2022). Interestingly, such studies employed protocols incorporating prolonged exercise stages (Oosthuyse et al., 2022). However, the menstrual cycle does not appear to affect PFO (Frandsen et al., 2020), and controlling for the menstrual cycle phase has not improved the repeatability of PFO assessment (Chrzanowski-Smith et al., 2020). As such, Oosthuyse et al. (2022) postulated that, for the menstrual cycle to have an impact on fat oxidation, the exercise duration would need to be sufficient to elicit significant stress on the use of endogenous energy stores.

2.1.3 Serum metabolome

Another way to study whole-body metabolism is through metabolomics, which involves identifying and measuring the small molecules the body produces and uses (X. Liu & Locasale, 2017). One efficient way to do this is by analyzing blood, specifically the serum, which is the cell-free and clotting protein–free fraction that carries most of the body's metabolites and into which all organs and tissues feed (Psychogios et al., 2011). The human serum metabolome consists of more than 4,000 metabolites, with the largest groups being various lipids carried by lipoproteins, while amino acids and glucose derivates are also abundant (Psychogios et al., 2011). Research has shown that the human serum metabolome is associated with differences in health and lifestyle (Soininen et al., 2015; Würtz et al., 2017), making it a useful tool for metabolism research.

The understanding of how metabolic deterioration affects the serum metabolome is still evolving. Much of the current knowledge has been gained through the use of a nuclear magnetic resonance (NMR) spectroscopy platform (Soininen et al., 2015; Tikkanen et al., 2021), which is what the current thesis has used. The platform quantifies 250 metabolite concentrations or ratios, including size-categorized lipoprotein particles and their lipid contents, fatty acids, glycolysis-related metabolites, amino acids, ketone bodies, and the inflammation marker GlycA. Thirty-seven measures have been validated for clinical use (Tikkanen et al., 2021). For practical reasons, this literature review focuses on the quantified metabolites and their connections with cardiometabolic health but acknowledges that this provides only a partial view of the topic. The typical metabolite changes associated with worsening metabolic health are described later in the text and summarized in FIGURE 1 at the end of the section.

2.1.3.1 Assessment of serum metabolome

Metabolomics methods can be divided into targeted and untargeted approaches. Targeted metabolomics aims to quantify the absolute concentrations of selected metabolites, while untargeted metabolomics focuses on detecting unknown metabolites and comparing their relative levels between groups of individuals or organisms (X. Liu & Locasale, 2017). The two main tools used in metabolomics are NMR and mass spectroscopy. Both have their strengths and weaknesses, and both can only detect a subset of serum metabolites (Psychogios et al., 2011).

The present thesis uses data obtained through targeted proton NMR metabolomics, a method relying on the principle that every molecule containing hydrogen ions emits a unique signal when placed in a particular magnetic field. The intensity of this signal reflects the concentration of the molecule in the sample (Soininen et al., 2015). This strength makes NMR metabolomics the most robust tool for quantitative metabolomics research because it can measure known metabolite concentrations with high accuracy (Psychogios et al., 2011). However, a drawback of the method is its limited sensitivity, which means it can only detect and quantify a subset of metabolites present in relatively high amounts in the sample (Emwas et al., 2019). Mass spectrometry techniques are the best choice for detecting and measuring specific metabolites present in low quantities, such as inflammation markers or hormones. Furthermore, when the sample volume is a limitation, mass spectrometry may be a more suitable option because it necessitates smaller volumes than NMR spectroscopy (Psychogios et al., 2011).

2.1.3.2 Lipoprotein particles

Lipoprotein particles have a lipid core consisting mainly of triglycerides and cholesterol, which is encapsulated in a phospholipid-protein monolayer. The ratio of lipids to proteins determines the density and diameter of the lipoprotein particle, which are used, together with the surface proteins apolipoprotein (apo) A-I and B, to categorize the particles into classes.

ApoB-containing lipoproteins. Each apoB-containing lipoprotein particle has one apoB protein; therefore, the apoB count can be used to determine the concentration of apoB-containing lipoprotein particles in a given sample (Sniderman et al., 2019). Chylomicrons and very low-density lipoproteins (VLDL) are the largest lipoprotein particles, and their primary function is to transport triglycerides (i.e., energy) from the small intestine or liver to the rest of the body (Packard et al., 2020). The fasting chylomicron concentration is low in healthy individuals (Björnson et al., 2020), such as the participants in the present thesis. Therefore, this review does not cover chylomicron metabolism.

The liver secretes two types of VLDL particles with partly different synthesis routes: larger $VLDL_1$ (50–80 nm, ~70% triglycerides) and smaller $VLDL_2$ particles (30–50 nm, ~30% triglycerides) (Packard et al., 2020). The $VLDL_1$ particle number determines serum triglyceride concentration, which is the clinical indicator of VLDL metabolism. A fasting triglyceride concentration of less than 1.2 mmol/l indicates its optimal efficiency (Ginsberg et al., 2021). Secretion and clearance rates regulate $VLDL_1$ particle number and triglyceride concentration

(Borén et al., 2015). Overnutrition and insulin resistance drive their secretion by increasing the delivery of NEFA to the liver and promoting *de novo* lipogenesis (Ginsberg et al., 2021). The clearance of $VLDL_1$ particles is primarily determined by the activity of lipoprotein lipase, which is influenced by genetic and lifestyle factors (Wu et al., 2021), as well as the hepatic removal of VLDL remnants (Ginsberg et al., 2021). In contrast, elevated liver cholesterol levels result in the abnormal production of $VLDL_2$ particles, particularly in conditions such as familial hypercholesterolemia (Packard et al., 2020).

As VLDL particles lose their triglyceride content and become less dense, they are reclassified as intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL). In healthy individuals, the proportion of VLDL, IDL, and LDL particles of the circulating apoB-containing lipoproteins is roughly 15%, 15%, and 70%, respectively (Ala-Korpela et al., 2022). This difference in concentration illustrates the fast processing and clearance of VLDL particles from circulation and explains why LDL particles are considered the primary drivers of atherosclerosis, despite the ability of VLDL particles with a diameter less than 70 nm also to enter the arterial wall (Sniderman et al., 2019). For this reason, LDL cholesterol is a good proxy of atherogenic lipoprotein particles in most individuals (Ference et al., 2017).

The relationship between apoB-containing lipoprotein measures and energy metabolism becomes clear through studies on overfeeding and energy restriction. Even short periods of positive energy balance increase VLDL particle synthesis (Sevastianova et al., 2012; G. I. Smith, Reeds, et al., 2014), while negative energy balance and FM loss decrease VLDL particle and triglyceride concentrations, even in female physique athletes (Sarin et al., 2019). T2D is associated with increased VLDL particle number, size, and triglyceride content (Ahola-Olli et al., 2019), but disease remission through severe energy restriction includes a decline in $VLDL_1$ particles and triglycerides (Al-Mrabeh et al., 2020). When triglyceride levels are elevated, VLDL overproduction drives the slower clearance of LDL particles, leading to a dominance of small LDL particles in circulation (Borén et al., 2020). In contrast, energy restriction reduces LDL particles, especially in the smallest subclass (Collins et al., 2023; Huffman et al., 2022). In addition to energy intake, the fatty acid composition of the diet affects VLDL and LDL particle levels. A preference for saturated over polyunsaturated fatty acids promotes hepatic triglyceride and VLDL production (Yki-Järvinen et al., 2021) and reduces LDL clearance by decreasing LDL receptor activity (Siri-Tarino et al., 2015).

ApoA-I-containing lipoproteins. High-density lipoproteins (HDL) are the smallest and most numerous circulating lipoprotein particles, comprising to 90% of all lipoproteins (Ala-Korpela et al., 2022). HDL particles typically contain two or three apoA-I proteins, so the concentrations of apoA-I and HDL particles are not equal (von Eckardstein et al., 2022). Historically, HDL particles were thought to be a homogenous group with the sole function of returning cholesterol from the periphery to the liver. However, both of these assumptions have been proven to be incorrect and have contributed to the ongoing confusion regarding the role

of HDL particles in health. Observational studies have consistently shown that HDL cholesterol has a robust protective association against CVD (Castelli et al., 1986; Emerging Risk Factors Collaboration et al., 2009; Sharrett et al., 2001) and T2D risk (Schmidt et al., 2005; Wilson et al., 2007). However, the relationship between HDL cholesterol and mortality is U-shaped because both low and extremely high concentrations of HDL cholesterol are associated with an increased risk of death (Madsen et al., 2017; Yi et al., 2021). In contrast, RCTs investigating HDL cholesterol-increasing drugs have shown no benefit (ACCORD Study Group et al., 2010; AIM-HIGH Investigators et al., 2011), and Mendelian randomization studies indicate that HDL cholesterol and apoA-I concentrations are not causally related to CVD (M. K. Karjalainen et al., 2020; T. G. Richardson et al., 2020; Voight et al., 2012).

In reality, HDL particles are structurally and functionally heterogeneous. Reverse cholesterol transport is just one of the many functions of HDL particles because they also regulate cellular functions throughout the body by modulating the cholesterol levels of cells and activating cell responses directly or indirectly by transporting signaling molecules (von Eckardstein et al., 2022). HDL particles also bind toxic substances and inactivate them directly or transport them to the liver for disposal (von Eckardstein et al., 2022). The differing health effects of HDL subcategories become evident when they are divided based on size. Larger HDL particles are negatively associated with apo-B-containing particle number, LDL cholesterol, and triglyceride concentrations, while smaller particles are positively associated with these items (Ala-Korpela et al., 2022). Only larger HDL particles have been found to be associated with a lower risk of CVD (Holmes et al., 2018; Würtz et al., 2015). In contrast, obesity is associated with increased numbers of small HDL particles (Juárez-Rojas et al., 2020), and weight loss shifts HDL size distribution toward larger particles (Coimbra et al., 2019; Collins et al., 2023; Huffman et al., 2022). Furthermore, small HDL particles are associated with insulin resistance (Garvey et al., 2003) and predict the development of T2D (Bragg et al., 2022).

Another important aspect of HDL particles is their lipid composition. As previously discussed, HDL cholesterol is traditionally considered health protective. On the other hand, HDL triglycerides have been found to be positively associated with cardiometabolic disease incidence (Bragg et al., 2022; Holmes et al., 2018; Würtz et al., 2015). A likely reason for this discrepancy is that overnutrition and insulin resistance upregulate triglyceride and VLDL particle production, increasing triglyceride transfer to HDL particles while also causing their increased glycation and catabolism (Vergès, 2015). These changes decrease the capacity of HDL for reverse cholesterol transport, which means that HDL cholesterol levels decline.

2.1.3.3 Other metabolites

Fatty acid ratios. The serum fatty acid pool is composed of fatty acids derived from dietary sources, as well as those modified or produced by the body. As hydrophobic molecules, fatty acids cannot travel freely in circulation. Esterified

fatty acids in apoB-containing lipoprotein particles form the largest constituent of serum fatty acids, while smaller amounts of NEFAs travel from the adipose tissue to the sites of oxidation that are bound to albumin. Given that the quantity of serum fatty acids varies between individuals, ratios of different fatty acid categories to total fatty acids reflect cardiometabolic health better than their absolute concentrations.

When studying circulating triglycerides in isolation, elevated ratios of monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) are associated with insulin resistance (Kotronen et al., 2009). Furthermore, higher total ratios of MUFAs and SFAs in circulation have been found to predict T2D (Ahola-Olli et al., 2019; Mahendran et al., 2013; Seah et al., 2022), and a higher MUFA ratio has been linked to an increased risk of CVD events (Würtz et al., 2015). It is important to note that circulating MUFAs do not correlate with dietary MUFA intake but instead reflect endogenous production (Hodson et al., 2008). Similarly, palmitate, which is a form of SFA, is the primary product of *de novo* lipogenesis and contributes to circulating SFA levels. Conversely, a higher ratio of circulating polyunsaturated fatty acids (PUFA) has been found to be protective against T2D (Ahola-Olli et al., 2019; Mahendran et al., 2013; Seah et al., 2022). Linoleic acid is the primary circulating PUFA. Unlike MUFAs, the body does not produce linoleic acid, and its concentration reflects dietary intake better (Hodson et al., 2008).

Glycolysis-related metabolites. Glucose is the substrate for glycolysis, and its fasting value is the primary clinical tool for prediabetes and diabetes screening. Fasting glucose has reasonable specificity but lower sensitivity because blood glucose levels are tightly controlled and do not change until the disease state progresses far enough (Barry et al., 2017). This has led to a search for alternative biomarkers in metabolomics studies for earlier identification of high-risk individuals (Ahola-Olli et al., 2019).

Pyruvate, which is the end product of glycolysis, can either be transported to the mitochondria for oxidation or transformed into lactate or glycerol-3-phosphate. Lactate, which has been previously thought to be mainly a waste product produced during high-intensity exercise, is actually a highly desired energy substrate for various tissues that cells also produce at rest (Brooks et al., 2022). Elevated lactate levels are seen in individuals with poor metabolic health and decrease after bariatric surgery (T. E. Jones et al., 2019). Interestingly, higher pyruvate and lactate—but not glucose—concentrations were associated with higher CVD event risk (Würtz et al., 2015). Higher lactate levels were also associated with incident T2D in the Chinese (Bragg et al., 2022) but not in the Finnish population (Ahola-Olli et al., 2019). Impaired aerobic metabolism has been suggested as a possible explanation for elevated fasting lactate levels (T. E. Jones et al., 2019); however, this is unlikely because oxidative capacity is not a rate-limiting factor of metabolism at rest (Holloszy, 2009). The increased pyruvate and lactate levels may simply represent increased energy availability.

Glycerol enters circulation following adipose tissue lipolysis and is commonly used as a proxy for lipolysis rate (Wolfe et al., 1987). The liver

primarily clears glycerol from the circulation, where it is used for gluconeogenesis or triglyceride synthesis (Kalhan et al., 2001). Elevated glycerol levels have been linked to higher fasting and postprandial glucose concentrations (Mahendran et al., 2013) and an increased risk of cardiometabolic disease (Ahola-Olli et al., 2019; Mahendran et al., 2013; Würtz et al., 2015).

Citrate, which is an intermediate in the tricarboxylic acid cycle, is primarily found in the bones, accounting for 90% of the body's citrate (Costello & Franklin, 2016). The regulation of circulating citrate is not well understood, and likely, the citrate present in soft tissues does not significantly contribute to the overall circulating citrate pool because citrate is mainly retained within mitochondria (Costello & Franklin, 2016). Epidemiological research has shown no clear role for circulating citrate as a cardiometabolic health marker. For example, although citrate has been found to have a positive association with all-cause mortality (Fischer et al., 2014), citrate also has a negative association with CVD events (Würtz et al., 2015) and no clear connection to T2D risk (Ahola-Olli et al., 2019; Bragg et al., 2022). A recent study also found that low citrate levels were associated with insulin resistance in individuals with obesity (Luukkonen et al., 2021), but citrate levels were not affected by energy restriction in individuals with or without excess weight (Huffman et al., 2022).

Amino acids. Branch-chained amino acids (BCAA; i.e., isoleucine, leucine, and valine) have now been established as biomarkers of metabolic functioning. Their concentrations are elevated in individuals with obesity (Newgard et al., 2009) and with insulin resistance (Palmer et al., 2015) and T2D (Vanweert et al., 2021), and they predict T2D development (Ahola-Olli et al., 2019; Bragg et al., 2022). In contrast, BCAA concentrations decrease following weight loss induced by bariatric surgery (Vaz et al., 2022) and energy restriction both in individuals with overweight or obesity (Collins et al., 2023) and in those with normal weight (Huffman et al., 2022). The reason for the increase in BCAAs in metabolic diseases is not entirely apparent. Perhaps the most convincing theory is that their levels rise because of insulin resistance (Mahendran et al., 2017; Q. Wang et al., 2017) because insulin stimulation is needed for amino acid breakdown. However, the current evidence suggests that defects in the BCAA catabolism may also play a role in insulin resistance development (Vanweert et al., 2022). Another theory is that insulin resistance is the cause of impaired BCAA catabolism in prediabetes, but increased BCAA levels may contribute to the progression of the disease to T2D (White et al., 2021).

The aromatic amino acids tyrosine and phenylalanine have received less attention than BCAAs but show similar associations with T2D (Morze et al., 2022). Conversely, glutamine and glycine concentrations are inversely associated with T2D (Morze et al., 2022). Their decreased levels are postulated as resulting from changes in BCAA metabolism. Glutamine is synthesized to transport amino groups from oxidative tissues to the liver; thus, its decreased levels could reflect declining BCAA catabolism (Holeček, 2020). However, White et al. (2020) suggested that, in individuals with obesity, elevated BCAA levels increase BCAA catabolism and the need for amino group transport from skeletal muscle to the

liver via the pyruvate-alanine cycle and glutamine synthesis. Glycine is a carbon donor for the pyruvate-alanine cycle, and the increase in BCAA flux could lead to glycine depletion (White et al., 2020). Further investigation is necessary to thoroughly understand the mechanisms linking amino acids and metabolic health.

Ketone bodies. Ketone bodies such as acetoacetate, acetone, and 3-hydroxybutyrate are small molecules produced in meaningful quantities by the liver, which can substitute glucose as a fuel in the central nervous system (Cotter et al., 2013). Hepatocytes convert acetyl coenzyme A to acetoacetate, which can then be further converted into 3-hydroxybutyrate, while smaller amounts of acetoacetate also spontaneously transform into acetone (Cotter et al., 2013). Ketone production increases when adipose tissue lipolysis is elevated, leading to a surge of NEFAs in the liver, here in the presence of low insulin and high glucagon levels (Cotter et al., 2013). However, ketone levels decrease after overfeeding without carbohydrate restriction (Dirlewanger et al., 2000) and increase during energy restriction (Huffman et al., 2022). Therefore, circulating ketone concentrations reflect the current energy balance and macronutrient intake of an individual.

The relationship between ketone concentrations and cardiometabolic health is complex. In healthy individuals, higher levels of acetoacetate and 3-hydroxybutyrate are positively associated with insulin sensitivity (Mahendran et al., 2013; Würtz et al., 2012) and lower risk of CVD events (Würtz et al., 2015). However, compared with individuals with normal fasting glucose, acetoacetate and 3-hydroxybutyrate are lower in individuals with impaired fasting glucose but higher in those with T2D (Mahendran et al., 2013). These findings suggest that ketone levels decrease during the early stages of metabolic deterioration because of insulin resistance, but when the disease progresses to T2D, ketone levels may increase as a result of impaired insulin secretion.

Circulating acetate, on the other hand, is mainly derived from the diet or produced by gut microbiota (Canfora & Blaak, 2017). Although animal and short-term human studies have suggested that acetate may have the potential to alter systemic metabolism through mechanisms such as increasing energy expenditure and improving insulin sensitivity, it remains unclear if these effects hold in real-life scenarios (Canfora & Blaak, 2017). Additionally, in prospective human studies, acetate has not been found to have as clear of an association with cardiometabolic disease risk (Ahola-Olli et al., 2019; Bragg et al., 2022; Würtz et al., 2015).

GlycA. GlycA is a biomarker of systemic inflammation that has garnered excitement in cardiometabolic disease research because metabolomics studies have found it to be one of the strongest predictors of CVD events (Akinkuolie et al., 2014, 2016; Würtz et al., 2015) and T2D (Ahola-Olli et al., 2019; Bragg et al., 2022). Unlike traditional single-molecule inflammation markers, GlycA is an NMR-derived composite signal of the concentration and glycosylation degree of serum acute phase proteins, reflecting the overall inflammation level in the body (Otvos et al., 2015). This makes it a more stable and comprehensive measure of

chronic inflammation (Otvos et al., 2015). For example, GlycA has been found to be more effective in explaining adverse cardiometabolic disease risk profiles in adolescents and young adults and predicting hypertension and metabolic syndrome development over a 10-year follow-up when compared with C-reactive protein (Chiesa et al., 2022). This suggests that GlycA has high utility, even in populations with low inflammation levels. Furthermore, changes in inflammation status can also be reflected in changes in GlycA levels when the energy balance is manipulated. Studies have shown that both bariatric surgery (Dadson et al., 2020) and energy restriction in individuals with and without overweight (Collins et al., 2023; Huffman et al., 2022) can lead to a decrease in GlycA levels.

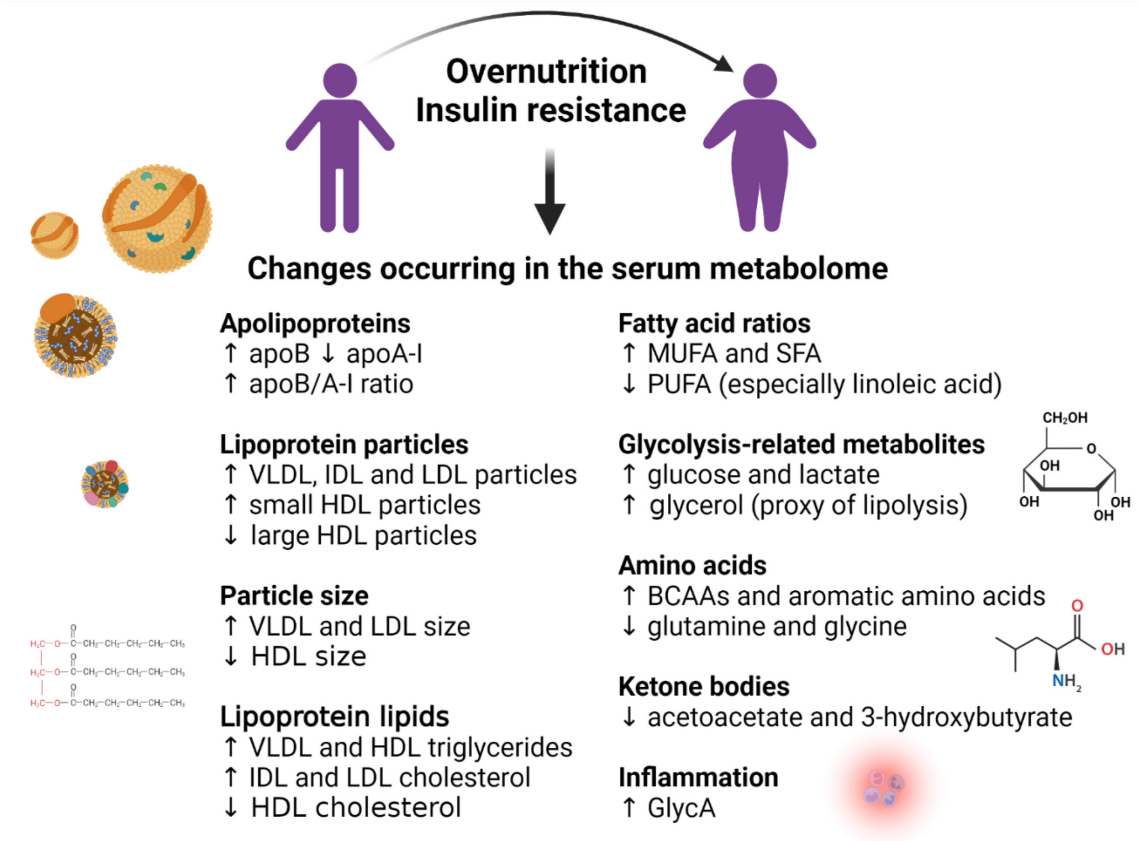

FIGURE 1 Changes in the serum metabolome in response to chronic overnutrition and resulting metabolic deterioration. When the energy intake chronically exceeds energy expenditure, metabolic health is impaired. Initially, this manifests as increased triglyceride production and decreased circulating ketone levels. The liver gradually increases the production of very low-density lipoprotein (VLDL) particles to relocate triglycerides to the adipose tissue to prevent fat accumulation. This also leads to an increase in other apoB-containing lipoprotein particles, particularly small low-density lipoprotein (LDL) particles, being in circulation. Over time, the liver, adipose tissue, and skeletal muscle lose insulin sensitivity, leading to further metabolic deterioration. Adipose tissue lipolysis increases and is reflected in elevated glycerol levels. As fatty acid availability in the liver increases, triglyceride levels continue to rise in all lipoprotein particle types, primarily consisting of monounsaturated and saturated fatty acids (MUFA and SFA) because of an increased dietary intake of SFA and de novo lipogenesis, resulting in a decreased polyunsaturated fatty acid (PUFA) ratio. High-density lipoprotein (HDL) particle size shifts toward smaller particles, and HDL cholesterol efflux capacity decreases, leading to lower HDL cholesterol levels. Branch-chained amino acid (BCAA) and aromatic amino acid catabolism are impaired, increasing their circulating levels and decreasing glutamine and glycine concentrations. The accumulation of BCAAs may further drive the progression of the disease. Lactate levels also increase in the early stages of disease progression, but fasting glucose levels remain normal until the disease progresses to prediabetes. In response to elevated inflammatory stimulus, GlycA increases.

2.2 Menopause

Menopause marks the end of the female reproductive lifespan, either as a spontaneous cessation of ovarian follicular activity (*natural menopause*) or as a result of medical procedures (*induced menopause*) (Shifren et al., 2014). Natural menopause is confirmed after 12 months of amenorrhea (Harlow et al., 2012) and is caused by the depletion of the ovarian follicle pool (S. J. Richardson et al., 1987). Both genetic and various environmental factors play a role in hypothalamic and ovarian aging, leading to follicle depletion (Davis et al., 2015). The age of natural menopause can have considerable variation and occur between the ages of 40 and 58 (Shifren et al., 2014). The worldwide mean age of natural menopause is 48.8 years, but the estimate is higher in wealthier nations (Schoenaker et al., 2014). In Finland, the median age of menopause is 51 years (Luoto et al., 1994; Pakarinen et al., 2010). Premature menopause is defined as confirmed menopause before the age of 40, and early menopause as menopause between the ages of 40 and 45 (Shifren et al., 2014).

Menopause status determination. The nomenclature for menopause staging (FIGURE 2A) follows the Stages of Reproductive Aging Workshop (STRAW) + 10 guidelines (Harlow et al., 2012). The reproductive phase of life (stages -5 to -3) begins after puberty. In their 40s, most women enter menopausal transition, which is divided into the early (-2) and late (-1) phases. In the early phase, menstrual cycle length varies persistently for at least 7 days, and in the late phase, amenorrhea for at least 60 days occurs. The duration of menopausal transition is highly variable but typically lasts from 1 to 3 years (Davis et al., 2015). The late menopausal transition ends with the final menstrual period. Reproductive endocrine functions stabilize during the early postmenopause (with more rapid changes in +1a and +1b, and stabilization in +1c), lasting approximately 5 to 8 years. In late postmenopause (+2), physiological changes are more related to biological aging than reproductive aging. It can be difficult to identify when the last menstrual period has occurred. A year after the final menstrual period marks the end of the waiting period when menopause can be diagnosed. In the current thesis, the term perimenopause is used interchangeably with menopausal transition. However, in reality, perimenopause encompasses a longer period, including the 12-month amenorrhea requirement, while menopausal transition refers explicitly to the period leading up to the final menstrual period (Harlow et al., 2012).

Menopausal hormonal changes. During the menopausal transition, the gradual cessation of ovarian follicular activity leads to a dramatic shift in the individual's sex hormone profile (FIGURE 2B). This is primarily characterized by a decrease in estradiol (E2) and increase in follicle-stimulating hormone (FSH) concentrations (Davis et al., 2015). However, it is important to note that other hormone changes also occur during menopause, such as a decrease in progesterone and increase in luteinizing hormone levels. The increase in lutenizing hormone is less pronounced compared with the changes in FSH

(Soares et al., 2020) and does not mirror the decline in E2 as closely (Overlie et al., 1999). E2 concentrations fluctuate during the menopausal transition, while FSH levels increase more consistently (Burger et al., 1999). For these reasons, FSH measurements are used in determining menopause status. In the early menopausal transition, FSH levels are variable but slightly elevated when measured on cycle days 2–5, while in the late phase, FSH levels are typically above 25 IU/l but may greatly fluctuate (Harlow et al., 2012). These hormonal changes continue to progress for about 2 years after the final menstrual period. After menopause, ovaries continue to secrete small amounts of estrogens and testosterone (Fogle et al., 2007), while adrenal glands produce mainly weak androgens, particularly dehydroepiandrosterone (Rege et al., 2013). After menopause, the primary source of systemic estrogens is the aromatization of androgens by the adipose tissue (Mohanty & Mohanty, 2021). Therefore, the estrogen–androgen ratio shifts toward an androgenic direction (Paschou et al., 2018).

The decline in E2 levels is primarily responsible for menopause symptoms, which are typically the most severe during late perimenopause and early postmenopause, when E2 changes are most pronounced (Davis et al., 2015). The most frequently reported symptoms are vasomotor symptoms, while changes in sleep, mood, and cognition, sexual functions, and increased urogenital and somatic pain experiences are also common (Monteleone et al., 2018; Woods & Mitchell, 2005).

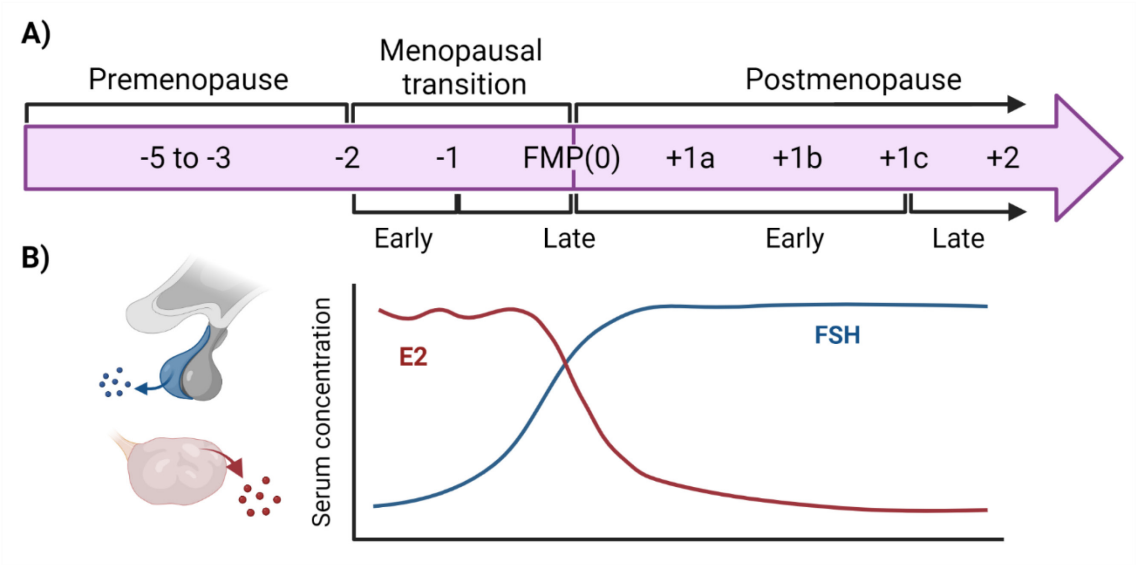

FIGURE 2 Stages of reproductive aging (A) and associated hormonal changes during menopause (B). The gradual loss of ovarian follicle activity results in fluctuations in estradiol (E2) levels during the early menopausal transition and a significant decline in E2 concentrations around the final menstrual period (FMP). Follicle-stimulating hormone (FSH) levels increase in response to ovarian E2 production decline. Hormone levels stabilize during early postmenopause.

As reviewed by Fuentes and Silveyra (2019), E2 exerts its functions mainly through two intracellular estrogen receptors (ERs)—ERα and Erβ—as well as

through a membrane-bound G protein-coupled ER 1. ERα and ERβ are expressed in nearly all tissues, but their ratios vary. However, making things even more complex, the functions of ERα and ERβ can sometimes be opposing. The effects of E2 mainly occur through gene regulation. In classic direct genomic signaling, E2 binds to ERs in the cytoplasm and causes the dimerization of receptors. This complex then travels to the nucleus and interacts with estrogen response elements in DNA, leading to changes in gene transcription. The complex can also cause indirect genomic signaling by interacting with other proteins that regulate gene expression. Furthermore, it has been proposed that all ERs can alter cellular function through nongenomic actions, such as modulating intracellular signaling pathways, which can lead to faster responses to E2 stimulation.

2.2.1 Menopause and body composition

Women often report changes in body composition during the menopausal transition that they cannot explain with lifestyle changes. Longitudinal studies have shown that during this time, women experience a loss of LM and an increase in FM, with a shift toward a more android fat distribution (Aloia et al., 1991; Juppi et al., 2020, 2022; Lee et al., 2009; Lovejoy et al., 2008; Sowers et al., 2007). Whether the changes result from aging or menopause has been debated (Al-Safi & Polotsky, 2015; Davis et al., 2012; Wildman & Sowers, 2011). Recent studies aiming to separate the effects of the phenomena indicated that menopause has a causal role (Clayton et al., 2022; Greendale et al., 2019; Hyvärinen et al., 2021). The exact reasons for the changes are not fully understood. Skeletal muscle mass decline explains the LM loss (Juppi et al., 2020), potentially because of E2 deficiency-induced changes in protein synthesis and degradation, apoptosis rate, and contractile protein structure and function (Pellegrino et al., 2022). Additionally, declining progesterone signaling may also play a role (Juppi et al., 2020; G. I. Smith, Yoshino, et al., 2014). The shift in FM distribution reflects the change toward a more androgenic sex hormone profile (Paschou et al., 2018), which is similar to what has been seen in transgender men receiving gender-affirming hormone therapy (Ford et al., 2022). Notably, this shift is also associated with increased visceral fat (Ambikairajah et al., 2019).

An increase in FM is always a product of a chronic positive energy balance. Menopause may change the energy balance by affecting energy intake, expenditure, or both. The effect of menopause on energy intake is not well understood, but it is believed that estrogen regulates hunger at the hypothalamic level, and its deficiency induces hyperphagia in animal models (Asarian & Geary, 2013). However, whether this effect is also present in humans is uncertain. Longitudinal studies using food frequency questionnaires (Grisotto et al., 2021; Macdonald et al., 2005) or 4-day food diaries (Lovejoy et al., 2008) have not found any changes in diet quality or estimated energy intake during the menopausal transition. A study assessing diet with 7-day food diaries even found that energy and carbohydrate intake declined in women transitioning into postmenopause during a 5-year follow-up compared with participants who did not (Duval et al., 2014). It is important to note that insufficient sleep may increase hunger and

energy intake by modulating appetite hormone levels (Chaput et al., 2023), and depressive mood has a bidirectional relationship with diet (Jacka, 2017), which may mediate the effects of menopause on energy intake. A recent study found that postmenopausal women had poorer sleep quality and higher sugar intake than age-matched premenopausal women (Bermingham et al., 2022), which supports this hypothesis.

Although the increase in FM associated with menopause has traditionally been explained by declining E2 concentrations, research has suggested that the rise in FSH may contribute independently to this phenomenon. In a mouse model, blocking FSH has been shown to reduce FM by increasing energy expenditure through the activation of brown adipose tissue and the process of adipocyte beiging (Han et al., 2020; P. Liu et al., 2017). However, it is unclear if these findings will translate to humans. The thermogenic capacity of brown adipose tissue is much greater in mice than in humans, and whether brown adipose tissue activation can increase energy expenditure in humans in meaningful quantities is still under investigation (Carpentier et al., 2018). Furthermore, observational studies examining the relationship between FSH and FM in humans have generally produced inconsistent results. For example, Mattick et al. (2022) found that FSH levels—but not E2 levels—were associated with lower FM in postmenopausal women aged 50 to 79 when both hormones were included in the same multivariable linear regression model. However, in longitudinal analyses, women who experienced a larger change in FSH levels gained more FM. Therefore, further research is needed to fully understand the role of FSH in the menopause-associated increase in FM and to clarify the conflicting evidence regarding whether menopause reduces energy expenditure (discussed in section 2.2.4.).

2.2.2 Menopause and cardiometabolic health

CVD is the primary cause of death in all women worldwide, while cancer deaths lead the statistics in women under the age of 65. However, this difference between cancer and CVD deaths in younger women has been decreasing (Khan et al., 2022). This narrowing of the mortality gap is likely because of an increase in CVD risk factors in the population, which are also influenced by menopause. Postmenopausal women have a higher risk of CVD than age-matched premenopausal women (Kannel et al., 1976), particularly if they have experienced menopause prematurely (Muka et al., 2016; Zhu et al., 2019). Additionally, women tend to develop CVD about 10 years later than men, and premenopausal women have a much lower CVD risk than age-matched men (Johnston et al., 2011). This sex difference starts to diminish after women reach menopause. For example, in Finland between 2016 and 2020, women represented 9%, 12%, and 16% of ischemic heart disease deaths in the 45–49, 50–54, and 55–59 age categories, respectively (Statistics Finland, 2022).

Regarding other categories of cardiometabolic diseases, the association with menopause is not as well established. The incidence of metabolic syndrome increases during menopausal transition and early postmenopause (Janssen et al.,

2008); there is some evidence that menopause might accelerate the development of nonalcoholic fatty liver disease (Pafili et al., 2022). Menopause is also considered a risk factor for T2D (Lambrinoudaki et al., 2022). Here, a meta-analysis of six prospective cohort studies found that older age at menopause was associated with decreased T2D risk (Guo et al., 2019). Additionally, women who underwent bilateral hysterectomy and oophorectomy had a higher risk of developing T2D than those without these procedures (Appiah et al., 2014). It is important to note that T2D also increases the risk of developing CVD, and the risk increase is more prominent in women than men (Peters et al., 2014; Y. Wang et al., 2019).

The menopause-associated increase in cardiometabolic disease risk can result from sex hormone changes directly or through mediating factors. Potential direct effects predisposing women to atherosclerosis development include, but are not restricted to, decreased LDL particle clearance through reduced activity of hepatocyte LDL receptors (A. Karjalainen et al., 2000; Parini et al., 1997) and endothelial dysfunction (Bechlioulis et al., 2010; Moreau et al., 2012). Indirect routes, such as changes in energy balance and resulting changes in body composition and metabolic functioning, may also play a role in the development of cardiometabolic diseases (Lambrinoudaki et al., 2022; Maas et al., 2021). A recent study (Mutie et al., 2023) using Mendelian randomization found that a higher body mass index (BMI) was causally related to an increased risk of T2D in all women, regardless of their menopause status. However, the study also found that a higher BMI only increased the risk of coronary artery disease in premenopausal women. This suggests that other risk factors may play a more significant role in postmenopausal women. It is tempting to posit that menopausal hormonal changes have a more direct effect on the development of CVD, while indirect routes may contribute more to the risk of T2D.

2.2.3 Menopausal hormone therapy

Menopausal hormone therapy (MHT) is primarily prescribed as either estrogen monotherapy or estrogen-progestin combination therapy, depending on whether the woman has had a hysterectomy. The estrogen component consists of E2 or conjugated equine estrogens (CEE, a mixture of estrogens), while the progestin component prevents endometrial hyperplasia in women with an intact uterus (Davis & Baber, 2022). MHT is usually administered orally or transdermally. The main difference between the administration routes is that oral MHT goes through the liver's first-pass metabolism before entering the systemic circulation (Davis & Baber, 2022).

MHT has one of the most controversial histories of any drug in medicine. Before the twenty-first century, MHT was prescribed liberally based on largely observational evidence for the treatment of menopausal symptoms and prevention of chronic diseases (Chester et al., 2018). This changed in 2002 with the publication of the first results from the Women's Health Initiative (WHI) trial, which showed a statistically significant increase in the risk of CVD events with the use of both oral CEE monotherapy and CEE and medroxyprogesterone acetate combination therapy when compared with the placebo (Rossouw et al.,

2002). Additionally, combination therapy was associated with an increased risk of breast cancer. As a result, the prescription of MHT plummeted (Ettinger et al., 2018).

Subsequent analyses of the WHI trial data have clarified the original findings. A 13-year follow-up found that the risks associated with combination therapy and incident CVD and breast cancer remained statistically significant (Manson et al., 2013). However, an 18-year follow-up reported that the combination therapy was not associated with all-cause, CVD, or cancer mortality (Manson et al., 2017). The fundamental limitation of the WHI trial is that the mean age of participants was 63 years and that most had experienced menopause more than 10 years prior to enrollment (Chester et al., 2018). Age-stratified analyses of the data revealed that the CVD risk dissipated in women aged 50–59 years at baseline (Manson et al., 2013).

Overall, the evidence from RCTs suggests that MHT is neutral or perhaps even beneficial for cardiovascular health when prescribed close to the final menstrual period (Marjoribanks et al., 2017). Additionally, MHT may offer some protection against T2D (Davis & Baber, 2022; Lambrinoudaki et al., 2022). Currently, MHT is prescribed after shared decision making for women with troublesome menopausal symptoms or severe fracture risk to reduce osteoporosis within 10 years after menopause. However, it is not recommended for the prevention of CVD or other chronic diseases (Davis & Baber, 2022).

The effect of MHT on preventing or reversing menopause-associated changes in body composition has been controversial. In a 3-year substudy of the WHI trial, the combination therapy group lost less LM than the placebo group (Chen et al., 2005). Additionally, long-term MHT users had better muscle power assessed with the vertical jump test than their nonuser monozygotic (MZ) cotwins (Ronkainen et al., 2009). Further studies also revealed differences in skeletal muscle transcriptome and proteome between the MHT-using and nonusing twins (Laakkonen, Soliymani, et al., 2017; Ronkainen et al., 2010). However, a meta-analysis of 12 RCTs found that MHT did not affect muscle mass in postmenopausal women compared with no treatment or placebo (Javed et al., 2019). Nevertheless, RCTs have suggested that at least oral E2 and norethisterone acetate combination therapy (Sipilä et al., 2001) and transdermal E2 monotherapy (Dam et al., 2020) may benefit muscle gain compared with a placebo when combined with a resistance training program designed to cause hypertrophy.

In terms of adiposity, MHT does not seem to decrease FM in postmenopausal women (Ambikairajah et al., 2019). However, it may restore more gynoid FM distribution, potentially resulting in less visceral fat (Salpeter et al., 2006). Indeed, in the aforementioned cotwin control study, MHT users had less body fat than nonuser cotwins, particularly in the abdominal area (Ahtiainen et al., 2012). However, in the already mentioned resistance training trial, women allocated to transdermal E2 monotherapy lost less total and visceral FM than those in the placebo group (Dam et al., 2021). It is important to note that the effects of MHT on body composition have not been extensively studied in women in menopausal transition using RCT design. However, in our longitudinal

studies, women who started MHT during the menopausal transition experienced similar LM loss and FM increase as those women without MHT use (Juppi et al., 2020, 2022).

2.2.4 Menopause and energy expenditure

The cause of menopause-associated increase in FM has often been attributed to reduced energy expenditure (Knight et al., 2021; Mauvais-Jarvis et al., 2013; Nappi et al., 2022), perhaps because it is easier to quantify than energy intake. As reviewed by Van Pelt et al. (2015), the mechanistic evidence for whether menopause modulates energy expenditure mainly comes from studies in rodents. Both female and male ERα—though not ERβ—knock-out mice consistently have more FM than wild-type controls. ERα knock-outs have also decreased physical activity and REE, indicating that ERα signaling is essential for systemic energetics. However, rodents do not experience menopause. Mechanistic studies in rodents induce menopause with ovariectomy and compare operated animals with sham-operated or E2-supplemented controls. Both ovariectomized mice and rats gain FM, particularly in the abdominal region, which is preventable with E2 supplementation. However, the mechanism behind FM increase differs between species. Food intake increases only in rats, while physical activity decreases in both species but is preventable with E2 supplementation (Van Pelt et al., 2015). A decline in REE seems to occur in mice (do Carmo et al., 2018; Rogers et al., 2009; Witte et al., 2010; Zidon et al., 2020) but less consistently in rats (Giles et al., 2010; Vieira-Potter et al., 2015; Witte et al., 2010).

These rodent studies, however, have several limitations. First, laboratories often house mice below their thermoneutral zone, which increases their energy expenditure because of cold stress (Ganeshan & Chawla, 2017). As ovariectomized animals gain FM, they become better insulated and less vulnerable to cold than their controls. Second, these studies have often failed to adjust appropriately for variations in body mass when analyzing energy expenditure data. The prevalent use of scaling leads to biased results when comparing those groups with different body masses. If the regression intercept between body mass and energy expenditure is positive—as it nearly always is—scaling will underestimate energy expenditure, and the error increases with increasing body mass (T. D. Müller et al., 2021). A third limitation is that the results from rodent studies are unlikely to be generalizable to other species, such as primates. In ovariectomized rhesus monkeys, neither 3-month selective estrogen receptor modulator supplementation nor its cessation altered REE despite increased FM (Sullivan et al., 2012). Moreover, in marmoset monkeys, ovariectomy and E2 supplementation did not change FM- or DLW-measured TEE (Kraynak et al., 2019). Finally, ovariectomy in rodents does not fully equate to oophorectomy in humans. Dehydroepiandrosterone is the main precursor of androgens and estrogens after ovarian removal. However, the primary producers of dehydroepiandrosterone in rodents are gonads, while in humans,

it is the adrenal glands. Therefore, the effects of ovary removal may be more severe in mice than in humans (Van Pelt et al., 2015).

In conclusion, to understand the effect of menopause on energy metabolism, it is important to study the phenomenon in women. The effects of induced menopause cannot be studied similarly to ovariectomy in other species because of ethical reasons. However, ovarian hormone production can be temporarily suppressed by blocking gonadotropin-releasing hormone secretion. Studies on premenopausal women (TABLE 1) have shown that acute and chronic ovarian hormone suppression may reduce REE by up to 60 kcal/d (Day et al., 2005; Gavin et al., 2020; Grant et al., 2022; Melanson et al., 2015). However, this estimate possesses considerable uncertainty, and statistically significant reductions were not observed in SEE or TEE compared with control groups (Gavin et al., 2020; Melanson et al., 2015). Moreover, two important distinctions exist between the sex hormone profile generated by ovarian hormone suppression and the changes that occur during menopause. First, the treatment suppresses the release of anterior pituitary gonadotropins, whereas their secretion increases during the menopausal transition. This is an important consideration if FSH indeed influences energy metabolism in humans. Second, ovarian hormone suppression also reduces testosterone levels. However, the significance of this is questionable because, at least in men, a 3-week gonadal hormone suppression did not decrease REE, and subsequent 3-week E2, testosterone, or their combined replacement did not increase it (Santosa et al., 2010).

It is clear that also the use of MHT as an intervention can provide valuable insights into the effects of female sex hormones on energy metabolism. However, none of the major MHT trials have used REE as an outcome. Despite this, four single-group studies (Anderson et al., 2001; Bessesen et al., 2015; dos Reis et al., 2003; Lwin et al., 2008) and one RCT with a cross-over design (Marlatt et al., 2020) suggested that MHT does not appear to have a clear impact on REE in postmenopausal women (TABLE 1). These studies, however, have limitations, such as small sample sizes and a high risk of bias. Additional evidence can be found in hormone therapy studies in Turner syndrome, a condition caused by a partial or complete absence of one of the X chromosomes, which manifests as ovarian insufficiency (Klein et al., 2018). Research has demonstrated that hormone therapy did not have a notable effect on REE or substrate use in girls (Torres-Santiago et al., 2013) or women (Gravholt et al., 2007). Additionally, no discernible differences were found between oral and transdermal administration of hormone therapy (Torres-Santiago et al., 2013).

TABLE 1 Intervention studies investigating the effects of ovarian hormone suppression in premenopausal women and the effects of menopausal hormone therapy in postmenopausal women on energy metabolism.

Study	Participants	Interventions	Result
Ovarian hormone suppression			
One-group pretest-posttest studies			
Day (2005) USA	14 PRE women, M = 29 y, SD = 5	6 days: $GnRH_{ANT}$	REE: ↓ 71 kcal/d (P = 0.002) RER: no change
Grant (2022) USA	9 PRE women, M = 30 y, SD = 6	5 days: $GnRH_{AG}$	REE: 4.8% (P = 0.055) RFO: ↓ 34.9% RER: ↑ 41.3%
Randomized controlled trials			
Melanson (2015) USA	70 PRE women, age 20–49 y	5 months: 1) $GnRH_{AG}$ + placebo, n = 35 2) $GnRH_{AG}$ + E2, n = 35	REE: ↓ -3.7% (P < 0.05) in group 1. SEE and TEE (metabolic chamber): no between-group differences
Gavin (2020) USA	34 PRE women, age 40–54 y	24 weeks: 1) $GnRH_{AG}$ + placebo, n = 9 2) $GnRH_{AG}$ + exercise, n = 11 3) $GnRH_{AG}$ + E2, n = 14	REE, group 1: ↓ 59 kcal/d TEE (DLW), group 1: ↓ 93 kcal/d. No statistically significant between-group differences.
Menopausal hormone therapy			
One-group pretest-posttest studies			
Anderson (2001) USA	18 POST women, age 45–55 y 15 POST women, age 70–80 y	2 months: Transdermal E2 for 1 month, then E2 + P4 for 1 month	REE: no change
dos Reis (2003) Brazil	23 women with menopausal symptoms and FSH > 30 IU/l, age 45–65 y	12 months: 1) Conjugated estrogens 2) Transdermal E2	REE: no change RFO: ↓ in group 1 than in group 2 at end measurement. (Reporting limits interpretation.)
Lwin (2007) USA	14 POST women M = 51, SD 4	2 months: Conjugated estrogens	TEE (metabolic chamber): no change Resting RER: no change 24-h RER: ↑ 0.865 to 0.876
Bessesen (2015) USA	12 POST women, M = 56 y, SD = 3	2 weeks: Transdermal E2	REE and RFO: no changes
Randomized cross-over trial			
Marlatt (2020) USA	8 POST women, age 50–60 y	8 weeks: 1) Placebo 2) Conjugated estrogens + bazedoxifene	REE and RER: no statistically significant between-treatment differences

AG, agonist; ANT, antagonist; DLW, doubly labeled water; E2, estradiol; GnRH, gonadotropin-releasing hormone; M, mean; P4, progesterone; POST, postmenopausal; PRE, premenopausal; REE, resting energy expenditure; RER, respiratory exchange ratio; RFO, resting fat oxidation; SD, standard deviation; SEE, sleeping energy expenditure; TEE, total energy expenditure

The majority of evidence on whether menopause affects REE comes from observational studies, which are primarily cross-sectional in design (TABLE 2). These studies have shown that young premenopausal women have higher REE than postmenopausal women (Reimer et al., 2005; Schattinger et al., 2021; Van Pelt et al., 1997). However, this difference disappears when the groups are more similar in age (Bessesen et al., 2015; Gould et al., 2022; Hodson et al., 2014; Monda et al., 2017). The only study that has measured TEE with DLW found no difference between premenopausal and postmenopausal women (Tooze et al., 2007). Studies comparing postmenopausal MHT users and nonusers (Aubertin-Leheudre et al., 2008; Monda et al., 2017; Reimer et al., 2005) have been limited by substantial tissue mass differences between groups, which were not considered in statistical analyses. Additionally, Aubertin-Leheudre et al. (2008) measured participants at varying times during the day, some only 3 hours after their last meal. These cross-sectional findings are consistent with the previously mentioned study by Pontzer et al. (2021), which found that FFM- and FM-adjusted REE or TEE trajectories did not differ between sexes in midlife.

To approach causality, it is essential to have longitudinal evidence on how REE behaves during the menopausal transition. Unfortunately, only two studies have investigated this issue so far. The study by Lovejoy et al. (2008), which is highly cited in the literature, followed 34 initially premenopausal women over a 4-year period. Half of the participants transitioned to postmenopause, while the remaining half remained in premenopause throughout the study. The study assessed energy metabolism during a 24-hour metabolic chamber stay, which included 2 hours of walking on a treadmill. The results indicated that both SEE and TEE decreased in both groups during the follow-up period without noticeable between-group differences. A later study (Duval et al., 2013) followed 91 women for 5 years, measuring REE annually. At the end of the follow-up period, 19 women were premenopausal or perimenopausal, 12 women had been postmenopausal for less than or equal to 12 months, and 25 had been postmenopausal for more than 12 months. Even though FFM decreased and FM increased in all women (Abdulnour et al., 2012), the measured REE did not decrease in women who reached postmenopause and even increased in women who were premenopausal or perimenopausal at the final follow-up.

TABLE 2 Cross-sectional studies comparing energy metabolism between postmenopausal women and premenopausal or perimenopausal women or menopausal hormone therapy users.

Study	Participants	Groups, n (age)	Results
Studies of young premenopausal and middle-aged postmenopausal women			
Van Pelt (1997) USA	Women representing different physical activity levels. Note: eight women in both POST groups were on MHT	1) PRE sedentary, *n* = 12 (M = 29, SE = 1) 2) POST sedentary, *n* = 15 (M = 61, SE = 2) 3) PRE runners, *n* = 13 (M = 31, SE = 1) 4) POST runners, *n* = 15 (M = 58, SE = 1)	FFM- and FM-adjusted REE: ↓ POST sedentary vs. PRE sedentary, but not in POST runners vs. PRE runners
Schattinger (2021) USA	Sedentary women with a BMI ≤ 35 kg/m²	1) PRE (M = 20, SD = 1), *n* = 7 2) POST (M = 57, SD = 5), *n* = 7	Measured REE: ↓ in POST vs. PRE. RER: no differences
Studies of middle-aged premenopausal and postmenopausal women			
Tooze (2007) USA	Women aged 40–69 y with a mean BMI of ~27 kg/m²	1) PRE, *n* = 90 (includes 7 PERI women) 2) POST, *n* = 110	FFM-adjusted TEE (doubly labeled water): no differences
Hodson (2014) UK	Women with a BMI of 18.5–35 kg/m²	1) PRE (M = 41, SE = 0.6), *n* = 26 2) POST (M = 58, SE = 0.5), *n* = 25	LM-adjusted REE: no differences
Bessesen (2015) USA	Women with a BMI < 30 kg/m²	1) PRE (M = 42, SD = 4), *n* = 23 2) POST (M = 55, SD = 4), *n* = 22	FFM-adjusted REE or RFO: no differences
Gould (2022) USA	Nonathlete women with a BMI of 18.5–39.9 kg/m²	1) PRE (M = 40, SD = 3.3), *n* = 24 2) PERI (M = 50, SD = 3.4), *n* = 24 3) POST (M = 55, SE = 3.5), *n* = 24	REE or RFO: no differences
Studies with a menopausal hormone therapy user group			
Reimer (2005) Canada	Women with a normal weight	1) PRE (M = 30, SD = 2), *n* = 11 2) POST (M = 53, SD = 2), *n* = 7 3) MHT (M = 55, SD = 1), *n* = 6	Measured REE: ↓ POST vs. PRE RER: ↓ MHT vs. other groups
Aubertin-Leheudre (2008) Canada	Women with a BMI of 18–30 kg/m²	1) POST (M = 60, SD = 3), *n* = 13 2) MHT (M = 59, SD = 3), *n* = 13	Measured REE: ↓ MHT vs. POST, likely because of 2 kg higher body mass?
Monda (2017) Italy	Sedentary women with obesity	1) PRE (M = 43, SD = 4), *n* = 40 2) POST (M = 51, SD = 4), *n* = 40 3) MHT (M = 51, SD = 3), *n* = 40	Measured REE ↑ MHT vs. PRE or POST, likely because of significantly higher FFM?

BMI, body mass index; FFM, fat-free mass; FM, fat mass; LM, lean mass; M, mean; MHT, menopausal hormone therapy; PERI, perimenopausal; POST, postmenopausal, PRE, premenopausal; REE, resting energy expenditure; RER, respiratory exchange ratio; RFO, resting fat oxidation; SD, standard deviation; SE, standard error; TEE, total energy expenditure

2.2.5 Menopause and fat oxidation

Resting fat oxidation. Decreased fat oxidation has been proposed as a potential mechanism by which menopause may contribute to the accumulation of FM (Lovejoy et al., 2008). However, as previously reviewed, the evidence supporting this theory is limited (Péronnet & Haman, 2019). Most human studies have not found differences in resting RER or RFO between menopausal groups (TABLE 2) or following ovarian hormone suppression or MHT (TABLE 1). In the study by Lovejoy et al. (2008), 24-hour fat oxidation decreased statistically significantly only in women experiencing menopause during the follow-up period. However, it is important to note that the lack of group and time interaction in the study limits the conclusions that can be drawn from these results. Additionally, 24-hour RER did not change, and women compensated for the decrease in fat oxidation by increasing protein oxidation.

Peak fat oxidation. Because fat oxidation is higher during submaximal exercise in premenopausal women than in men (Cano et al., 2022), menopause can be assumed to decrease PFO. Whether the sex difference disappears after menopause is uncertain. A study by Frandsen, Amaro-Gahete, et al. (2021) investigated the relationship between age, sex, and PFO by dividing 247 men and 188 women into three age categories: under 45, 45–55, and over 55 years old. A sex difference was present only in the youngest age category. Conversely, a study by Numao et al. (2009) found that 10 postmenopausal women with obesity had higher FFM adjusted fat oxidation rates while cycling at 50% of $\dot{V}O_{2PEAK}$ compared with men matched for age, BMI, and cardiorespiratory fitness. It is worth noting that the groups also had comparable E2 levels.

Studies that compare fat oxidation during exercise between women in different menopausal states have been scarce, and none have used PFO as an outcome measure. In a seminal study, Abildgaard et al. (2013) compared fat oxidation rates between 11 premenopausal and seven postmenopausal women while cycling at 50% of $\dot{V}O_{2MAX}$ intensity for 45 min. They found that absolute fat oxidation rates were 0.31 g/min (SD = 0.03) in premenopausal and 0.21 g/min (SD = 0.07) in postmenopausal women, but after adjusting for LM, the difference between the groups was not statistically significant (P = 0.05). Additionally, the fraction of energy expenditure fueled by fat oxidation did not differ notably between groups (52% vs 43%, P > 0.05). A more recent study (Gould et al., 2022) compared substrate use at rest and during graded exercise between premenopausal, perimenopausal, and postmenopausal women (see the details in TABLE 2). However, the analysis strategy used makes the results difficult to interpret. The authors pooled all volume of oxygen ($\dot{V}O_2$) uptake and volume of CO_2 ($\dot{V}CO_2$) output data without identifying steady states to represent low, moderate, and high exercise intensities, which violate the assumptions of indirect calorimetry (Jeukendrup & Wallis, 2005). The results suggested that premenopausal women had 0.09 g/min (SE = 0.03, P = 0.045) higher absolute fat oxidation than postmenopausal women at a moderate-intensity exercise. However, the premenopausal group had almost 6 kg more FFM. No results accounting for FFM were reported. The only study to have compared substrate

use during exercise between postmenopausal women and postmenopausal MHT users was Johnson et al. (2002). They found no significant differences between groups in treadmill exercise at 85% of $\dot{V}O_{2MAX}$; however, the selected testing intensity violated the valid use of indirect calorimetry (Jeukendrup & Wallis, 2005).

2.2.6 Menopause and serum metabolome

The associations between menopause and circulating metabolites have been studied by mainly using traditional clinical biomarkers as outcomes. The results from longitudinal studies have varied, likely because of differences in study populations and designs. The most consistent findings have been increased serum triglyceride, total cholesterol, and LDL cholesterol levels (Ca et al., 2009; Hyvärinen et al., 2021; Jensen et al., 1990; Karvinen et al., 2019; Matthews et al., 2009, 2021). HDL cholesterol has been reported to decrease (Do et al., 2000; Jensen et al., 1990) peak before (Matthews et al., 2009) or increase (Abdulnour et al., 2012; Ca et al., 2009; Hyvärinen et al., 2021; Karvinen et al., 2019; Matthews et al., 2021) around menopause. Only our recent study found fasting glucose to increase (Hyvärinen et al., 2021), while others found glucose levels to decrease (Abdulnour et al., 2012; Jensen et al., 1990).

The effects of MHT depend on the selected estrogen and progestin components and administration route (Godsland, 2001). Oral CEE monotherapy increases triglycerides and HDL cholesterol while decreasing total and LDL cholesterol. Oral E2 monotherapy has similar effects on total and LDL cholesterol as CEE, but it increases HDL cholesterol and triglycerides less. Transdermal E2 increases HDL cholesterol less than oral administration but has the potential to lower triglycerides. Different progestins have different effects, but typically, they modulate the effects of estrogens on triglycerides and HDL cholesterol. MHT may also lower insulin resistance as calculated from fasting glucose and insulin concentrations, and oral MHT can increase C-reactive protein (Salpeter et al., 2006).

Prior to the present thesis, two studies have used the same NMR technology to investigate the associations between menopause and circulating metabolites but have done so using different designs. Auro et al. (2014) examined the differences in metabolite levels in relation to age, sex, and menopause status using cross-sectional data from 16,107 Finns (age range 24–75 years; 8,321 women) and 9,958 Estonians (age range 18–103 years; 6,416 women). The study found that in women aged 25–75 years (n = 10,083), postmenopausal status was associated with increased numbers of larger VLDL, IDL, and LDL particles, as well as increased concentrations of total and LDL cholesterol, glutamine, isoleucine, and tyrosine. When the analyses were restricted to women aged 40–55 years (n = 3,240), postmenopause was found to be associated with higher apoB/A-I ratio, MUFA, SFA, glutamine, and glycine concentrations. The study also found that, in age- and sex-stratified analyses, a proatherogenic lipoprotein particle shift occurred in men in early middle age, while in women, it occurred around age 45. A sensitivity analysis was also conducted on the data of MHT users (n = 1,053),

and it was found that they had similar metabolite profiles with premenopausal women but lower concentrations of apoB, VLDL, and LDL particles in some subclasses, along with HDL, triglycerides, and GlycA, than postmenopausal women. Additionally, MHT users had higher large HDL particle concentrations.

More recently, Q. Wang et al. (2018) analyzed cross-sectional data from 3,312 British women, of whom 1,492 also experienced menopausal status change during a 2.5-year follow-up. The study found that menopause was associated with an increased number of apoB, very small VLDL, IDL, and LDL particles, and increased concentrations of IDL and LDL cholesterol in both cross-sectional and longitudinal analyses. Perhaps surprisingly, the study also found that menopause was associated with increased glutamine concentrations and decreased SFA ratios in both analyses. The GlycA concentration rose only in the cross-sectional analysis and apoA-I in the longitudinal analysis. Additionally, LDL particle size decreased in women who were followed over menopausal transition.

Synthesis of metabolomics studies can be challenging because of the high number of outcomes. However, broadly speaking, these two prior studies indicated that menopause is associated with increases in apoB-containing lipoprotein particles and cholesterol concentrations. However, the findings concerning triglycerides, fatty acid ratios, and nonlipid metabolites were less consistent but generally indicated a trend toward elevated BCAA and aromatic amino acid levels.

2.3 Physical activity

Physical activity, which here can be defined as the movement produced by skeletal muscle that results in energy expenditure, can be broadly classified into three categories: activities performed during sleep, work, and leisure time (Caspersen et al., 1985). Exercise, on the other hand, is a subcategory of physical activity specifically intended to improve health and/or physical fitness (Caspersen et al., 1985). When discussing energy expenditure caused by physical activity, nonexercise activity thermogenesis (NEAT) refers to the AEE that cannot be attributed to exercise. NEAT encompasses a wide range of physical activities that may occur in various settings, such as fidgeting, tapping one's toes, and speaking during work or leisure time (Levine, 2004). Like REE, AEE is influenced by an individual's body mass and composition. To make AEE comparable between individuals, it is common practice to express it as a physical activity level by dividing TEE by REE (Westerterp, 2018).

2.3.1 Assessment of physical activity

Physical activity has four dimensions: mode or type, frequency, duration, and intensity (Warren et al., 2010). The latter three are essential when quantifying the volume of physical activity, which is commonly expressed as kilocalories or the

metabolic equivalent of task (MET) units (Strath et al., 2013). One MET represents the average oxygen consumption (3.5 ml/kg/min) or energy expenditure (1 kcal/kg/h) while sitting (Ainsworth et al., 1993). Therefore, the measures are interchangeable. For example, the MET value of jogging is 7.0 (Ainsworth et al., 1993), indicating that a person weighing 70 kg will expend approximately 7 MET × 70 kg × 1 h = 490 kcal during an hour-long jog.

Physical activity can be assessed using various methods, including direct, self-reported, and device-based techniques. Direct methods measure AEE. The gold standard techniques for measuring AEE are indirect calorimetry in laboratory settings and DLW in free-living conditions (Strath et al., 2013). However, these methods are costly and require specialized equipment, making them practical for studies with smaller sample sizes. Self-reported methods such as questionnaires, recalls, and diaries are commonly used in epidemiological research (Warren et al., 2010). In particular, questionnaires are frequent because they are quick to fill and easy to analyze into data; however, they suffer from recall and social desirability bias (Strath et al., 2013). Of the device-based methods, accelerometers are the most common because they provide information on all three fundamental physical activity domains and have a low burden for participants (Warren et al., 2010). Accelerometers measure body acceleration and deceleration, typically in units of acceleration because of gravity, which can then be analyzed using various algorithms to determine physical activity volume and intensity (Strath et al., 2013).

The physical activity measures obtained through questionnaires and accelerometers tend to have a low-to-moderate correlation (Prince et al., 2008). This is because the two methods assess different aspects of physical activity. For example, questionnaires are less effective in capturing information on NEAT (Strath et al., 2013), while accelerometers may not accurately measure activities that take place in water or certain types of land-based physical activities, such as climbing, lifting loads, or cycling (Warren et al., 2010). Additionally, accelerometer measurements are often short, and wearing the device may affect physical activity behavior. Although accelerometers are commonly used to estimate TEE, they tend to produce large errors compared with DLW (O'Driscoll et al., 2020).

2.3.2 Physical activity and energy expenditure

Three models (FIGURE 3) have been proposed to explain how REE responds to increases in AEE (Careau et al., 2021). The *additive* model posits that AEE and REE are independent variables, such that an increase in AEE results in a corresponding rise in TEE. The *performance* model suggests that AEE increases REE—and, subsequently, TEE—more than expected because of the increased recovery and maintenance needs. Finally, the *constrained* model predicts that a decrease in REE compensates for an increase in AEE, resulting in TEE remaining unchanged because the energy budget of an organism is limited, and any investment in AEE requires the reallocation of energy from other physiological processes.

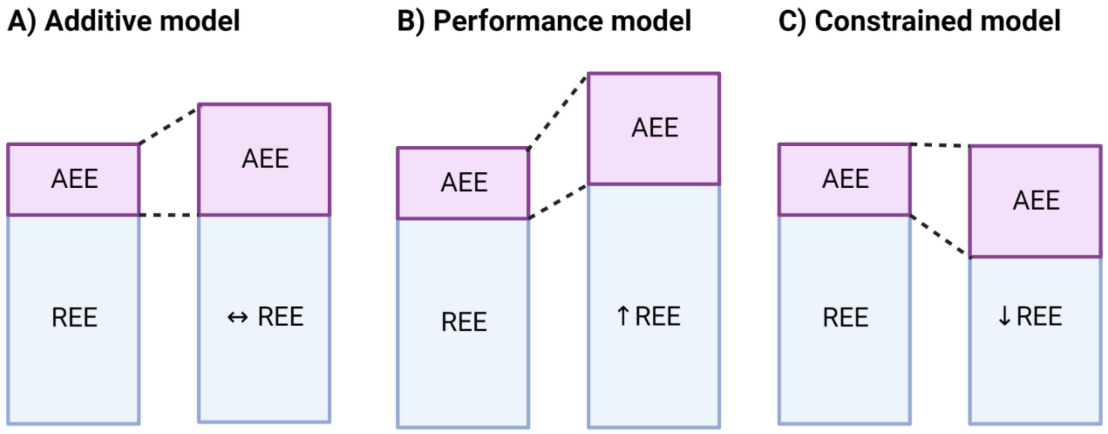

FIGURE 3 The three models explaining the relationship between activity energy expenditure (AEE) and resting energy expenditure (REE). Model A (Additive) assumes REE remains unchanged with increasing AEE, model B (Performance) expects REE to increase, and model C (Constrained) expects REE to decrease with increasing AEE. Figure modified from Careau et al. (2021).

The main argument supporting the constrained model is that, despite having markedly higher physical activity levels, hunter-gatherers and other small-scale populations have similar TEEs to those living in industrialized nations (Gurven et al., 2016; Pontzer et al., 2012). This finding is consistent with the data from other primate species, where animals living in captivity have similar TEE to those living in the wilderness (Pontzer et al., 2014). Energy compensation has also been documented in humans in response to increased AEE when the increase is extreme. An example is the Race Across the USA, a 140-day transcontinental event where participants run marathons on average for 6 days a week. Thurber et al. (2019) found that the postrace TEE of five runners was approximately 596 kcal/d lower than predicted, even though mean body mass and FFM losses were only 3.7 and 0.9 kg, respectively. Pooling of the data from other extreme endurance events with varying durations showed that the estimated physical activity level humans could sustain decreases as a function of time and levels to a maximum of about 2.5 × REE. Moreover, analysis of overfeeding data suggested that the human body's ability to sustain physical activity is limited by the efficiency of the alimentary tract in extracting energy from food. Finally, studies using exercise as a weight loss intervention have consistently reported poorer results than expected based on prescribed AEE, suggesting that some compensatory mechanisms may be at play (Riou et al., 2015). However, when interventions aim for weight loss, it is challenging to differentiate the effects of exercise from the impact of negative energy balance because energy restriction and subsequent tissue mass loss can induce adaptive thermogenesis without added AEE (Nunes et al., 2022). Furthermore, an increase in AEE often causes a compensatory increase in energy intake (Martin et al., 2019), particularly among nonresponders (Herrmann et al., 2015).

The constrained model suggests that, when AEE increases, humans reallocate energy from modifiable components of REE, such as reproductive and

bodily defense functions, to maintain TEE at a homeostatic level (Pontzer, 2018). These trade-offs can explain why moderate exercise volume improves health and overtraining impairs it. Observational studies in industrialized populations have reported mixed findings concerning potential changes in REE. Although the TEE of The Race Across the USA runners declined, their REE (n = 3) remained unchanged (Thurber et al., 2019). However, a recent large cross-sectional study by Careau et al. (2021) supported the constrained model. Among the 1,754 participants, REE was positively associated with TEE (β = 0.72; 95% CI, 0.63–0.82) when adjusted for sex, age, FFM, and FM. The estimate shows that, when TEE increased by one standardized unit in the data, the REE increase was only 72% of that. The < 1 slope indicates a compensatory relationship between AEE and REE. The study also reanalyzed data from 68 individuals aged 70–90 (Cooper et al., 2013), finding that any increases in AEE were offset almost entirely by decreases in REE during a 7-year follow-up period.

There is, however, limited evidence for a decline in REE because of exercise interventions in humans. A seminal study by Westerterp et al. (1992) found some indication of REE decline in previously sedentary participants (n = 23) who trained four times a week for 44 months to complete a half marathon. The participants lost approximately 2 kg of FM and gained 3 kg of FFM during the study. TEE measured by DLW (n = 13) increased from baseline to 8 weeks but remained unchanged throughout the rest of the study, despite a gradual increase in the training volume. SEE measured during a stay in a metabolic chamber declined statistically significantly in the 11 male participants. A later reanalysis of the data reinforced the decline in SEE as a result of increased AEE, incorporating a more advanced modeling strategy that accounted for confounders and both between- and within-individual correlations (Careau, 2017). However, the decline in SEE was modest and unable to fully account for the conservation of TEE. A recent meta-analysis of 18 studies found no evidence of decreased REE after exercise interventions (MacKenzie-Shalders et al., 2020). It should be noted that the studies included in the meta-analysis had a high risk of bias, and the meta-analysis included some miscalculations. Additionally, adjusting the results for body composition changes was impossible because of differences in reporting. Furthermore, REE decline did not occur, even in the two highest-quality trials where exercise was administered to induce weight loss (Martin et al., 2019; Willis et al., 2014).

Another mechanism for TEE constraint is that exercising will decrease NEAT, meaning that the adaptation occurs inside the AEE component. However, current evidence does not support this theory (Halsey, 2021; Melanson, 2017). For example, it is unrealistic that decreased NEAT accounts for the nearly 600 kcal/d TEE compensation, as indicated in the study by Thurber et al. (2019). New theories for energy conservation mechanisms have now emerged, such as reduced circadian variation of REE and decreased mitochondrial proton leak (Halsey, 2021). A study by Urlacher et al. (2019) compared energy expenditures between Shuar children and children from the United States and the United Kingdom. Despite having a 25% higher accelerometer-measured physical

activity level and 20% greater REE, Shuar children had a similar TEE to their industrialized counterparts. The elevated REE can be explained by the fact that the Shuar are Amazonian forager-horticulturalists with elevated immune system activation because of high parasite burden. A modeling exercise found that TEE similarity was because of higher AEE in the industrialized children, resulting from higher body mass and poorer movement efficiency and lower circadian variation of REE in the Shuar children. However, an 8% discrepancy remained, which the authors suggested may be because of higher proton leak or thermoregulatory needs in industrialized children.

2.3.3 Physical activity and fat oxidation

Resting fat oxidation. When energy balance is maintained, acute exercise does not increase 24-hour fat oxidation (Melanson et al., 2009). Additionally, long-term exercise training has been found to not elevate RFO (Martin et al., 2019; Scharhag-Rosenberger et al., 2010). These findings align with the laws of energy conservation and mass (Péronnet & Haman, 2019). However, some studies have reported an increase in RFO the morning after a single exercise session (Kang et al., 2020) or after exercise intervention compared with nonexercising controls (Amaro-Gahete, Acosta, et al., 2020). These findings were likely confounded by changes in energy balance.

Peak fat oxidation. It is reasonable to assume that engaging in physical activity will increase PFO because PFO is closely related to cardiorespiratory fitness that is well-known to respond to it. Two large-scale cross-sectional studies identified self-reported physical activity as a PFO determinant (G. Fletcher et al., 2017; M. C. Venables et al., 2005), while one did not (Chrzanowski-Smith, Edinburgh, Thomas, et al., 2021). The differing results may be because of the different covariates used in the studies. A limitation of the PFO explanatory models used in studies is that they include physical activity (exposure) and $\dot{V}O_{2MAX}$ (partial mediator) as covariates. Therefore, it is not surprising that the association between physical activity and PFO would diminish when adjusting for $\dot{V}O_{2MAX}$ (Amaro-Gahete, Acosta, et al., 2020).

Most intervention studies on PFO have used single-group designs or compared two exercise interventions without a nonexercising control group (Maunder et al., 2018). The longest single-group study (Scharhag-Rosenberger et al., 2010) found that the PFO of 17 untrained men and women increased by 0.07 g/min after 12 months of self-monitored walking/jogging. The statistical significance remained even when using PFO scaled to FFM as the outcome. However, the results from RCTs comparing exercise and control interventions have been mixed. Two 12-week studies conducted at the University of Copenhagen have investigated the effects of exercise on PFO in sedentary adult men (n = 50 and 44) with overweight, here showing positive results. The first study (Rosenkilde et al., 2015) randomized participants into groups that engaged in daily endurance training (300 or 600 kcal/d) or a control group. Both training groups demonstrated improved PFO compared with the control group. The second study (Nordby et al., 2015) combined the more intensive endurance

training protocol (600 kcal/d) with no dietary intervention, an energy-restricted diet, or an energy-increased diet before then comparing the results to a control group. PFO improved compared with the control group only in the group without any dietary modification. When summarized, the 600 kcal/day exercise-only intervention lasting for 3 months improved PFO by 0.12 and 0.16 g/min and FFM-scaled PFO by 1.8 and 2.2 mg/kg FFM/min. Another 12-week study (Amaro-Gahete, De-la-O, et al., 2020) examined the effects of lower-dose exercise in 89 sedentary middle-aged men and women. The three studied interventions included a training regime dosed according to physical activity recommendations (150 min endurance exercise and 60 min resistance training per week) or HIIT training without or with electrical stimulation twice a week. None of the interventions resulted in an improvement in LM-scaled PFO compared with the control group. Overall, these RCT findings have suggested that even sedentary individuals would need to significantly increase their exercise volume to see improvements in PFO.

2.3.4 Physical activity and serum metabolome

The studies forming the current PhD thesis did not directly examine the associations between physical activity and the serum metabolome; however, an understanding the nature of this relationship is essential for comprehending the adaptability of the circulating metabolome. In a seminal study, Kujala et al. (2013) applied the same NMR technology as in the present thesis to compare serum metabolites between 16 same-sex twins (age range 50–74 years) who had over 30-year physical activity discordance and 1,037 age- and sex-matched individuals (age range 30–62 years) with at least a 5-year physical activity difference. The results showed that physical activity was associated with a distinctly opposite serum metabolite profile compared with the changes related to metabolic deterioration, as shown in FIGURE 1. The participants who were more physically active had lower levels of apoB-containing particles in virtually all subclasses than their less active counterparts. They also had smaller average VLDL and LDL particle sizes and lower VLDL triglyceride and LDL cholesterol concentrations. Additionally, their average HDL size was larger, HDL cholesterol concentration was higher, and their serum fatty acid profile was more favorable, with higher levels of PUFAs and lower levels of MUFAs and SFAs. Physical activity was also associated with lower glucose, isoleucine, phenylalanine, and GlycA concentrations, as well as higher acetoacetate concentrations.

Subsequent studies have revealed the potential mediators between physical activity and serum metabolite profile. Kujala et al. (2019) found that the profile differences of young men with high and low cardiorespiratory fitness were similar to the differences observed between more and less physically active individuals, as expected because of the bidirectional relationship and shared genetic factors between physical activity and cardiorespiratory fitness (Mustelin et al., 2011). However, adjusting for body fat percentage weakened the associations between cardiorespiratory fitness and most metabolite levels (Kujala et al., 2019), suggesting that body composition plays a significant role as a

mediator. A more recent study (Lehtovirta et al., 2022) observed similar associations between physical activity and serum metabolome in adolescents and young adults, finding an inverse association between physical activity and fasting insulin, here with a larger association size compared with any of the NMR-measured metabolites. The study did not perform mediation analyses, but the findings implied that insulin sensitivity may also mediate the associations between physical activity and metabolite levels.

It is important to note that observational studies may be limited by unmeasured and residual confounding that could partly explain the results. However, studies using both device-measured and self-reported physical activity have shown consistent results, though the associations between device-measured physical activity and serum metabolites are smaller and less certain (P. R. Jones et al., 2022; Vaara et al., 2022). Intervention studies have also provided consistent results, with exercise training leading to decreases in triglycerides and increases in HDL cholesterol levels, while LDL cholesterol levels tend to respond less (Durstine et al., 2001; Sarzynski et al., 2022). Exercise also leads to decreases in large VLDL particles and increases in large LDL and HDL particles, resulting in improvements to their size profile (Sarzynski et al., 2015). In summary, observational and intervention studies have provided consistent results, highlighting the importance of physical activity for maintaining a favorable serum metabolite profile.

2.4 Fat oxidation and blood glucose regulation

An estimated 537 million people globally have diabetes, primarily T2D, a number projected to increase 46% by 2045. This trend in T2D prevalence is linked to modern environmental factors such as dietary changes, sedentary behavior, and obesity (Sun et al., 2022). In contrast, T2D, elevated fasting glucose levels, and obesity are extremely rare among hunter-gatherers and other small-scale cultures living in traditional or preindustrial environments (Eaton et al., 1988; Pontzer et al., 2018). This highlights the causal role of long-term energy balance in the development of T2D, as demonstrated by the ability of energy restriction and subsequent weight loss to reverse the condition (Churuangsuk et al., 2022).

T2D develops when the pancreas no longer produces sufficient amounts of insulin to counteract the inability of other tissues to respond to its stimulation, resulting in hyperglycemia. The inability of the body's cells to respond normally to insulin stimulation is referred to as insulin resistance and is considered a primary event in the development of T2D, which often progresses for decades before disease onset (Roden & Shulman, 2019). Insulin resistance also increases the risk of other chronic diseases, such as CVD (Paneni et al., 2013), nonalcoholic liver disease (Loomba et al., 2021), several cancers (Gallagher & LeRoith, 2015), and Alzheimer's disease (Candeias et al., 2012). Although the dysregulation of glucose metabolism is a defining feature of insulin resistance, it is essential to recognize how lipid metabolism plays an equally vital role in this complex

condition (Roden & Shulman, 2019). In light of the topic of the current thesis, understanding these interconnections is essential.

2.4.1 Insulin resistance pathogenesis

The mechanisms underlying the development of insulin resistance remain unclear. Nevertheless, two of the most widely accepted theories propose that insulin resistance is connected to mitochondrial function either through reactive lipid metabolites or reactive oxygen species (Frangos et al., 2021). A review (Roden & Shulman, 2019) provided an integrative explanation for the development of insulin resistance in humans, here based on the reactive lipid theory. According to the theory, a sustained positive energy balance leads to the expansion of adipose tissue, eventually overwhelming its capacity to store fat and causing hypoxia, which then triggers apoptosis and cell death. The infiltration of macrophages into the adipose tissue causes localized inflammation, leading to increased lipolysis and the eventual spreading to low-grade systemic inflammation. The accumulation of reactive lipids may also disrupt insulin signaling, further increasing lipolysis (Lyu et al., 2021). As a result, lipids begin to spillover to other organs, such as the liver and skeletal muscle. In the liver, the excess of NEFA and glycerol stimulates the synthesis of triglycerides and gluconeogenesis. When mitochondrial function is insufficient, blood glucose levels rise, triglycerides accumulate, and the production of reactive lipids further impairs insulin signaling. In the skeletal muscle, the increased availability of NEFA leads to the accumulation of lipids and the formation of reactive lipids similar to the liver. The subsequent loss of insulin sensitivity lowers glucose uptake and glycogen storing, further increasing circulating glucose levels because skeletal muscle is the primary glucose sink in the body. The pancreas initially compensates for these alterations by increasing insulin secretion, hence maintaining normal blood glucose levels. However, as the disease state progresses and the pancreas's ability to secrete insulin declines, T2D develops, resulting in hyperglycemia.

In this theory, the central hypothesis is that insulin signaling is disturbed by the accumulation of lipotoxic metabolites such as diglycerides and ceramides (Sangwung et al., 2020). Triglyceride storage alone is not problematic because research has shown that endurance athletes with high insulin sensitivity have similar levels of intramyocellular triglycerides as those with T2D (Goodpaster et al., 2001). Of particular concern is the accumulation of sn-1,2 diglycerides in the plasma membrane, which can activate novel forms of protein kinase Cs, which, in turn, inhibit the insulin signaling cascade both in skeletal muscle and liver (Sangwung et al., 2020) and perhaps also in the white adipose tissue (Lyu et al., 2021). However, it is worth mentioning that most of the literature is centered on researching the phenomenon in skeletal muscle, including the evidence presented below.

The second key point of the theory is that inherited or acquired mitochondrial dysfunction may predispose individuals to the accumulation of reactive lipids and insulin resistance (Sangwung et al., 2020). Initially, the defect

was believed to lay specifically in the fatty acid oxidation pathway (Lowell & Shulman, 2005). In a pioneering study, through the use of limb indirect calorimetry, Kelley and Simoneau (1994) discovered that individuals with non-insulin-dependent T2D had higher fasting RER compared with age and BMI-matched controls, and their RER did not increase similarly after a test meal, indicating defects in fat oxidation ability, insulin sensitivity, and substrate-switching in leg muscles. Later, the findings were repeated by comparing individuals with normal weight and individuals with obesity (Kelley et al., 1999). The study also included a 4-month weight loss intervention, which improved insulin sensitivity but showed no change in fasting RER, leading to the idea that poor fat oxidation capacity is the primary cause predisposing an individual to insulin resistance and obesity. The concept has also been extensively studied in rodents using high-fat-feeding but with conflicting results (Frangos et al., 2021; Muoio & Neufer, 2012).

The reactive lipid theory has recently dissociated itself from the concept that explicitly poor mitochondrial fat oxidation capacity leads to insulin resistance. One study (Song et al., 2020) employed advanced tracer experiments to examine the relationship between skeletal muscle mitochondrial substrate use and insulin sensitivity in both mice and humans. The findings revealed no discernible difference in the energy substrate preference or ability to use fatty acids between insulin-resistant and insulin-sensitive muscles in a fasted state. However, insulin-resistant muscles demonstrated a decreased ability to increase glucose use in response to insulin stimulation compared with insulin-sensitive muscles. This suggests that poor fat oxidation capacity does not cause insulin resistance but that insulin resistance can impair metabolic flexibility, i.e., the ability to adapt to changing substrate availability or energy demands (Goodpaster & Sparks, 2017). Furthermore, research has indicated that increasing flux through beta-oxidation may even promote insulin resistance (C. D. Smith et al., 2021). Thereby, the theory now suggests that a low overall mitochondrial capacity, rather than an inability to oxidize fatty acids, may contribute to insulin resistance in some instances (Sangwung et al., 2020; Song et al., 2020).

The reactive oxygen species theory has been based on mitochondrial bioenergetics and posits that the balance between energy supply and demand is the crucial factor through the modulation of redox stress in determining insulin sensitivity. As reviewed by Fisher-Wellman and Neufer (2012), the transport of electrons through the electron transport chain is an automatic process tightly coupled with proton pumping. The rate at which the system operates is determined by the energy expenditure (i.e., *pull*) rather than the energy supply (i.e., *push*). When the *push* exceeds the *pull*, the mitochondria become overpressurized as protons accumulate on the outer surface of the inner membrane. This creates a back pressure that slows proton pumping, in turn slowing electron transport and oxygen consumption. To alleviate this pressure, electrons leak from the chain to oxygen, increasing the emission of reactive oxygen species, particularly hydrogen peroxide. The production of reactive species is particularly elevated when the push is generated by beta-oxidation.

Subsequent sift in the cellular redox environment modulates the function of metabolic enzymes and other proteins, leading to reduced sensitivity for insulin stimulation. The theory argues against the idea that mitochondrial dysfunction causes insulin resistance because mitochondrial function, or the *pull*, is rarely at its maximum during everyday living (Muoio & Neufer, 2012). However, the theory supports the idea that mitochondrial function can deteriorate when the disease state progresses, hence limiting exercise performance (Muoio & Neufer, 2012).

Reconciling the two theories of insulin resistance can be a complex task, but one thing is clear—both views agree that a sustained excess energy supply with high-lipid cellular environment in oxidative tissues leads to trouble (Fisher-Wellman & Neufer, 2012; Roden & Shulman, 2019). They also agree that poor fat oxidation capacity or metabolic inflexibility is not the root cause of insulin resistance (Muoio & Neufer, 2012; Song et al., 2020). However, where both theories differ is in identifying the primary mechanism that disrupts insulin signaling and whether intrinsic mitochondrial characteristics play a role in predisposition to insulin resistance. Ultimately, it is likely that both mechanisms contribute to insulin resistance with varying proportions in different scenarios (Frangos et al., 2021). Notably, both theories recognize the efficacy of E2 signaling and exercise in promoting insulin sensitivity by relieving lipid-induced (J. P. Camporez et al., 2019; J. P. G. Camporez et al., 2013; Sangwung et al., 2020) and redox stress (Muoio & Neufer, 2012; Torres, Kew, et al., 2018; Torres, Ryan, et al., 2018).

2.4.2 Connections between whole-body fat oxidation and blood glucose regulation

When I first began working on this PhD project, I believed that RFO could explain the metabolic health differences between individuals because this was a commonly held assumption in research. However, after reading the comprehensive review by Péronnet and Haman (2019), my perspective shifted. As previously discussed, fasting substrate use is primarily dependent on energy balance and diet macronutrient distribution (Goris & Westerterp, 2000; Miles-Chan et al., 2015) and does not accurately reflect maximal fat oxidation capacity (Holloszy, 2009) or serve as a causal factor in the development of insulin resistance (Muoio & Neufer, 2012; Song et al., 2020). However, insulin resistance and other consequences of myocellular lipid overload may damage mitochondrial function (Cheng et al., 2010; Ruegsegger et al., 2018), which may become evident in particular during exercise (Muoio & Neufer, 2012). Therefore, PFO has more rationale as a marker of the ability to regulate blood glucose levels than RFO.

Most studies investigating the associations between fat oxidation and blood glucose regulation have used fasting glucose- and insulin-derived surrogates as outcomes, such as the homeostatic model of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) indexes. These indexes primarily reflect hepatic rather than peripheral insulin resistance (Borai et al.,

2011). Although the rationale for using RFO as a metabolic health marker is not convincing, Amaro-Gahete, Jurado-Fasoli, et al. (2020) found that higher RFO was associated with a lower HOMA-IR in 71 middle-aged men and women. However, it is important to consider that, as fasting duration increases, whole-body fat oxidation increases and insulin concentrations decline (Horton & Hill, 2001), here following changes in energy availability. Therefore, energy balance is the likely confounder behind the observed association. In a later study, exercise test data from the same middle-aged participants were pooled with data from 119 younger adults (Amaro-Gahete et al., 2021). The results showed that PFO was not associated with fasting glucose, insulin, or HOMA-IR, contrary to the findings of Robinson et al. (2015, 2016), who were the first to show that PFO was positively correlated with the QUICKI index and negatively with fasting insulin in 57 young men. However, the correlations found by Robinson et al. (2015, 2016) were small (0.33 for QUICKI and -0.29 for fasting insulin) and not adjusted for LM.

Compared with fasting indexes, the oral glucose tolerance test (OGTT) provides a more dynamic reflection of glycemic control, and insulin sensitivity surrogates derived from the OGTT are typically better correlated with the direct measure of insulin resistance, that is, the hyperinsulinemic-euglycemic clamp (Borai et al., 2011). Before the current thesis, the relationship between RFO and OGTT responses had been examined by only one study, likely because of the more time-consuming nature of the OGTT. Kunz et al. (2000) found that, in 166 participants with hypertension and obesity, FM, glucose area under the curve (AUC), and female sex were positively associated with RFO and explained 40% of its variation. These findings challenge the idea that RFO is a marker of improved glycemic control. Furthermore, no study had previously investigated the associations between PFO and OGTT outcomes, highlighting the need for further research.

3 PURPOSE OF THE STUDY

The primary aim of the present PhD thesis was to investigate the associations between menopause, physical activity, and whole-body metabolism, here with a particular focus on REE, fat oxidation at rest and during exercise, and serum metabolome. Additionally, as a secondary aim, the current thesis examined whether differences in RFO and PFO could explain the variations in the ability to regulate blood glucose after glucose ingestion. These research aims are illustrated in FIGURE 4.

To achieve these objectives, the following research questions were formulated:

1. Is menopausal transition associated with changes in serum metabolome, and are these changes explained by changes in serum E2 and FSH concentrations? (*Study I*)

2. Do REE (*Study II*), RFO, and PFO (*Study IV*) vary according to menopausal status, and are serum E2 and FSH concentrations associated with these outcomes in middle-aged women?

3. Are there differences in REE, RFO, and PFO among male MZ cotwins who have long-term discordant leisure-time physical activity levels (*Study III*), and is physical activity level associated with RFO and PFO (*Studies III* and *IV*)?

4. Are RFO and PFO associated with proxies of glycemic control and insulin sensitivity? (*Studies III* and *IV*)

4 MATERIALS AND METHODS

To achieve the aims of the present PhD thesis, four studies were conducted using a combination of longitudinal, cross-sectional, and cotwin control study designs (FIGURE 4). The participants were women in *Studies I*, *II*, and *IV* and men in *Study III*. The primary outcomes were assessed using NMR metabolomics in *Study I* and indirect calorimetry in *Studies II–IV*. Menopause was the main exposure studied in *Studies I*, *II*, and *IV*, while *Study III* enabled more detailed examination of associations related to physical activity The secondary aim of the present thesis—the relationship between fat oxidation and blood glucose regulation—was examined in *Studies III* and *IV*. Including data from both sexes was chosen to enhance the generalizability of the findings.

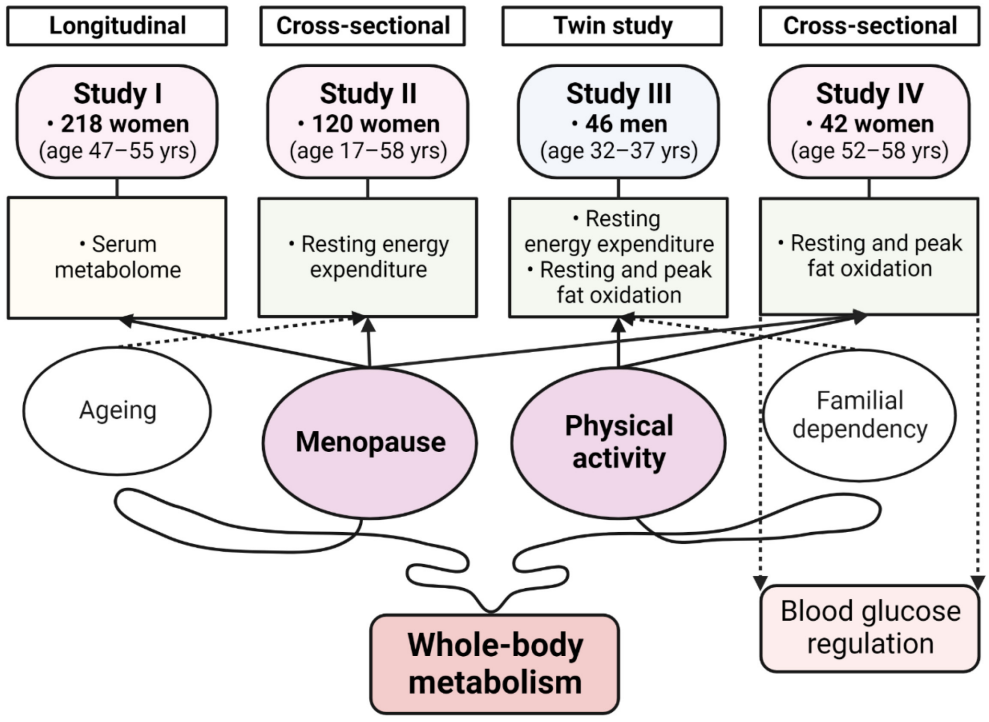

FIGURE 4 Key characteristics of the studies in this PhD thesis.

4.1 Study designs and participants

4.1.1 Study I

Study I (FIGURE 5) employed data and samples from 218 women in the longitudinal core-ERMA (Estrogenic Regulation of Muscle Apoptosis) study (Kovanen et al., 2018) collected between 2015 and 2019. The primary aim of *Study I* was to investigate whether menopausal transition alters the circulating metabolome and to explore whether changes in E2 and FSH levels may explain the observed changes in metabolites.

The ERMA cohort was established by randomly selecting 47–55-year-old women from the Jyväskylä area from the Population Information System (n = 6,878) and sending them a study invitation and a prequestionnaire. Of the women, 3,229 (47%) responded, and 1,627 were called for menopausal status determination. Women with a self-reported BMI of over 35 kg/m^2 or a condition or medication affecting the ovaries, the immune system, or daily functioning were excluded. Finally, the menopausal status of 1,393 women was determined, and 1,158 participated in ERMA laboratory measurements. The perimenopausal women were invited to participate in the longitudinal phase of the study, where they were followed to early postmenopause. The aim was to gain an understanding of what occurs in women's physiology during the phase of the most significant hormonal changes. The menopausal transition was monitored with menstrual diaries and FSH measurements every 3 to 6 months, depending on the participant's menopause progression.

Of the 381 women who began the follow-up, 234 reached early postmenopause during the study. One participant died, and 26 dropped out. Additionally, some did not reach postmenopause (n = 69), or their menopausal status remained unclear at the last measurements (n = 48). For the purpose of *Study I*, women who had received a cancer diagnosis (n = 4) or were using lipid- or glucose-lowering medication (n = 12) were also excluded, resulting in a final sample size of 218 participants. Of these women, 35 (15%) began using MHT during the follow-up. This ratio is consistent with the general Finnish population: in 2020, 12% of Finnish women aged 45 years or older were MHT users (Finnish Medicines Agency Fimea, 2021). Those participants who began MHT participated in the final measurements after 6 months of treatment. Based on questionnaire responses, one participant had started MHT for 17 days, and another participant 2 days before the last measurement. However, only the first woman was classified as an MHT starter because the two-day use of low-dose transdermal MHT by the latter participant was unlikely to have affected the studied parameters. This assumption was confirmed by comparing her metabolite concentrations with women who refrained from MHT. Of the 35 MHT starters, 27 used oral products containing E2 only (n = 7) or E2 combined with a progestogen (n = 20, including 16 dydrogesterone and four norethisterone acetate users). The remaining MHT starters used E2 only (n = 7) or E2 and norethisterone acetate-containing patches (n = 1).

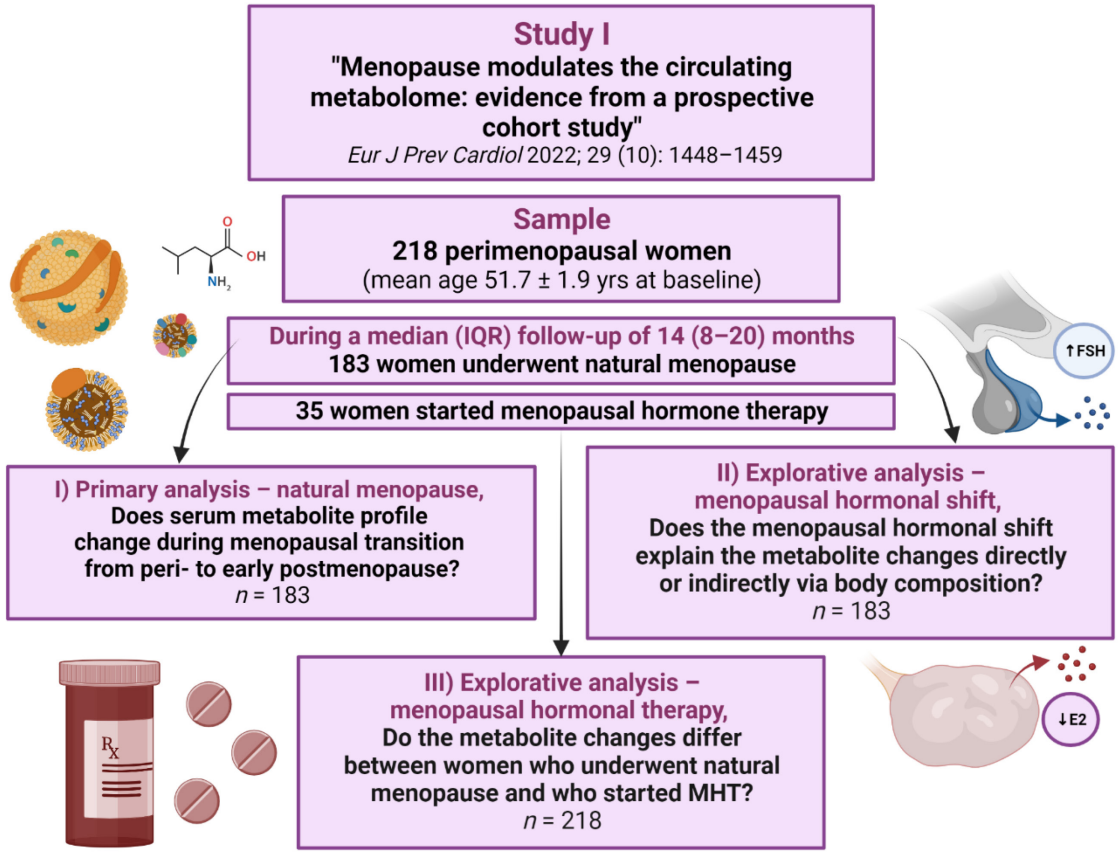

FIGURE 5 The aims of *Study I* and sample sizes in statistical analyses. E2, estradiol; FSH, follicle-stimulating hormone; MHT, menopausal hormone therapy.

4.1.2 Study II

Study II (FIGURE 6) aimed to investigate the associations between age and menopausal status on REE by using a cross-sectional sample of 120 participants, including 59 middle-aged women. The participants were from four studies conducted between 2008 and 2022 at the Faculty of Sport and Health Sciences at the University of Jyväskylä: EsmiRs (Estrogen and microRNAs as Modulators of Women's Metabolism), Calex, Physique, and Athletic Performance and Nutrition (NO-REDS). All participants were required to be free of any known diseases or medications that may affect energy expenditure, but the use of hormonal contraception and MHT were allowed. The participants were categorized into three age groups, and middle-aged women were further divided into menopause groups: premenopausal or perimenopausal (PRE/PERI), postmenopausal (POST), and postmenopausal MHT-using women (MHT). In a sensitivity analysis, perimenopausal women were excluded from the data.

The EsmiRs study is described in detail in section 4.1.4 of *Study IV*. The Calex study (Ojanen et al., 2021) aimed to examine how lifestyle factors influenced functional gene and protein expression of muscle and adipose tissue by recruiting mother-father-daughter families with varying adiposity levels. The measurements were performed between 2008 and 2011. From this study, REE

data from 17 middle-aged (ages 42–58 years) and 21 younger women (ages 17–21 years) were used. These participants formed a subsample of 16 mother–daughter pairs, where the analyses were partially controlled for genotype. Ten pairs also had TEE measured with the DLW method. The Physique study (Isola et al., 2023) aimed to investigate how weight reduction for a bodybuilding competition influenced the physiology of normal-weighed men and women. *Study II* used REE data from 23 women (ages 22–38 years) collected in 2019 prior to the weight loss. The Athletic Performance and Nutrition study is an ongoing project designed to assess the physiological adaptations of elite winter sports athletes throughout consecutive training and competition seasons. *Study II* used REE data from 17 women (ages 18–35 years) collected in 2021 during a transition season when the athletes' training volume was at its lowest.

In all studies, REE measurements were performed with the same metabolic cart in the same thermoneutral laboratory. There were slight variations in the measurement protocols, as detailed in section 4.2.5. The participants were required to fast overnight and avoid physical activity at least the day before measurements.

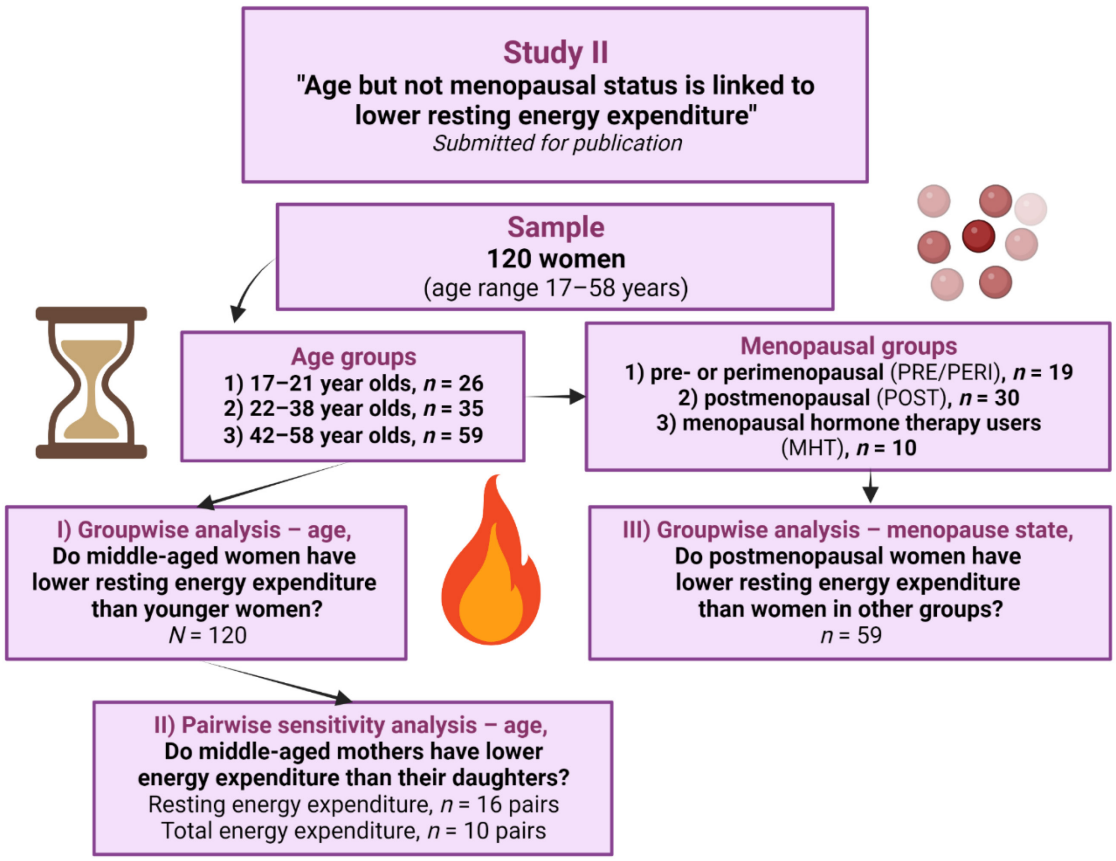

FIGURE 6 The aims of *Study II* and sample sizes in statistical analyses.

4.1.3 Study III

Study III (FIGURE 7) used data from the FITFATTWIN study, which were collected between 2011 and 2012 (Rottensteiner et al., 2015). The primary aim of *Study III* was to compare REE, RFO, and PFO between members of male MZ twin pairs who exhibited marked differences in leisure-time physical activity over the preceding 3 years. Additionally, the twin design allowed for the examination of familial dependence on the outcomes. However, direct heritability estimates could not be calculated because the sample did not include dizygotic twin pairs. *Study III* also investigated the associations between fat oxidation outcomes and blood glucose regulation during an OGTT.

The 46 participants were recruited from the 202 MZ twin pairs of the Finntwin16 cohort. Through responses in the fifth Finntwin16 data collection wave questionnaire, cotwins of 39 pairs were identified as potentially discordant for leisure-time physical activity. Following a telephone interview, 20 twin pairs were invited to the FITFATTWIN study, and 17 accepted the invitation. Six additional twin pairs were recruited from the cohort, knowing their members were not discordant for leisure-time physical activity. After a detailed physical activity history characterization during the laboratory measurements (explained in section 4.2.2), the cotwins of 10 pairs were classified as discordant for leisure-time physical activity over the past 3 years. These cotwins exhibited a minimum difference of 1 MET-h/d in the past 3-year short MET index and 1.5 MET-h/d difference in the past 12-month MET index. Additionally, the less active cotwin had a less than 5 MET-h/d in the 12-month MET index, and the active cotwin had a higher Baecke questionnaire sports score than the other cotwin.

The metabolism measurements were conducted over the course of 2 consecutive days. On the first day, exercise metabolism tests were performed at noon. The participants were instructed to abstain from vigorous exercise and alcohol consumption for 48 hours before the measurements and refrain from eating for 2 hours before testing. PFO was measured from 41 twin individuals; however, five participants could not participate in testing because of medical reasons. On the second day, body composition and resting metabolism measurements were performed in an overnight fasted state. To minimize any potential impact of physical activity on the results, the participants were transported to the laboratory via car. Resting metabolism measurements were successfully conducted on 43 twin individuals. However, one twin pair declined participation, and data from one participant were excluded because of hyperventilation. After the resting metabolism measurement, OGTT was performed. One twin pair did not participate in the OGTT.

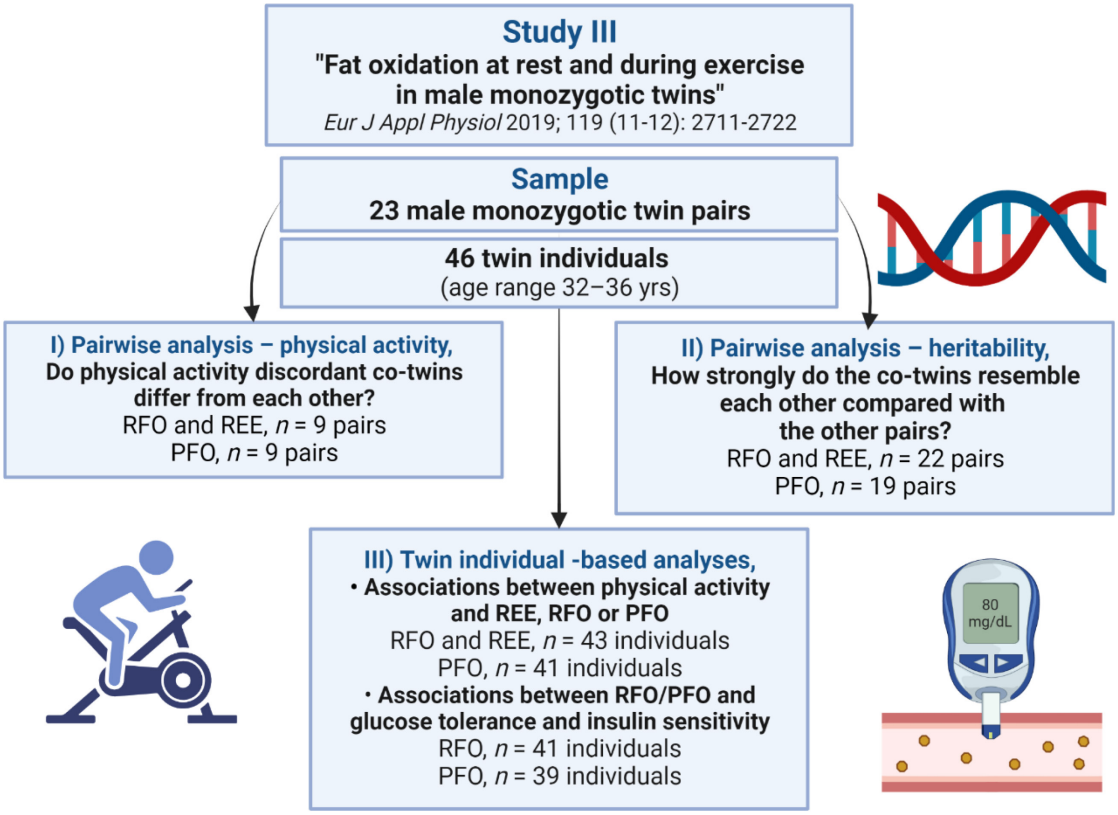

FIGURE 7 The aims of *Study III* and sample sizes in statistical analyses. PFO, peak fat oxidation; REE, resting energy expenditure; RFO, resting fat oxidation.

4.1.4 Study IV

Study IV (FIGURE 8) was conducted as a part of the EsmiRs study (Hyvärinen et al., 2021), which represents the fourth-year follow-up of the ERMA cohort. Data were collected during the years 2019 and 2020. The primary aim of *Study IV* was to compare RFO and PFO across women of the same age who differed in menopausal status. The study also aimed to examine whether circulating levels of E2 and FSH could explain the differences in fat oxidation outcomes. The groups being compared were the same as in *Study II*. The premise of the study was that the postmenopausal group would exhibit a lower PFO than the other groups owing to lower E2 levels. Additionally, it was hypothesized that E2 levels would positively associate with PFO. Like in *Study III*, a secondary aim was to investigate the associations between fat oxidation measures and blood glucose regulation during an OGTT.

The target sample size was calculated using the data from a previously described study (Abildgaard et al., 2013). The mean difference between premenopausal and postmenopausal participants in fat oxidation was 1.67 (pooled SD = 1.40) mg/kg LM/min. The effect size, as measured by Cohen's *d*, was 1.19. It is important to note that this effect size is large for a biological phenomenon. A power calculation showed that a minimum of 12 participants per group would be needed to detect a statistically significant between-group

difference with an alpha level of 0.05 and 80% power. Considering potential dropouts and measurement difficulties, the recruitment goal was set at 20 women per group.

The sample for *Study IV* was recruited from the 304 women who participated in the laboratory measurements of the EsmiRs study. The study nurse was responsible for determining the participation eligibility and initial group allocations. The eligibility criteria were as follows: 1) BMI of ≥18 and <30 kg/m^2, 2) intact ovaries and uterus, 3) absence of any diseases or medication known to affect metabolism or exercise responses, 4) no hormonal contraception, and 5) no regular smoking. Because of the premature ending of recruitment as a result of the COVID-19 pandemic, the final sample consisted of 13 participants in the PRE/PERI group, 20 participants in the POST group, and nine participants in the MHT group. After the final menopausal status determination, six participants from the PRE/PERI group were allocated to the POST group. Four participants from the POST group had E2 concentrations that were over four times higher (0.27–0.54 nmol/l) than the group's third quartile (0.09 nmol/l) and, therefore, were excluded from group comparisons owing to the study's premise. All MHT-using participants used a combination MHT containing E2 and progestin. Seven used oral and two transdermal preparation. In an exploratory analysis, the PRE/PERI and MHT groups were combined as a HIGH E2 group and compared against the POST group.

The metabolism measurements were performed on two visits, which were separated by a median of 2 weeks (interquartile range 1–3 weeks) for most participants, but for five participants, the time between visits was longer (13–18 weeks) because of metabolic cart malfunction or COVID-19 restrictions. During the first visit, the women participated in resting metabolism and OGTT measurements. They also underwent a comprehensive medical examination to ensure safe exercise metabolism testing on the second visit. The participants were advised to avoid any lifestyle changes throughout the study. They kept food diaries for 2 days before the measurements and matched their diets for the preceding 24 hours. They also refrained from alcohol and caffeine use for 24 hours and 12 hours before measurements, respectively. The participants ate their last meal on the previous evening between 8 PM and 10 PM and drank 100 ml of water after awakening. Regarding physical activity, the participants were instructed to avoid moderate-to-vigorous physical activity for 48 hours before measurements, limit physical activity on the measurement morning, and arrive at the laboratory by car.

Valid RFO measurements were obtained from 39 participants. However, data from three women—two from PRE/PERI group and one from the POST group—were excluded from *Study IV* because of potential slight hyperventilation. The PFO measurements of 40 participants were successful. However, data from two MHT group women were excluded because of metabolic cart failure or difficulty in wearing the gas mask. OGTT data were complete for 35 participants. Data from at least three sampling points were obtained from three participants, while three participants provided only fasting and 120-minute samples.

Unfortunately, one participant could not give the final blood sample, and her data were not included in the statistical analyses.

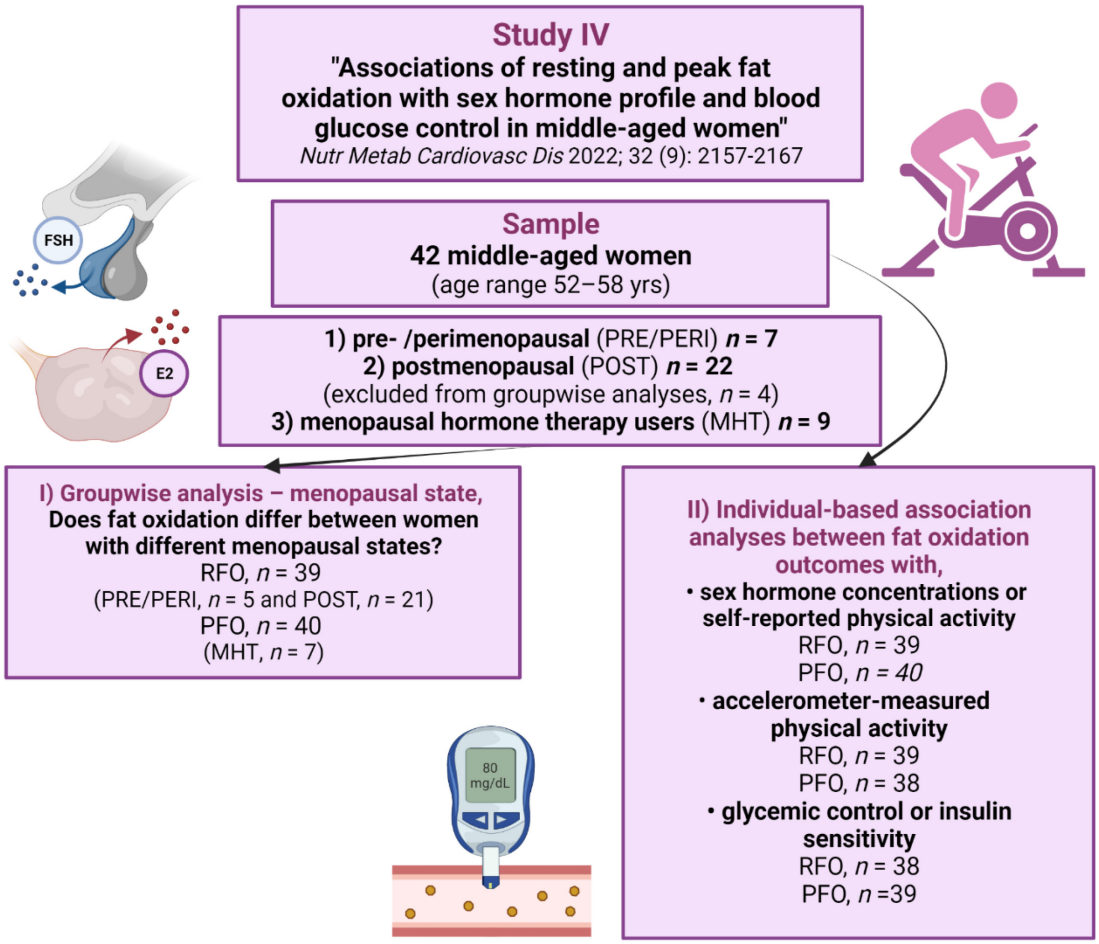

FIGURE 8 The aims of *Study IV* and sample sizes in statistical analyses. PFO, peak fat oxidation; RFO, resting fat oxidation.

4.2 Assessment of exposures

4.2.1 Menopause status and sex hormone levels

Menopausal status. Menopausal status was determined using the Stages of Reproductive Aging Workshop + 10 guidelines (Harlow et al., 2012)—with some exceptions—and menstruation and FSH data. The different studies employed slightly different methods in menopausal status assessment.

Study I. During the initial menopausal status determination, the participants kept menstrual diaries for a minimum of 3 months, and perimenopausal women were identified as those with irregular or absent menstrual bleeding and FSH levels between 17 and 30 IU/l. During follow-up, early postmenopause was determined by 6 months of amenorrhea and two

consecutive FSH measurements above 30 IU/l. Postmenopause in participants with hysterectomy (n = 11) or uncertain bleeding reports (n = 14) was determined by FSH measurements alone. The amenorrhea criterion was shortened from 12 months to optimize the number of women who completed the study within funding constraints. The median amenorrhea duration was 8.1 months (interquartile range 6.5–10.4). However, 28 participants did not meet the 6-month amenorrhea criterion, and sensitivity analyses were performed without these women to account for the protocol deviation.

Study II. In the Calex study questionnaire, the participants answered how many menstrual periods they had during the past year and the time from their last menses. This information was supplemented with one FSH measurement to determine their menopausal status. The EsmiRs women were categorized as explained below.

Study IV. During the EsmiRs study recruitment, allocation into the study groups was based on self-assessment. In the study questionnaire, the participants estimated their menopausal status using a short description of menopause-related menstrual changes. They also wrote down the time of their last menses. In the final menopausal status determination, the questionnaire responses were supplemented with FSH measurements from both metabolism measurement visits.

Menopausal hormone therapy. In the ERMA (*Study I*) and EsmiRs studies (*Studies II* and *IV*), MHT use was determined through a questionnaire in which the participants reported the MHT products they had used in their lifetime, including the start and end dates of use. In the Calex study (*Study II*), medication use was queried as a part of the study questionnaire.

Serum sex hormone concentrations. Measurements were performed using IMMULITE 2000 XPi (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA) from fasted serum samples stored at -80 °C. However, accurately measuring low concentrations of sex steroids with immunoassay technologies can be challenging (Rosner et al., 2013). The CV for the used E2 immunoassay kit was 9.7%, and the limit of detection was 0.055 nmol/l. The immunoassay method used in our laboratory was compared against liquid chromatography-mass spectrometry using 166 samples and had a reasonably good agreement (r = 0.91). However, the agreement was poor (r = 0.42) in 76 samples, with E2 concentrations less than 100 pmol/l, here as measured with liquid chromatography-mass spectrometry.

4.2.2 Physical activity

Self-reported physical activity. In *Study III*, physical activity was assessed using a combination of a short and long interview and the Baecke questionnaire (Baecke et al., 1982). The short interview was used to evaluate past 3-year leisure-time physical activity at 1-year intervals. The interview followed a 4-item questionnaire (Kujala et al., 1998) that was also used in *Studies I* and *IV* to assess the participants' current physical activity. The first question addressed the frequency of monthly leisure-time physical activity, and the options ranged from less than once per month to over 20 times per month. The second question

focused on the intensity of the physical activity by asking whether it typically resembled walking, walking with periodical jogging, jogging, or running. The answers to this question were assigned a MET value, with walking being assigned 4 METs, walking with periodical jogging assigned 6 METs, jogging assigned 8 METs, and running assigned 13 METs (Ainsworth et al., 1993). The third question addressed the average duration of the physical activity, with options ranging from under 15 minutes to over 2 hours. The leisure-time physical activity volume was then calculated using the following formula:

Leisure-time physical activity (MET-h/d) = (monthly frequency × MET-intensity × minute duration) / 30 d

Finally, daily active commuting habits were queried, with options ranging from no active commuting and I do not currently work to an hour or longer. The active commuting volume was calculated using the standard intensity (4 METs) and frequency (5 days per week):

Active commuting (MET-h/d) = (daily duration x 4 METs x 5 per week) / 7 d

To obtain the total physical activity volume, the leisure-time physical activity and active commuting volumes were combined. This variable is referred to as the short MET index.

In *Study III*, a comprehensive characterization of leisure-time physical activity for the past 12 months was assessed using a longer interview that followed the Kuopio Ischemic Heart Disease Risk Factor Study (Lakka & Salonen, 1992). The participants were asked about their engagement in 20 set physical activities, including the frequency, duration, and intensity of each activity. The participants were also allowed to mention other physical activities not included in the selection. The total physical activity volume was calculated as MET-h/d and is referred to as the 12-month MET index.

Accelerometer-measured physical activity. In *Study IV*, physical activity was also assessed using hip-worn GT3X+ and wGT3X+ Actigraph accelerometers (Pensacola, Florida, USA). The measurement period was 7 days, and the participants were required to wear the device for at least 10 hours on 3 days (Hyvärinen et al., 2020). The participants also logged their wake-up times, working hours, and nonwearing periods of more than 30 minutes. Raw acceleration data were collected at 60 Hz, filtered, and then converted into 60-second epoch counts. Tri-axial vector magnitude cutoff points of 450, 2,690, and 6,166 counts per minute were used to differentiate between light, moderate, and vigorous intensities of physical activity, respectively (Laakkonen, Kulmala, et al., 2017; Sasaki et al., 2011).

4.2.3 Age

In the ERMA cohort, the date of birth was derived from the Population Information System, while in the other studies, the participants reported their

birth dates. The age of the participants at the time of measurement was determined by calculating the time between birth and the measurement date.

In *Study II*, age categories were created following Pontzer et al. (2021), where it was found that FFM- and FM-adjusted REE and TEE declined to adult levels at 18.0 (95% CI, 16.8–19.2) and 20.5 years of age (95% CI, 19.8–21.2), respectively. The decline then began again after 46.5 (95% CI, 40.6–52.4) and 63.0 years of age (95% CI, 60.1–65.9), respectively. Therefore, participants aged 18~21 years old were assigned to group I, participants aged 22~39 years old were assigned to group II, and participants aged 40~60 years old were assigned to group III.

4.2.4 Serum metabolome

In *Study I*, 250 metabolite measures were quantified from the same fasting blood samples as the sex hormones using high-throughput proton NMR spectroscopy (Nightingale Health Plc, Helsinki, Finland; biomarker quantification version 2020). The quantification process has been described previously (Soininen et al., 2015; Würtz et al., 2017). Of the 250 metabolites, 180 were selected as the primary outcomes for the present study. Lipoprotein lipid ratios were excluded because they were found to contain partially overlapping information with lipoprotein lipid concentrations and did not add significant new information to the study's objectives. This decision was made to maximize statistical power when analyzing metabolite concentration changes during the menopausal transition, given the relatively small sample size of the study.

4.2.5 Resting energy expenditure

All indirect calorimetry data were collected in a thermoneutral laboratory using the Vmax Encore 92 metabolic cart (Sensormedics, Yorba Linda, CA, USA). The cart was calibrated following the manufacturer's instructions before each measurement. The resting measurements were conducted using the same ventilated hood. The participants were instructed to lie still, keep their eyes open, and the laboratory lighting was dimmed. Resting and measurement periods varied between studies, with resting periods ranging from 0 to 30 minutes and measurement periods ranging from 15 to 30 minutes. Data from the first 5–10 minutes were excluded. In all studies, REE was calculated using data from at least 5-minute steady-state period, where the CV of $\dot{V}O_2$ and $\dot{V}CO_2$ was no more than 10% (Fullmer et al., 2015). REE was calculated with the modified Weir equation (Weir, 1990):

$$\text{REE (kcal/d)} = (3.941 \times \dot{V}O_2 + 1.106 \times \dot{V}CO_2) \times 1440$$

REE was made comparable between individuals using the residual method. A linear regression model was applied, with REE as the outcome and FFM/LM and FM as the explanatory variables. No *ln*-transformation was required for FFM/LM and FM because all participants were within the range where FFM/LM

and REE have a linear relationship, and this was also visually confirmed. The residuals were obtained for each participant, showing the deviation of measured REE from estimated values. The models generated, including the regression coefficients, were as follows:

$$\textit{Study II:}\ \text{REE (kcal/d)} = 413.4 + 20.2 \times \text{FFM (kg)} + 1.7 \times \text{FM (kg)}$$

$$\textit{Study III:}\ \text{REE (kcal/d)} = 374.4 + 20.6 \times \text{LM (kg)} + 7.6 \times \text{FM (kg)}$$

4.2.6 Fat oxidation at rest and during exercise

In *Studies III* and *IV*, RFO was calculated from the same gas exchange data used for the REE calculation, here by using the Frayn equation, with the assumption that nitrogen excretion is negligible (Frayn, 1983):

$$\text{Fat oxidation (g/min)} = 1.67 \times \dot{V}O_2 - 1.67 \times \dot{V}CO_2$$

As can be observed by comparing the Weir and Frayn equations, the Frayn equation is more sensitive to factors influencing the relationship between oxygen uptake and CO_2 excretion than the Weir equation. In healthy individuals, RER should fall below 0.91 while fasting (Fullmer et al., 2015). Thus, this value was used as an RFO measurement acceptance criterion in *Studies III* and *IV*. Three women were excluded from *Study IV* and were included in *Study II* because slight hyperventilation does not affect REE as much as RFO.

The exercise tests in *Studies III* and *IV* were conducted using the same bicycle ergometer (Ergoselect 200, Ergoline GmbH, Germany) while continuously monitoring participants' electrocardiograms to ensure their safety; however, the test protocols varied between the studies. In *Study III*, testing began with 2-minute workloads at 20 and 25 W, followed by an increment of 25 W every 2 minutes until exhaustion. Data from the last 30 seconds of each stage were used for calculations. Steady-state achievement in gas exchange was determined by comparing $\dot{V}O_2$ and $\dot{V}CO_2$ between 1:30 and 1:45 and 1:46 and 2:00 minutes. Three participants who did not reach a steady state were excluded from a sensitivity analysis, which indicated that their inclusion in the primary analyses did not significantly affect the results.

In *Study IV*, the test protocol was designed using evidence from participants with low cardiorespiratory fitness (Chrzanowski-Smith et al., 2018; Dandanell et al., 2017) and was fine-tuned through pilot testing. The test began at 20 W, and the workload was increased by 20 W every 4 minutes until the participants reached RER 1.0 or the seventh stage. Gas exchange data from the last 60 seconds of each stage were extracted, and steady-state achievement was confirmed by comparing $\dot{V}O_2$ and $\dot{V}CO_2$ between 3:00 and 3:30 and 3:30 and 4:00 minutes. Fat oxidation was calculated for stages where the steady-state assumption was met with the Frayn equation. In both studies, the highest measured fat oxidation rate was selected as the PFO.

4.2.7 Total energy expenditure

In *Study II*, TEE data were obtained from a subset of participants from the Calex study. On the first assessment day, the participants provided fasted urine samples, ingested a DLW dose of 1 g/kg, and provided a second sample 4–6 hours later. The third sample was collected 14 days later after overnight fasting. Sample analysis was conducted in triplicate at the University of Alabama (Birmingham, USA) using a Metabolic Solutions Inc. mass spectrometer (Merrimack, NH, USA). TEE was calculated following a methodology described previously (Schoeller et al., 1986; Schoeller & Hnilicka, 1996).

4.2.8 Glycemic control and insulin sensitivity

In *Studies III* and *IV*, a 2-hour OGTT protocol was used with blood samples taken every 30 minutes, with the exception that *Study III* did not collect a 90-minute sample. The participants were required to fast before providing blood samples, following which they ingested a solution containing 75 g of glucose (GlucosePro, Comed Oy, Ylöjärvi, Finland). The plasma glucose concentrations were analyzed using the Konelab 20 XT (Thermo Fisher Scientific, Vantaa, Finland). The serum insulin concentrations were analyzed in *Study III* using the IMMULITE 1000 and in *Study IV* using the IMMULITE 2000XPi (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The primary outcomes were insulin sensitivity and glycemic control. Insulin sensitivity was assessed using the Matsuda index (Matsuda & DeFronzo, 1999), which is a surrogate outcome that uses both fasting and postprandial glucose and insulin concentrations. The index was calculated using an available Microsoft Excel file (http://mmatsuda.diabetes-smc.jp). Glycemic control was assessed by calculating the total glucose AUC using the trapezoidal method. The AUC was also calculated for insulin.

4.3 Assessment of covariates

4.3.1 Body composition

In *Studies II–IV*, body composition was assessed using a dual-energy X-ray absorptiometry scanner (DXA Prodigy, GE Lunar Corp., Madison, WI, USA). In *Study I*, body fat percentage was measured using bioelectrical impedance analysis (InBody720, Biospace, Seoul, Korea). *Study IV* used the same equipment to identify body composition changes between laboratory visits.

4.3.2 Cardiorespiratory fitness

In *Studies III* and *IV*, cardiorespiratory fitness and PFO were assessed during the same exercise testing session. *Study III* participants continued the 2-minute workloads with 25 W increments until volitional exhaustion. Gas exchange was

recorded using 30-second rolling averages, and the highest $\dot{V}O_2$ recording was selected as the $\dot{V}O_{2PEAK}$. The maximal test performance criteria were a rate of perceived exertion of 19–20 or RER of greater than 1.1 at the final stage. Four participants were unable to complete the maximal test because of health reasons, and their $\dot{V}O_{2PEAK}$ was extrapolated based on $\dot{V}O_2$, heart rate recordings and estimated maximal heart rate.

In *Study IV*, the exercise test consisted of two phases: PFO and $\dot{V}O_{2PEAK}$. The participants transitioned to the $\dot{V}O_{2PEAK}$ phase immediately after the PFO phase. The ramp method was chosen to minimize the fatiguing effect of increasing workloads. The phase started at 100 W, and the workload was increased by 1 W every 3 seconds until volitional exhaustion or until the participant could not maintain the instructed cadence of 70 ± 5 rpm. Gas exchange was recorded using 10-second rolling averages, and $\dot{V}O_{2PEAK}$ was determined as the 30-second period with the highest $\dot{V}O_2$. Additionally, the maximal work capacity (W_{MAX}) the participant reached was recorded as an alternative measure of cardiorespiratory fitness. The participants reached their maximum well based on a RER_{MAX} cutoff of ≥ 1.10 and an age-predicted maximal heart rate (210–age) with a cutoff of ≥ 99% (Wagner et al., 2020), with the exception of one PRE/PERI woman who did not quite reach the criteria.

4.3.3 Dietary intake and diet quality

In *Study IV*, the participants filled food diaries for 2 days prior to the resting and exercise metabolism measurements. Energy and macronutrient intake were subsequently calculated using the AivoDiet software (Aivo Finland Oy, Turku, Finland). The food quotient was employed to describe the metabolizable RER of the diet and calculated as per (Elia, 1991):

$$\text{Food quotient} = [0.71 \times \text{fat intake (kcal/d)} + 0.835 \times \text{protein intake (kcal/d)} + 1.0 \times \text{carbohydrate intake (kcal/d)}] / \text{energy intake (kcal/d)}$$

In *Study I*, dietary habits were assessed at the beginning and end of the follow-up period using a food frequency questionnaire comprising 45 foods commonly consumed in Finland. An 11-item scale was employed to evaluate diet quality, as modified from a validated tool (Masip et al., 2019) and based on the Nordic Nutrition Recommendations (Nordic Council of Ministers, 2012). The scale awarded points for regular consumption of whole grains, low-fat dairy, vegetables, fruits and berries, nuts and seeds, and fish, while infrequent consumption of highly processed baked goods, sweet and salty snack foods, fast food, sugary beverages, and processed meats also earned points. The maximum possible score was 11.

4.3.4 Lipolysis-related metabolites

In *Study IV*, concentrations of NEFA were analyzed using the Indiko clinical chemistry analyzer (Thermo Fisher Scientific, Vantaa, Finland). In *Studies III* and

IV, concentrations of glycerol and 3-hydroxybutyrate were analyzed using the NMR metabolomics technology (Nightingale Health Plc, Helsinki, Finland), as previously described.

4.3.5 Education level, smoking status, and use of alcohol

In *Study I*, the participants' education level, smoking status, and alcohol use were assessed using a structured questionnaire. The questionnaire was completed at every follow-up visit. Education was assessed with a single question and categorized into primary, secondary, and tertiary levels. In the statistical analyses, the two lowest categories were combined because of the small sample size of the first category.

Regarding smoking status, the participants were first asked if they had ever smoked more than 5–10 cigarettes in their lifetime. Those who answered no were classified as never smokers. Those who answered yes were then asked if they currently smoked regularly. If they answered no, they were classified as a quitter; if they answered yes, they were classified as a current smoker. For the statistical analyses, dummy variables were created to indicate whether the participant had ever smoked and whether they currently smoked.

Alcohol use was assessed by asking participants about their weekly consumption of three different types of alcohol-containing beverages (beer, cider, long drinks; wine; spirits) with seven answer options. Alcohol portions (12 g of alcohol per portion) were calculated and added together to express the participant's alcohol consumption per week.

4.4 Ethical considerations

All studies in the present thesis were conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee of the Central Finland Health Care District (Calex, memo 22/8/2008 and 5/2009; ERMA, 8U/2014; EsmiRs, 9U/2018; FITFATTWIN, 4U/2011; Physique, 19U/2918) or the Ethics Committee of the University of Jyväskylä (NO RED-S, 514/13.00.04.00/2021) for all studies. The participants provided informed consent, and their anonymity and safety were protected throughout all phases of the studies.

4.5 Statistical analyses

The R statistical environment (R Core Team, 2022) version 4.0.5 or higher was used for statistical analyses, unless otherwise stated. Descriptive data are presented as the means with standard deviations, medians with interquartile ranges, or frequencies with percentiles. Before accepting the results, the assumptions of the

modeling approaches were confirmed. The R code used to generate results in *Study II* can be accessed at https://doi.org/10.5281/zenodo.7446075 and for *Study IV* at https://osf.io/afmu7/. To enhance readability, the findings are presented using point estimates and corresponding *P*-values. The CIs for the key results can be found in the original publications.

4.5.1 Data transformations

The distributions of hormone and metabolite concentrations are often skewed, and the variables typically produce non-normally distributed residuals. In *Study I*, the metabolite measures were Box–Cox transformed using the lambda parameter estimated for each variable separately with the *MASS* package version 7.3–54 (W. N. Venables & Ripley, 2002). The metabolite measures were standardized to their baseline mean and SD to facilitate a comparison of the results between metabolites with different units and concentrations. In *Study IV*, E2 concentrations and OGTT-derived variables were *ln*-transformed to meet the residual normality assumption.

4.5.2 Longitudinal analyses

In *Study I*, linear mixed effects models were employed to investigate changes in metabolite concentrations over time, with participant identification as a random effect. The *nlme* package (Pinheiro et al., 2022) was used for all linear mixed effect modeling. The associations between MHT and metabolite measures were examined by incorporating time and MHT interaction as the exposure. The impact of the menopausal hormonal shift, either directly or indirectly through changes in fat percentage, on changes in metabolite outcomes was examined using latent change score modeling in Mplus (version 7.4) (FIGURE 9). The model can be viewed as an extension of the paired *t*-test, where the metabolite change is controlled for the first measurement value. The model was applied for each metabolite separately, with the results showing the proportion of the metabolite measure change that can be attributed to the menopausal hormonal shift.

Because of the large number of metabolite outcomes, multiple statistical testing was required. However, many metabolite measures were highly correlated, and classical multiple correction methods such as the Bonferroni correction are too conservative in this scenario (Würtz et al., 2017). To address multiple testing when analyzing the influence of natural menopause and MHT, the Keff–Šidák correction (Moskvina & Schmidt, 2008) was used because it performed the best in the simulation experiments. False discovery rate correction was used when studying the associations between menopausal hormonal shift and metabolite changes.

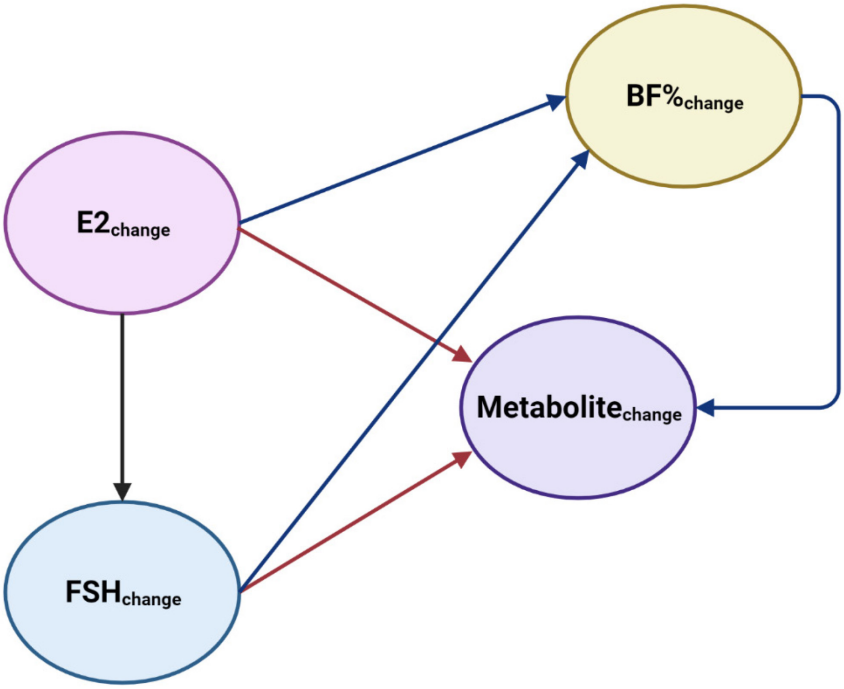

FIGURE 9 Effects of interest in the conceptual model between estradiol (E2) and follicle-stimulating hormone (FSH) concentrations, body fat percentage (BF%), and metabolite measure change.

4.5.3 Cross-sectional analyses

In *Study II*, differences in residual REE between middle-aged women and younger age groups were tested while accounting for the familial relationship between Calex participants, here by using a linear mixed effects model with family identification as a random effect and the middle-aged group as the reference. Residual REE differences between menopause groups were compared using linear regression with postmenopausal women as the reference. Since tissue mass variables may explain REE differently among women of varying ages, additional regression models were generated using measured REE as the outcome, menopause status or sex hormone concentrations as the explanatory variable, and FFM, FM, and age as covariates.

In *Study III*, the original publication compared the differences between physically active discordant cotwins using a paired samples *t*-test in IBM SPSS Statistics 24.0. However, this method does not allow for covariate adjustment. Therefore, the energy metabolism outcomes were divided by LM to analyze the mass-relative differences. However, ratio scaling produces accurate results only when the regression line between the outcome and exposure variables passes the origin (Tanner, 1949). During the writing of the present thesis, the analyses were repeated using linear mixed effects models with twin pair identification as a random effect. The reanalyses did not change the results, which is understandable because the cotwins had relatively similar tissue masses. Therefore, the results from the original publication are presented here. Twin individual-based analyses were performed with linear regression in Stata 15.0.

The violation of independence assumption was taken into account with the clustering option of Stata using twin pair identification as the clustering variable.

Studies II and *III* also analyzed familial dependence on the metabolism outcomes by estimating intraclass correlation coefficients (ICCs) using one-way random-effects models generated with the *psych* package (Revelle, 2022). This method compares intrapair variation to interpair variation. The ICC is 0 when an individual resembles a member of another pair as much as their family member and 1 when family members are identical in the outcome studied (Bogardus et al., 1986).

In *Study IV*, the differences in fat oxidation between the menopause groups were compared using an analysis of variance or covariance models generated with the *jmv* package (Ravi et al., 2020). Exploratory analysis between the HIGH E2 and POST groups and association analyses between continuous variables were performed using linear regression.

5 RESULTS

5.1 Participants

Study I involved participants who were between 47 and 55 years old at the beginning of the study (TABLE 3). Those participants who began using MHT during the follow-up were found to be younger and had lower serum FSH concentrations compared with those who did not start MHT. The follow-up period for each participant was individualized and ranged from 4 months to 3.5 years. During this time, the participants decreased their alcohol consumption, and their body fat percentage increased on average by 1%. The changes in body fat percentage were similar for participants who did and did not start using MHT. Detailed information on body composition changes can be found in Juppi et al. (2020, 2022).

TABLE 3 Participant characteristics at the beginning and end of the follow-up in *Study I*.

	Natural menopause (*n* = 183)		**MHT starters (*n* = 35)**	
	Baseline	Follow-up	Baseline	Follow-up
Age, y	51.9 (1.9)	53.1 (1.9)	50.6 (1.8)	52.0 (1.9)
Follow-up duration, d		397 (243–603)		464 (334–707)
HT use duration, d				220 (191–242)
Sex hormones				
E2, nmol/l	0.25 (0.17–0.41)	0.16 (0.11–0.27)	0.25 (0.18–0.42)	0.28 (0.20–0.61)
FSH, IU/l	30 (23–49)	70 (47–90)	23 (17–36)	36 (18–60)
Education				
Primary	5 (3%)	5 (3%)	1 (3%)	1 (3%)
Secondary	100 (55%)	100 (55%)	15 (43%)	15 (43%)
Tertiary	78 (42%)	78 (43%)	19 (54%)	19 (54%)
Body composition				
Height, cm	165.1 (5.7)	-	166.3 (5.5)	-
Body mass, kg	69.5 (11.3)	70.2 (11.6)	70.4 (10.2)	70.5 (10.8)
Body mass index, kg/m^2				
< 25	92 (50%)	85 (46%)	20 (57%)	19 (54%)
25–29.9	66 (36%)	71 (39%)	10 (29%)	12 (34%)
≥ 30	25 (14%)	27 (15%)	5 (14%)	4 (11%)
Percent body fat, %	30.9 (8.3)	32.1 (8.0)	30.5 (7.1)	31.4 (6.3)
Smoking				
Never	122 (67%)	122 (67%)	26 (74%)	26 (74%)
Quitter	49 (27%)	50 (28%)	6 (17%)	5 (14%)
Smoker	12 (7%)	11 (6%)	3 (9%)	4 (11%)
Alcohol use, portions/wk	3.0 (1.0–5.0)	2.5 (1.0–5.0)	4.5 (2.5–5.8)	2.5 (1.0–4.9)
Diet quality score, 0–11	5.7 (2.3)	5.5 (2.1)	5.5 (2.4)	5.6 (2.4)
Physical activity, MET-h/d	3.6 (1.7–4.8)	3.8 (2.3–5.6)	4.5 (1.8–7.5)	4.8 (2.5–7.5)

Data as means (standard deviation), medians (interquartile range), or counts (%).
E2, estradiol; FSH, follicle-stimulating hormone; MET, metabolic equivalent of task; MHT, menopausal hormone therapy

Study II included women who were between the ages of 17 and 58 (TABLE 4). Of these participants, 59 were middle-aged and represented different menopause groups. The PRE/PERI group women were slightly younger than those in the POST and MHT groups.

The participant characteristics of *Studies III* and *IV*, which included assessments of fat oxidation, are combined in TABLE 5. The participants in *Study III* were men between the ages of 32 and 37. The cotwins discordant for physical activity had a 205 kcal/d ($P < 0.001$) difference in AEE over the past 12 months. The more active cotwins had 1.4 kg higher LM (95% CI, -0.3 to 3.0) and 3.3 kg lower FM (95% CI -6.7 to 0.2) than their less active cotwins. When adjusted for LM, the more active cotwins also had 357 ml higher $\dot{V}O_{2PEAK}$ ($P < 0.001$) than the less active cotwins. As reported in Rottensteiner et al. (2015), Matsuda index was also higher among the more active co-twins ($P = 0.021$).

In *Study IV*, the participants were women between the ages of 52 and 58, representing the different menopause groups, as in *Study II*. These groups did not differ in terms of age ($P = 0.56$), self-reported physical activity ($P = 0.74$), or body fat percentage ($P = 0.46$). As expected, the POST group had lower E2 and higher FSH concentrations compared with the PRE/PERI (both hormones $P < 0.001$) and MHT groups (E2; $P < 0.001$, FSH; $P = 0.003$). No notable changes in body composition were observed during the study period.

TABLE 4 Participant characteristics and energy expenditures across age and menopause groups in *Study II*.

	Age groups (*N* = 120)			**Menopause groups (*n* = 59)**		
	I	**II**	**III**	**PRE/PERI**	**MHT**	**POST**
	***n* = 26**	***n* = 35**	***n* = 59**	***n* = 19**	***n* = 10**	***n* = 30**
Age, y	19.8 (1.1)	28.2 (4.3)	53.8 (3.5)	50.7 (4.3)	55.1 (1.7)	55.3 (1.9)
Sex hormones						
E2, nmol/l				0.29 (0.18–0.55)	0.29 (0.17–0.38)	0.09 (0.06–0.12)
FSH, IU/l				8 (7–25)	38 (34–61)	80 (71–102)
Body composition						
Height, cm	165.6 (5.6)	165.3 (5.8)	166.4 (5.0)	168.1 (4.6)	166.5 (5.6)	165.3 (4.8)
Total mass, kg	65.0 (10.5)	64.5 (7.9)	68.5 (9.1)	69.1 (9.0)	71.3 (12.3)	67.1 (7.9)
Fat-free mass, kg	44.2 (5.0)	49.8 (5.3)	44.2 (4.2)	45.6 (4.1)	43.4 (4.2)	43.6 (4.1)
Fat mass, kg	20.9 (8.2)	14.7 (5.8)	24.2 (7.3)	23.6 (7.8)	27.9 (8.4)	23.4 (6.4)
Body fat %	31 (8)	23 (7)	36 (7)	35 (8)	40 (5)	36 (6)
Resting energy expenditure						
Measured, kcal/d	1,430 (110)	1,474 (146)	1,286 (124)	1,323 (105)	1,249 (143)	1,276 (128)
Predicted, kcal/d¤	1,339 (104)	1,442 (107)	1,345 (87)	1,372 (84)	1,335 (98)	1,332 (85)
Predicted, kcal/d$	1,440 (103)	1,467 (94)	1,286 (92)	1,322 (86)	1,283 (115)	1,264 (84)
Total energy expenditure						
Measured, kcal/d	2,162 (310), *n* = 10		2,148 (236), *n* = 10			

Data as means (standard deviation) or medians (interquartile range)

¤ FFM and FM, $ FFM, FM, and age

E2, estradiol; FFM, fat-free mass; FM, fat mass; FSH, follicle-stimulating hormone; MHT, menopausal hormone therapy; POST, postmenopause; PRE/PERI, premenopause or perimenopause

TABLE 5 Participant characteristics and energy metabolism outcomes in *Studies III* and *IV*.

	Study III			***Study IV***			
	All (*N* = 46)	**More active (*n* = 10)**	**Less active (*n* = 10)**	**All (*N* = 42)**	**PRE/PERI (*n* = 7)**	**POST (*n* = 22)**	**MHT (*n* = 9)**
Age, y	34.5 (1.5)	33.9 (1.3)	33.9 (1.3)	55.3 (1.6)	54.9 (1.5)	55.6 (1.6)	55.4 (1.4)
E2, nmol/l				0.11 (0.06–0.25)	0.18 (0.15–0.22)	0.06 (0.05–0.09)	0.27 (0.25–0.42)
FSH, IU/l				66 (37)	21 (14)	89 (32)	50 (26)
Body composition							
Height, cm	178 (7.5)	180 (5.4)	179 (5.2)	166.0 (4.9)	166.7 (4.6)	165.8 (5.0)	166.6 (6.0)
Body mass, kg	76.7 (9.8)	76.0 (8.5)	78.0 (12.7)	67.8 (8.1)	69.9 (8.9)	67.2 (7.7)	68.9 (10.1)
Percent body fat, %	21.4 (6.7)	20.7 (4.0)	24.0 (4.6)	36.5 (5.5)	36.4 (5.1)	36.3 (5.8)	38.9 (4.4)
Physical activity							
Short index, MET-h/d	3.0 (1.2–6.5)	4.3 (3.0–6.2)	1.2 (1.1–1.7)	4.5 (2.3–7.5)	4.5 (3.4–6.8)	4.4 (2.3–7.5)	2.9 (1.5–4.5)
12 mo index, MET-h/d	3.0 (1.4–5.0)	3.9 (3.2–4.4)	0.7 (0.5–1.9)				
ACCEL, MVPA min/d				49.0 (26.0)	71.5 (28.8)	49.2 (23.6)	32.4 (15.8)
Glucose regulation	***n* = 44**						
Glucose AUC, mmol/l/h	11.6 (9.8–12.9)	4.6 (4.1–5.3)	5.5 (3.8–5.7)	12.6 (11.3–14.5)	13.8 (12.0–15.0)	12.3 (10.8–13.5)	16.0 (13.8–16.4)
Matsuda index	10.2 (7.9–23.4)	11.2 (9.0–41.6)	9.1 (8.1–9.6)	7.3 (5.4–9.4)	8.5 (6.9–10.7)	7.2 (5.4–11.1)	6.3 (3.7–8.8)
Resting metabolism	***n* = 43**	***n* = 9**	***n* = 9**	***n* = 39**	***n* = 5**	***n* = 21**	
REE, kcal/d	1685 (190)	1735 (187)	1675 (191)				
RER	0.82 (0.04)	0.82 (0.03)	0.80 (0.03)	0.83 (0.03)	0.83 (0.03)	0.84 (0.03)	0.81 (0.02)
RFO, g/min	0.075 (0.017)	0.075 (0.017)	0.079 (0.016)	0.050 (0.010)	0.053 (0.009)	0.048 (0.010)	0.054 (0.009)
RFO, mg/kg LM/min	1.31 (0.29)	1.31 (0.29)	1.40 (0.29)	1.22 (0.23)	1.25 (0.19)	1.18 (0.25)	1.34 (0.18)
Exercise metabolism	***n* = 41**	***n* = 9**	***n* = 9**	***n* = 40**			***n* = 7**
$\dot{V}O_{2PEAK}$, ml/kg LM/min	55 (8)	58 (5)	52 (5)	52 (7)	51 (5)	53 (8)	48 (6)
PFO, g/min	0.39 (0.14)	0.46 (0.20)	0.38 (0.12)	0.22 (0.07)	0.23 (0.10)	0.22 (0.06)	0.21 (0.05)
PFO, mg/kg LM/min	6.8 (2.2)	8.0 (3.1)	6.9 (1.8)	5.3 (1.4)	5.4 (2.1)	5.3 (1.3)	5.0 (1.2)
FAT_{MAX}, (%$\dot{V}O_{2PEAK}$)	40 (10)	40 (9)	43 (8)	34 (9)	37 (15)	33 (8)	33 (5)

Data as means (standard deviation) or medians (interquartile ranges)

ACCEL, accelerometery; AUC, area under the curve; E2, estradiol; FSH, follicle-stimulating hormone; LM, lean mass; MET, metabolic equivalent of task; MHT, menopausal hormone therapy; MVPA, moderate-to-vigorous physical activity; PFO, peak fat oxidation; POST, postmenopause; PRE/PERI, premenopause or perimenopause; REE, resting energy expenditure; RER, respiratory equation ratio; RFO, resting fat oxidation; $\dot{V}O_{2PEAK}$, peak oxygen uptake

5.2 Serum metabolome (Study I)

5.2.1 Natural menopause

The women who experienced natural menopause had a statistically significant change in 85 of the 180 metabolite measures that were studied. The key findings are presented in FIGURE 10. The number of apoB-containing particles increased, reflecting increases in VLDL and LDL particles. Additionally, VLDL particle size and the amount of VLDL-carried triglycerides and cholesterol increased. IDL and LDL cholesterol also increased. The results concerning HDL measures were uncertain, with the exception of HDL triglycerides, which increased. The profile of serum fatty acids changed in favor of SFAs over PUFAs. At the concentration level, linoleic acid (-0.32 SD, P = 0.001) and docosahexaenoic acid (-0.21 SD, P = 0.023) decreased. Glycerol and glutamine concentrations increased, while citrate, leucine, acetoacetate, and 3-hydroxybutyrate concentrations decreased.

Adjusting for body fat percentage reduced the association sizes of lipoprotein measures and glycerol and amino acid concentrations by 0.03–0.05 SDs. On the other hand, the associations between menopause, fatty acid ratios, and concentrations of citrate and ketone bodies were unaffected by the adjustment. In a sensitivity analysis, data from 28 participants whose amenorrhea had lasted less than 6 months were excluded. All reported associations strengthened. Notably, the association between menopause and an increased small HDL particle number (0.24 SD, P = 0.037) reached statistical significance.

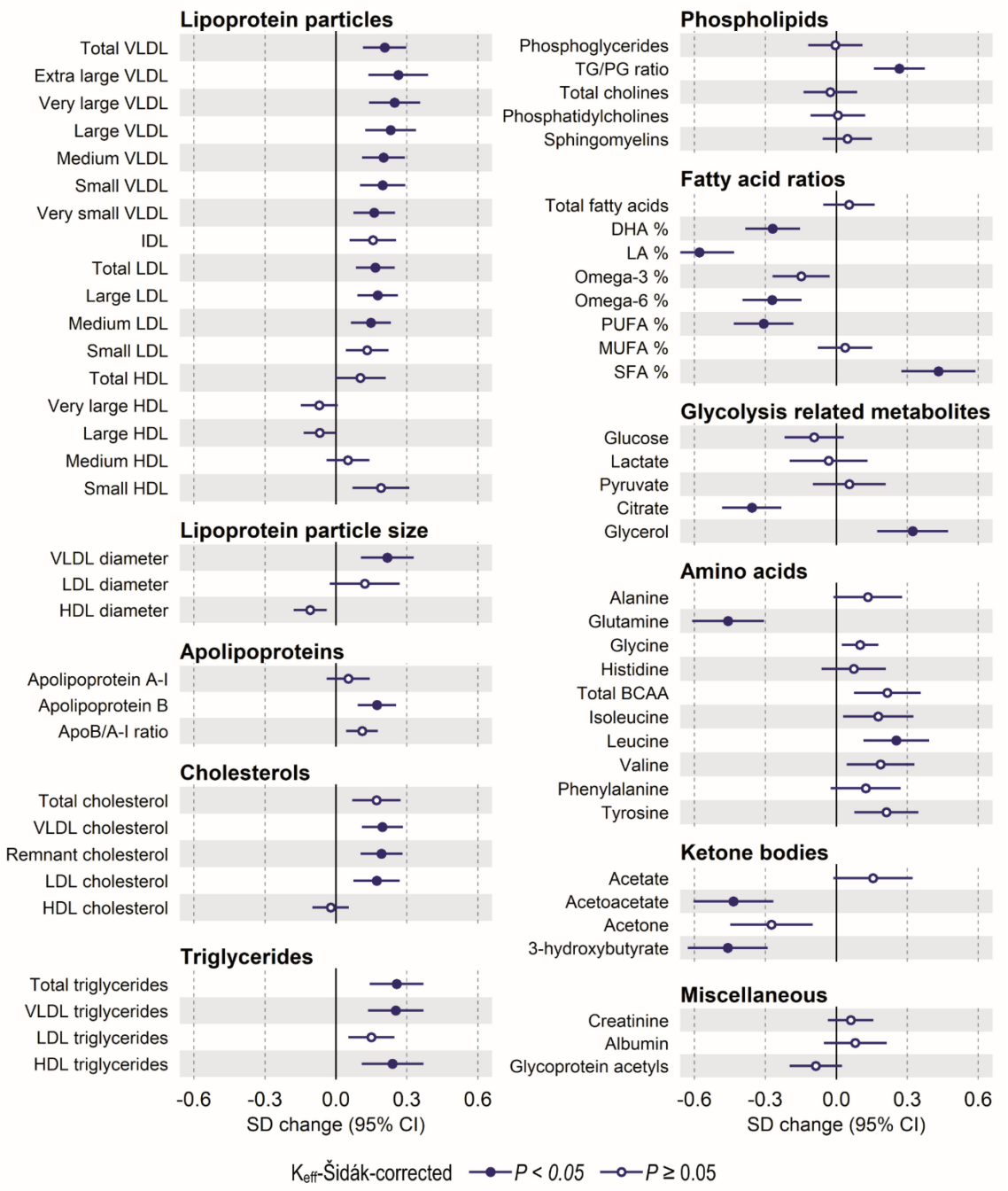

FIGURE 10 The associations of menopause with key metabolite outcomes (n = 183). The dots represent metabolite changes during follow-up in standard deviation (SD) units and whiskers 95% confidence intervals (CI). The metabolite changes are adjusted for age at the first measurement, follow-up duration, education level, smoking status, alcohol use, physical activity level, and diet quality. The figure is republished from Karppinen et al. (2022) under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/). BCAA; branch-chained amino acid; DHA, docosahexaenoic acid; HDL, high-density lipoprotein; LA, linoleic acid; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TG/PG, triglyceride/phosphoglyceride; VLDL, very low-density lipoprotein.

5.2.2 Menopausal hormonal shift

Of the 85 menopause-associated metabolite changes observed in the primary analysis, 64 were found to be directly linked to the menopausal hormonal shift. The results of key metabolites are shown in FIGURE 11. The shift explained 7.4% of the increase in apoB, 8.5% of the increase in LDL particles, and 10.6% of the increase in LDL cholesterol. Despite the noticeable change in VLDL measures during the follow-up, the shift only explained 4.0% of the increase in VLDL particle number and 2.9% of the increase in VLDL triglyceride concentration. Additionally, the menopausal hormonal shift explained the rise in glutamine (2.4%), the decreases in citrate (5.0%), 3-hydroxybutyrate (4.4%), and acetoacetate (3.4%) concentrations, as well as the decrease in the linoleic acid ratio (3.4%). However, no associations between menopausal hormonal shift and metabolite changes were observed through body fat percentage mediation. This occurred because the changes in sex hormone concentrations were not associated with changes in body fat percentage, which would require the pathway (FIGURE 9) to be causal.

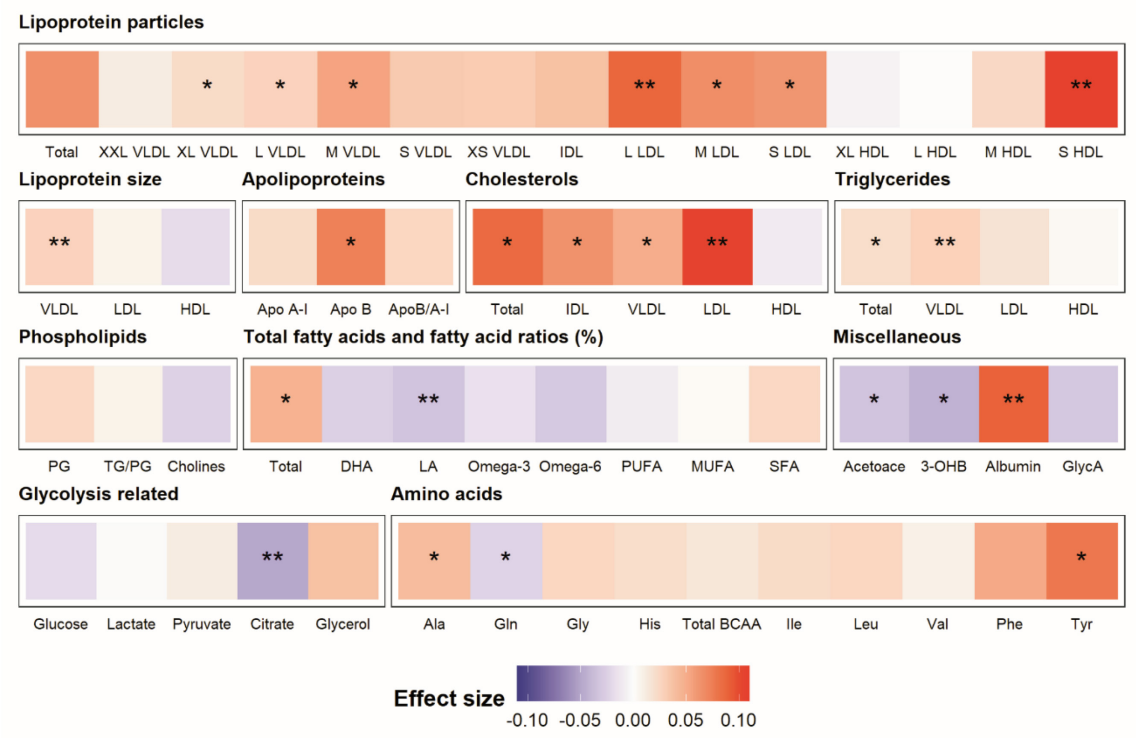

FIGURE 11 The associations of menopausal hormonal shift with key metabolite changes adjusted for age at the first measurement, follow-up duration, education, smoking, alcohol use, physical activity, and diet quality. 3-OHB, 3-hydroxybutyrate; Ala, alanine; BCAA; branch-chained amino acid; DHA, docosahexaenoic acid; Gln, glutamine; Gly, glycine; HDL, high-density lipoprotein; His, histidine; Ile, isoleucine; LA, linoleic acid; LDL, low-density lipoprotein; Leu, leucine; MUFA, monounsaturated fatty acid; Phe, phenylalanine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TG/PG, triglyceride/phosphoglyceride; Tyr, tyrosine; Val, valine; VLDL, very low density lipoprotein. * $P < 0.05$; ** $P < 0.01$ are false discovery rate corrected. The figure is republished from Karppinen et al. (2022) under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

5.2.3 Menopausal hormone therapy

MHT was primarily associated with changes in lipoprotein measures (TABLE 6). Specifically, the use of MHT was linked to an increase in the apoB/A-I ratio, which was achieved through an increase in larger HDL particles and a decrease in smaller LDL particles. Additionally, MHT was also associated with a reduction in glycine concentration. Notably here, adjusting for body fat percentage did not impact these results.

TABLE 6 Menopause and menopausal hormone therapy interactions with key metabolite outcomes. The table is republished from Karppinen et al. (2022) under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

	Crude model			Adjusted model #		
Metabolite	**Int**	**99.95% CI**	***P***	**Int**	**99.95% CI**	***P***
ApoB/apoA-I ratio	-0.54	-0.86 to -0.21	< 0.001	-0.55	-0.88 to -0.22	< 0.001
Apolipoprotein A-I	0.51	0.08 to 0.93	0.005	0.55	0.13 to 0.97	0.001
Glycine	-0.46	-0.80 to -0.11	0.001	-0.46	-0.80 to -0.12	0.001
M HDL particles	0.54	0.10 to 0.97	0.003	0.58	0.15 to 1.01	0.001
L HDL particles	0.35	0.06 to 0.64	0.004	0.36	0.07 to 0.66	0.002
HDL cholesterol	0.36	0.02 to 0.70	0.028	0.39	0.05 to 0.74	0.008
HDL diameter	0.35	0.05 to 0.65	0.007	0.35	0.04 to 0.74	0.009
S LDL particles	-0.41	-0.76 to -0.05	0.005	-0.44	-0.83 to -0.05	0.011
LDL particles	0.31	-0.76 to -0.05	0.010	-0.39	-0.75 to -0.03	0.020
M LDL particles	-0.39	-0.74 to -0.03	0.017	-0.37	-0.77 to -0.01	0.039

age at the first measurement, follow-up duration, education level, smoking status, alcohol use, physical activity, and diet quality.
HDL, high-density lipoprotein; Int, interaction; L, large; LDL, low-density lipoprotein; M, medium; S, small. The confidence intervals and *P*-values were Keff-Šidák-corrected for multiple comparisons.

5.3 Resting energy expenditure (Studies II and III)

Residuals refer to those derived from the FFM/LM- and FM-adjusted models unless otherwise specified.

5.3.1 Determinants

In *Studies II* and *III*, the proportion of variance in REE explained by FFM or LM and FM was 47% and 61%, respectively. When age was included in the analysis, the adjusted R^2 in *Study II* improved to 0.68. The ICCs for measured REE and residual REE in 16 mother–daughter pairs were 0.22 ($P = 0.19$) and 0.05 ($P = 0.42$), respectively, and the ICC for FFM, FM, and age-adjusted residual REE was 0.41 ($P = 0.046$). Additionally, ICCs for measured TEE and residual TEE in 10 daughter–mother pairs were 0.83 and 0.92, respectively ($P < 0.001$ for both

coefficients). In *Study III*, ICCs for measured REE, LM-relative REE, and residual REE in 21 MZ twin pairs were 0.58 (P = 0.002), 0.59 (P = 0.001), and 0.36 (P = 0.039), respectively.

5.3.2 Age

In *Study II*, age was inversely associated with residual REE (B = -3.9, P < 0.001), with the oldest group having 126 and 89 kcal/d lower REE than the younger groups I and II, respectively (FIGURE 12A). Furthermore, middle-aged mothers were found to have significantly lower residual REE compared with their daughters (-100 kcal/d; P < 0.001; n = 16 pairs), but no clear difference in TEE was observed (B = -26 kcal/d; P = 0.82; n = 10 pairs).

5.3.3 Menopause and sex hormones levels

The POST group did not have lower residual REE than the PRE/PERI or MHT groups (FIGURE 12B). This conclusion remained unchanged when using the middle-aged subsample-specific models that controlled for FFM, FM, and age. The MHT group had even lower REE by 58.4 kcal/d (P = 0.025) compared with the POST group. In *Study II* middle-aged women, neither serum E2 (B = 5.2, P = 0.89) nor FSH concentrations (B = 0.2, P = 0.43) were found to be significantly associated with REE, as shown by the results adjusted for FFM, FM, and age.

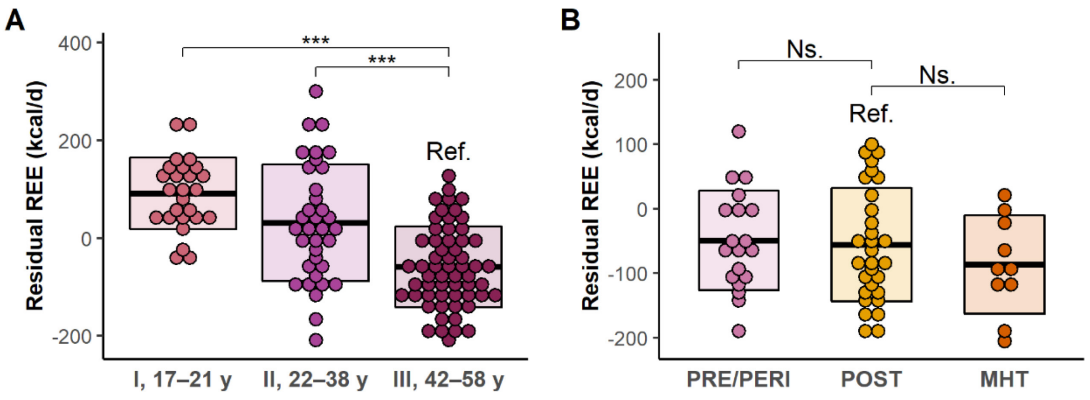

FIGURE 12 Comparison of fat-free mass and fat mass-adjusted residual resting energy expenditure (REE) among (A) age and (B) menopause groups. Box plots show group means and standard deviations. MHT, menopausal hormone therapy; Ns, nonsignificant; POST, postmenopausal; PRE/PERI, premenopausal or perimenopausal; Ref, reference; *** P < 0.001.

5.3.4 Physical activity

In the physical activity discordant MZ twin pairs of *Study III*, there were no apparent differences observed in measured or residual REE between the cotwins (FIGURE 13). Additionally, neither the 12-month MET index (β = 0.089, P = 0.60)

nor the 3-year short MET index (β = -0.032, P = 0.82) were found to have a clear association with residual REE.

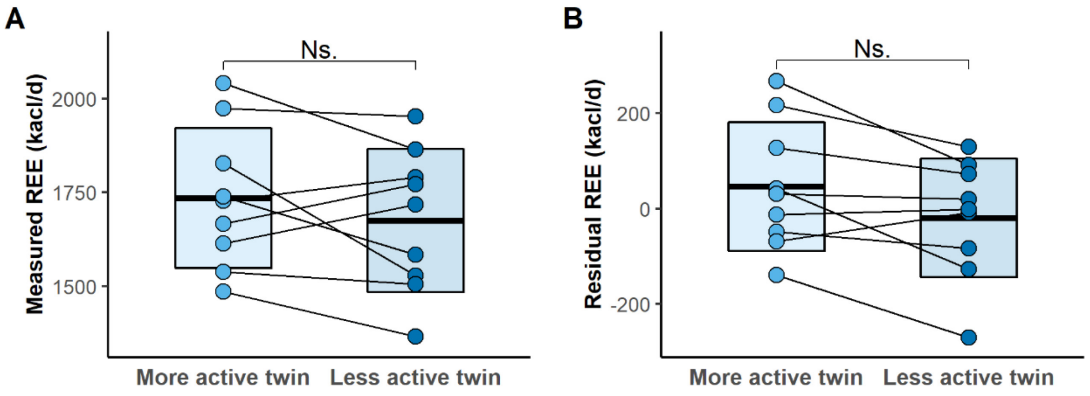

FIGURE 13 Comparison of (A) measured and (B) fat-free mass and fat mass-adjusted residual resting energy expenditure (REE) between physical activity discordant cotwins. The boxplots show group means with standard deviations. Ns., non-significant.

5.4 Fat oxidation at rest and during exercise (Studies III and IV)

All the results presented have been adjusted for LM unless otherwise specified.

5.4.1 Resting fat oxidation determinants

In *Study III*, ICCs for RFO calculated as g/min and g/kg LM/min were 0.54 (P = 0.004) and 0.57 (P = 0.003), respectively. Although no statistically significant associations between body composition and RFO were observed in the men in *Study III*, *Study IV* found that a higher FM was positively associated with RFO in middle-aged women (β = 0.44; P = 0.006). Diet was not recorded in *Study III*. However, in *Study IV*, the energy intake on the 2 previous days before the measurement was negatively associated with RFO (β = -0.40; P = 0.019). Together, LM, FM, and energy intake explained 40% of the variance in RFO (adjusted R^2 = 0.35; P < 0.001). These variables were considered the main determinants of RFO.

Both studies found that higher serum 3-hydroxybutyrate concentrations were positively associated with RFO (*Study III*: β = 0.54; P < 0.001 and *Study IV*: β = 0.52; P < 0.001). *Study IV* also measured serum NEFA concentrations, which were found to be positively associated with RFO (β = 0.46; P < 0.003).

5.4.2 Peak fat oxidation determinants

In *Study III*, ICCs for PFO calculated as g/min and g/kg LM/min were 0.67 (P < 0.001) and 0.59 (P = 0.002), respectively. Both studies showed positive associations between LM and PFO (*Study III*: β = 0.41; P = 0.007 and *Study IV*: β = 0.49; P

= 0.002). The studies also found an association between cardiorespiratory fitness and PFO. In *Study III*, $\dot{V}O_{2PEAK}$ was positively associated with absolute PFO (β = 0.51; P = 0.028); however, the association was not statistically significant when adjusted for LM (β = 0.43; P = 0.13). In *Study IV*, both $\dot{V}O_{2PEAK}$ (β = 0.47; P = 0.006) and W_{MAX} (β = 0.59; P = 0.002) were positively associated with PFO.

In *Study IV*, the association between PFO and serum metabolites was evaluated both before and after exercise testing. The results showed that pretest concentrations of NEFA (β = 0.33; P = 0.024) and glycerol (β = 0.31; P = 0.024) were positively associated with PFO. Additionally, posttest glycerol concentration was positively associated with PFO (β = 0.42; P = 0.003) even after adjusting for pretest values.

5.4.3 Physical activity

In *Study III*, no apparent differences were observed in RFO or PFO between the physically more and less active MZ twins (FIGURE 14). When adjusted for LM, more active twins had -0.004 g/min (P = 0.38) lower RFO and 0.06 g/min (P = 0.24) higher PFO than their cotwins. Additionally, none of the physical activity variables were found to be associated with RFO in *Studies III* or *IV*. In *Study III*, a positive association was found between the 12-month MET index and PFO (β = 0.26, P = 0.034). However, this association attenuated after adjusting for LM. In *Study IV*, both self-reported short MET index (β = 0.37, P = 0.020) and accelerometer-measured physical activity (β = 0.35, P = 0.024) were positively associated with PFO. Including the self-reported short MET index in the PFO explanatory model along with LM and W_{MAX} slightly improved the adjusted R^2 from 0.37 to 0.38 (P < 0.001 for both models). These variables were considered the main determinants of PFO.

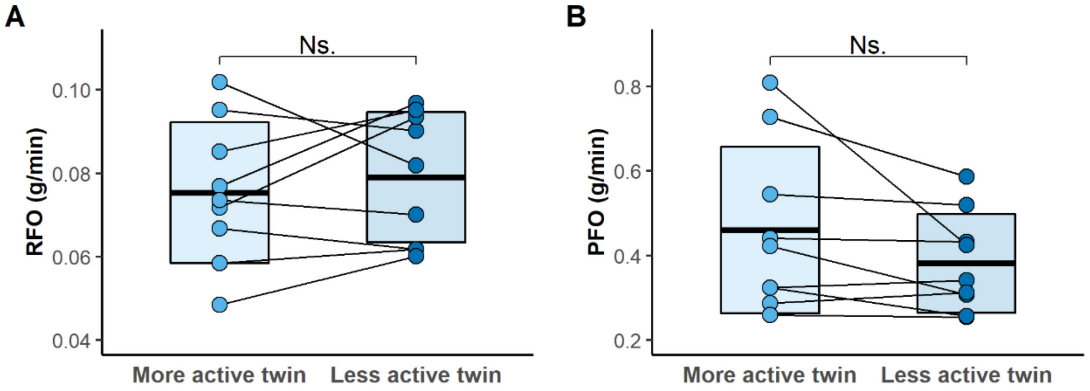

FIGURE 14 Comparison of (A) resting fat oxidation (RFO) and (B) peak fat oxidation (PFO) between physical activity discordant cotwins. The boxplots show group means with standard deviations. Ns., nonsignificant.

5.4.4 Menopause and sex hormone levels

In *Study IV*, menopausal status was not found to be associated with absolute RFO or PFO (FIGURE 15). These results remained unchanged when adjusted for LM (RFO: F[2,31] = 1.7; P = 0.20, PFO: F[2,32] = 0.18; P = 0.84) or their main determinants (RFO: F[2,29] = 0.25; P = 0.78, PFO: F[2,29] = 0.01; P = 0.99). Explorative analysis between the HIGH E2 and POST groups did not change the results. Furthermore, neither serum E2 concentration (β = 0.22, P = 0.17) nor serum FSH concentration (β = -0.21, P = 0.20) were found to be associated with RFO, and the same was true for PFO (E2: β = 0.01, P = 0.95, FSH: β = 0.16, P = 0.28). The inclusion of these hormones in the explanatory models for RFO and PFO did not improve the adjusted R^2 values beyond the main determinants of RFO and PFO.

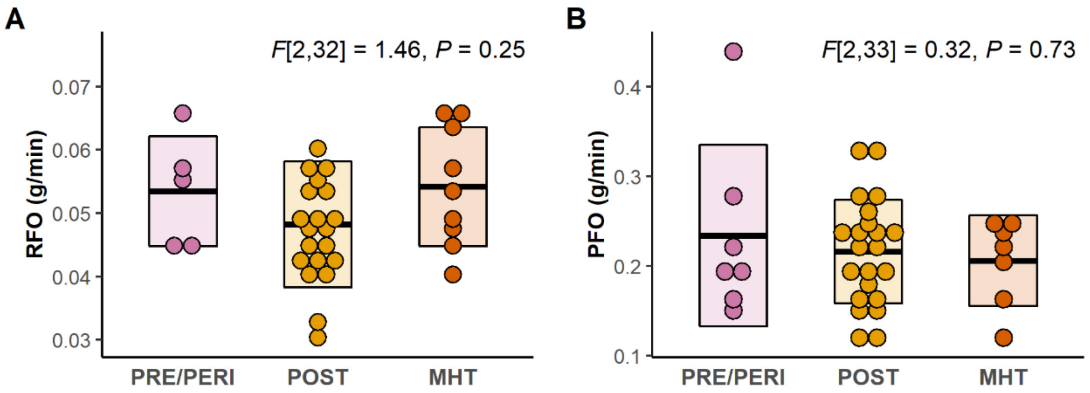

FIGURE 15 Comparison of (A) resting fat oxidation (RFO) and (B) peak fat oxidation (PFO) among menopause groups. MHT, menopausal hormone therapy; POST, post-menopausal; PRE/PERI, premenopausal or perimenopausal. The boxplots show group means with standard deviations.

5.4.5 Blood glucose regulation

The results concerning the primary outcomes are illustrated in FIGURE 16A–16D. No clear association was found between either RFO or PFO and the Matsuda index in *Studies III* or *IV*. However, in *Study IV*, a positive association was found between RFO and glucose AUC (β = 0.52, P = 0.002) as well as with insulin AUC (β = 0.42, P = 0.012). These associations remained unchanged after adjusting for fasting concentrations of NEFA, 3-hydroxybutyrate, glucose, or insulin (data not shown). In a sensitivity analysis of those participants with complete OGTT data, the association between RFO and glucose AUC remained statistically significant (β = 0.51, P = 0.007). In the men in *Study III*, a negative relationship was observed between insulin AUC and absolute PFO (β = -0.42, P = 0.015); however, this association was not statistically significant after adjusting for LM.

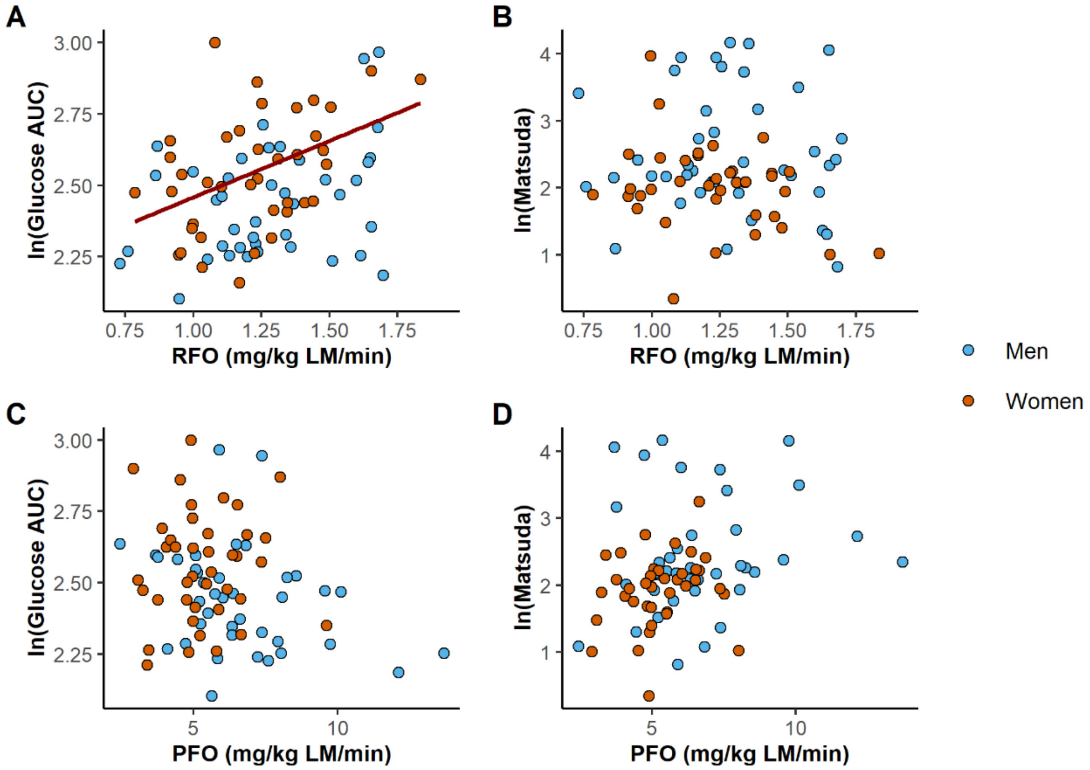

FIGURE 16 The associations between lean mass-relative resting fat oxidation (RFO) and peak fat oxidation (PFO) with glycemic control (A and C) and insulin sensitivity (B and D) in the studied men and women. Oral glucose tolerance test outcomes were *ln*-transformed to meet the residual normality assumption.

6 DISCUSSION

The present PhD thesis primarily investigated the associations between menopause and physical activity with whole-body metabolism, as measured by REE, fat oxidation at rest and during exercise, and the serum metabolome. The main finding was that the menopausal transition is associated with changes in the serum metabolome that are indicative of worsening cardiometabolic health. For the first time, the current thesis directly connected these changes in the serum metabolome to shifting sex hormone levels. A cross-sectional examination revealed that, even after adjusting for FFM and FM, REE declines in women from young to middle adulthood. However, neither middle-aged women with varying menopausal states nor long-term physically more and less active male MZ twins showed differences in REE or fat oxidation rates at rest or during exercise. Despite this, higher physical activity was found to be associated with higher PFO in middle-aged women.

The secondary aim of the current thesis was to investigate the assumption that higher RFO and PFO indicate a better ability to regulate blood glucose levels. The results did not support this premise. RFO was found to reflect the energy balance of the participants and, in middle-aged women, even predicted poorer glycemic control during a glucose challenge. No clear relationship was observed between PFO and glycemic control or insulin sensitivity. Overall, the present thesis highlights the complex interplay between menopause, physical activity, and whole-body metabolism, along with the need for further research.

6.1 Menopause and serum metabolome

Menopause marks the end of the female reproductive life phase, resulting in the cessation of fertility and changes in hormonal balance that affect the functioning of cells beyond reproduction. Through *Study I*, where the women were followed from perimenopause to early postmenopause, the current thesis has demonstrated that menopause modulates metabolism, here at the level of the

serum metabolome. The changes in the metabolome were consistent with those associated with metabolic impairment and may partly explain the increased risk of cardiometabolic diseases such as CVD (Kannel et al., 1976; Muka et al., 2016; Zhu et al., 2019) and T2D (Guo et al., 2019; Muka et al., 2017) observed in postmenopausal women in industrialized populations.

The findings can be summarized by the changes in both causal and system biomarkers. First, the increased number of apoB-containing lipoprotein particles, a known causal biomarker for CVD, has suggested that menopause may accelerate the development of atherosclerosis by increasing the propensity of these particles to enter and become trapped in arterial walls (Sniderman et al., 2019). This finding is consistent with previous research, such as the Study of Women's Health Across the Nation, which found that the number of apoB particles did not change in reproductive women until sharply increasing in the years leading up to and immediately following the final menstrual period (Matthews et al., 2021). Additionally, previous studies using the same NMR metabolomics technology have observed an association between menopause and increase in apoB particle number (Auro et al., 2014; Q. Wang et al., 2018). Of the apoB-containing lipoproteins, the menopausal hormonal shift was found to be particularly responsible for the increase in LDL particle numbers, while MHT protected against this increase. However, the same was not seen as clearly for VLDL particle numbers, suggesting that menopause impairs LDL particle clearance, possibly through mechanisms such as decreased LDL receptor activity (A. Karjalainen et al., 2000; Parini et al., 1997).

Second, system biomarkers provided evidence of wider-scale alterations in the metabolic state of women. The most noticeable change was the increase in VLDL particle number, size, and triglyceride load. These changes were accompanied by an increase in glycerol and decrease in ketone concentrations. Overall, this indicates that menopausal transition may cause an increase in adipose tissue lipolysis rate, perhaps because of decreasing insulin sensitivity, which can lead to an increase in NEFA availability in the liver. The NEFA seems to be primarily used for triglyceride synthesis rather than oxidation. Premenopausal women are more efficient at directing NEFA into oxidation and ketone production than men (Marinou et al., 2011), but this ability may be lost after menopause. The decrease in ketogenesis may also result from increased liver triglyceride accumulation (J. A. Fletcher et al., 2019). However, limitations of the study's body composition analysis have prevented further examination of these mechanisms at the organ level. Energy balance changes may also explain these findings, which will be discussed later.

Menopause was also associated with an increase in HDL triglyceride concentration, and the largest observed association was between menopausal hormonal shift and an increase in small HDL particles. This phenomenon has also been observed in the Study of Women's Health Across the Nation (El Khoudary et al., 2021), where the shift toward smaller HDL particle size was related to the amount of visceral fat and was partly mediated by insulin resistance (Nasr et al., 2022). The increase in HDL triglycerides likely reflects

triglyceride spillover from VLDL particles, which can reduce the capacity of HDL to remove cholesterol from the bloodstream (Vergès, 2015). Additionally, MHT was associated with an increase in larger HDL particle number and HDL cholesterol. Overall, these findings suggest that some of the protective functions of HDL may be lost after menopause.

The findings also indicate that menopause is associated with changes in specific amino acid concentrations, such as increased leucine and decreased glutamine. Together with the other mentioned system biomarkers, these changes align with the overall picture of overnutrition and metabolic deterioration. Indeed, the study participants gained an average of 0.8 kg of FM during the follow-up period (Juppi et al., 2022), indicating that most were in a sustained positive energy balance. However, the current thesis cannot establish the role of body composition in mediating the effects of menopause on serum metabolome because a hormonal shift was not associated with a change in body fat percentage. The use of visceral fat as a mediator could have produced different results, as menopause particularly seems to redistribute FM to central areas (Greendale et al., 2019; Juppi et al., 2022). This limitation of the modeling strategy should be noted. Nevertheless, the findings highlight that the impact of sex hormones may be more apparent at the molecular level than at the phenotypical level.

The question arising from the observed positive energy balance is whether it resulted from an increase in energy intake, a decrease in energy expenditure, or a combination of both. Although the effect of menopause on energy intake remains inconclusive, *Study I* offered some insights into the possibility of dietary changes during the menopausal transition. The study did not collect food diaries, and the crude food frequency-based scale indicated no changes in diet quality during follow-up. However, a shift in the serum fatty acid profile from a polyunsaturated to saturated direction was observed, primarily because of a decrease in linoleic acid concentration. Because the body does not synthesize linoleic acid and its circulating concentration reflects dietary intake (Hodson et al., 2008), it can be proposed that the diet of the participants changed to include more processed foods, which are typically high in SFA and energy density (Vellinga et al., 2022). This potential dietary change could explain the increase in FM, contributing to the observed changes in serum metabolome.

In contrast to menopause, physical activity protects cardiometabolic health (Pedersen & Saltin, 2015). Although the current thesis did not investigate whether engaging in physical activity could prevent menopause-associated changes in the serum metabolome, adjusting for self-reported physical activity had a negligible effect on the results from *Study I*. Additionally, other studies using clinical chemistry data from the ERMA women have shown that higher physical activity levels are not able to prevent the menopause-associated changes in lipid measures and fasting glucose (Hyvärinen et al., 2021; Karvinen et al., 2019). However, it remains to be seen whether the changes can be ameliorated if physical activity increases substantially during the menopausal transition.

6.2 Menopause, physical activity, and resting energy expenditure

With a cross-sectional examination of women of varying ages and menopause states, *Study II* aimed to determine whether the observed changes in serum metabolome and body composition in the women in *Study I* could be partly explained by reduced REE. The results showed that FFM- and FM-adjusted REE was lower in midlife than in earlier adulthood. This is consistent with previous studies showing that young premenopausal women have higher REE than postmenopausal women (Schattinger et al., 2021; Van Pelt et al., 1997). Furthermore, the adjusted REE difference was present when comparing middle-aged mothers with their daughters, suggesting that an age-associated decline in REE was also present when partially controlling for genetic factors. The present thesis and previous cross-sectional evidence from both industrialized (Geisler et al., 2016b; M. J. Müller et al., 2004; Siervo et al., 2015) and gatherer-horticulturalists populations (Gurven et al., 2016) have shown that, even when considering differences in FFM and FM, REE begins to decline earlier than 60 years of age, as suggested recently (Pontzer et al., 2021). It is important to note that the decline in REE may not necessarily represent a decrease in cellular metabolic rate; however, it may result from limitations in adjusting for differences in tissue quality (M. J. Müller et al., 2018). As people age, the proportion of metabolically active cells in tissues decreases (Fragala et al., 2015; Giorgio et al., 2010), leading to a likely overestimation of the decline in REE. In fact, age-associated REE decline was not present when adjusted for total body potassium analysis-measured FFM cell mass (Roubenoff et al., 2000).

Despite middle-aged women having lower adjusted REE than younger women, there was no apparent difference in adjusted REE between the menopause group, which is consistent with previous literature (Bessesen et al., 2015; Duval et al., 2013; Gould et al., 2022; Hodson et al., 2014). While the middle-aged subsample-specific model indicated that the MHT group had a lower FFM-, FM-, and age-adjusted REE than the postmenopausal group, it should be noted that the MHT group was the smallest in the study. Therefore, high uncertainty exists whether this difference can be attributed to MHT use.

It may seem counterintuitive that the loss of ovarian hormone stimulation would not result in energy savings. However, there are several potential explanations for this phenomenon. One possibility is that the effects of sex hormones on REE may be relatively small, making it difficult to detect any decline in REE after menopause, especially when taking into account the limitations of indirect calorimetry and body composition assessment methods (Alcantara et al., 2022; M. J. Müller et al., 2018). Long-term ovarian hormone suppression has been shown to result in a decline of approximately 60 kcal/day compared with placebo or simultaneous estrogen supplementation (Gavin et al., 2020; Melanson et al., 2015). If menopause were to decrease REE by a similar amount, this reduction would be within the range of day-to-day variation (Bader

et al., 2005). Furthermore, aging progresses differently among individuals, so the effects of menopause and aging cannot be easily disentangled.

An alternative explanation for the lack of difference in REE between menopause groups is that women may be reallocating the energy freed from reproduction inside the REE component. This theoretical concept (FIGURE 17) builds on the active grandparent hypothesis (Lieberman et al., 2021), which combines elements from the grandmother hypothesis (Hawkes, 2003) and constrained TEE model (Pontzer, 2018). The central premise is that TEE is homeostatically kept at the same level, perhaps until age 60, when aging-related changes begin to manifest (Pontzer et al., 2021). The cessation of reproductive functions frees energy from the TEE budget, which women ideally use to increase AEE. Hazda hunter-gatherer women, for example, remain highly physically active after their reproductive years (Raichlen et al., 2017) and even increase the time spent foraging to provide resources for their offspring (Hawkes et al., 1997). Notably, Hazda women do not exhibit an increase in body fat percentage or cardiometabolic disease risk in old age (Pontzer et al., 2018). *Study II* found that, even though middle-aged mothers and their daughters differed in adjusted REE, they had very similar adjusted TEE, which could indicate that the mothers compensated for REE decline by increasing AEE. However, reverse causation is equally possible. Typically though, physical activity is proposed to decrease in women living in industrialized nations in middle age around menopause (Duval et al., 2013; Lovejoy et al., 2008), which is similar to animal experiments (Leeners et al., 2017; Van Pelt et al., 2015). However, our studies have not observed this trend (Hyvärinen et al., 2021; Juppi et al., 2022).

If not used for the movement or maintenance of a body that encounters demanding daily physical challenges, women may redirect the freed energy from reproduction toward building energy storage and bodily defenses (Lieberman et al., 2021). In this scenario, REE does not decline as expected because of factors such as increased activity of the pituitary-adrenal axis and the immune and sympathetic nervous systems. Studies have shown that higher systolic blood pressure (Bosy-Westphal et al., 2008; Brock et al., 2012; Creber et al., 2018; Luke et al., 2004; Sriram et al., 2014), insulin resistance (Bosy-Westphal et al., 2008), and C-reactive protein concentrations (Geisler et al., 2016a) are all associated with elevated REE. These factors may offset the expected decline in REE, leading to an increased risk of cardiometabolic diseases after menopause.

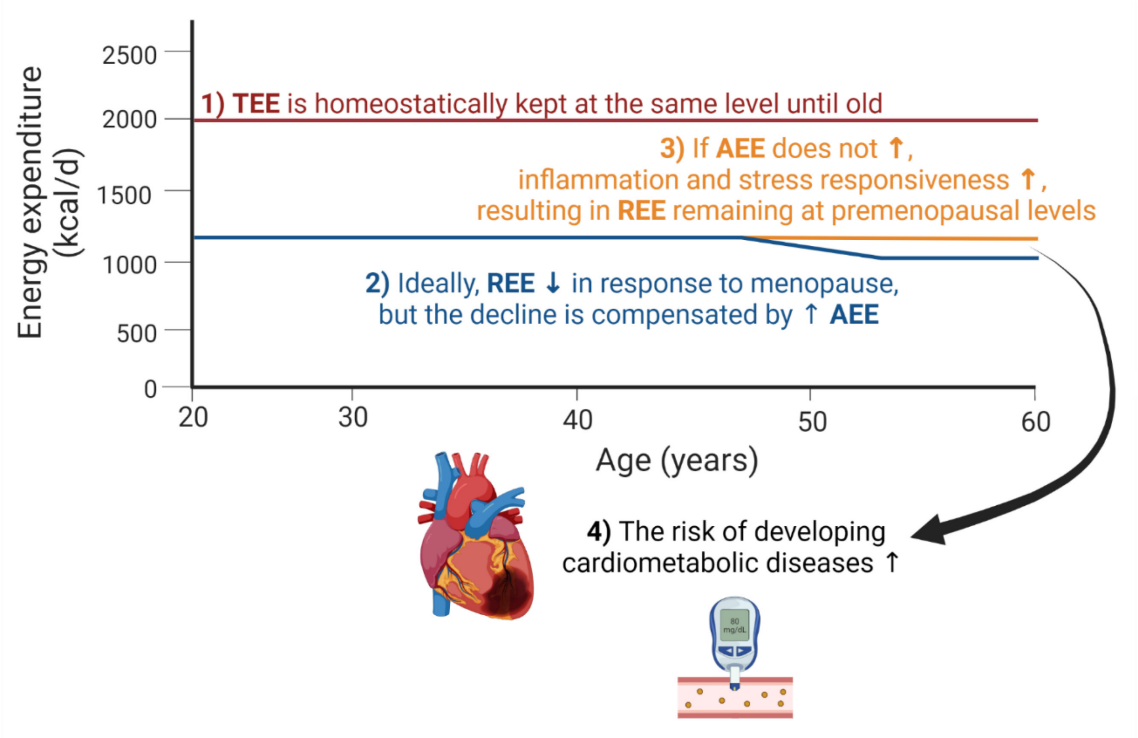

FIGURE 17 Illustrating the impact of menopause on energy resource allocation: a figure modified from Lieberman et al. (2021). The theory posits that daily total energy expenditure (TEE) remains constant until old age (1). Ideally, the energy freed from reproduction after menopause is used for activity energy expenditure (AEE) and the maintenance of a body capable of varied movement (2). However, if this is not the case, the excess energy may be stored as fat, potentially leading to an overactive immune system and heightened stress responsiveness (3). These alterations, which maintain resting energy expenditure (REE) at premenopausal levels, increase the risk of developing cardiometabolic diseases (4).

For the presented theory to hold, evidence of an association between menopause and the REE-elevating mechanisms described above is needed. Determining whether menopause increases systemic inflammation has been challenging also because of the lack of perfect biomarkers (Furman et al., 2019). However, no longitudinal studies have reported increases in traditional inflammation markers, such as C-reactive protein or interleukin 6, following menopause. In *Study I*, no increase in GlycA, which is a composite biomarker of chronic inflammation, was observed. However, a preliminary analysis of 51 women who returned for the cohort fourth-year follow-up has shown a substantial increase in GlycA concentration as the women progressed in postmenopause. This suggests that a menopausal hormonal shift may not directly increase systemic inflammation, but it may develop later as a secondary consequence. The impact of menopause on the hypothalamic-pituitary-adrenal axis or sympathetic nervous system is unclear. Urinary cortisol levels peak at late perimenopause and are positively associated with FSH levels (Woods et al., 2006). The sympathetic nervous system regulates blood pressure acutely and chronically, and the effect is more significant in men and postmenopausal women than in premenopausal women (Barnes et al., 2014). Systolic blood

pressure increases with age in women, but menopause does not seem to accelerate this (Clayton et al., 2022; El Khoudary et al., 2020; Hyvärinen et al., 2021). These observations suggest that neuroendocrine changes may be less likely to be the destination of energy reallocation after menopause than energy storage and inflammation.

The present thesis also examined the adaptability of REE, specifically in relation to physical activity. Menopause and physical activity may have a somewhat related response because physical activity is proposed as downregulating REE by also repressing sex hormone concentrations in both men and women (Pontzer, 2018). However, no significant differences in absolute or LM- and FM-adjusted REE were observed between physically active and inactive cotwins in *Study III*. This contradicts the constrained TEE model. Additionally, self-reported physical activity measures were not associated with adjusted REE. However, among all twin pairs, familial dependency in REE was slightly lower than what has been reported earlier (Bouchard et al., 1989), possibly because of the selection of twins based on potential physical activity discordance. These results align with the majority of observational and intervention studies that have shown that REE remains unchanged after exercise training (MacKenzie-Shalders et al., 2020; Thurber et al., 2019) or that the decline is so modest that it cannot fully explain TEE conservation (Westerterp et al., 1992). Ultimately, the constrained TEE model remains a hypothesis, and more evidence of its existence, especially in the general population, along with its mechanisms, are needed before its acceptance (Halsey, 2021; Melanson, 2017).

6.3 Menopause, physical activity, and fat oxidation

To gain a more comprehensive understanding of energy metabolism, the current thesis also examined the adaptability of fat oxidation at rest and during exercise in response to menopause status and physical activity. Therefore, in *Studies III* and *IV*, RFO and PFO were compared between physically active and inactive male cotwins and middle-aged women representing different menopause states. In *Study IV*, the relationship between physical activity, here as measured by both self-report and accelerometer, and fat oxidation outcomes was also examined among middle-aged women.

The nature and value of RFO as an outcome in metabolism research is a topic of debate (Galgani & Fernández-Verdejo, 2021). However, the findings of the present thesis, as well as previous studies (Goris & Westerterp, 2000), have indicated that REE is primarily a marker of energy balance. Therefore, the lack of association between menopause status or physical activity level and RFO were anticipated. However, some studies have reported lower RFO after ovarian hormone suppression (Grant et al., 2022) or menopause transition (Lovejoy et al., 2008), lower RER in MHT users (Reimer et al., 2005), or higher RFO after exercise training (Amaro-Gahete, De-la-O, et al., 2020). These studies are exceptions, and the results most likely reflect the differences in energy balance between groups

or time points. To demonstrate the validity of the argument, most studies have not observed changes in the resting substrate after ovarian hormone suppression (Day et al., 2005), MHT use (Bessesen et al., 2015; Lwin et al., 2008; Marlatt et al., 2020; O'Sullivan et al., 1998), between menopause groups (Bessesen et al., 2015; Gould et al., 2022; Schattinger et al., 2021), or following exercise interventions (Martin et al., 2019; Scharhag-Rosenberger et al., 2010; Wilmore et al., 1998).

The current thesis can offer two examples of how not considering the role of energy balance as a determinant of RFO can lead to biased conclusions. First, the strongest predictor of RFO was found to be serum 3-hydroxybutyrate concentration, perhaps because 3-hydroxybutyrate is highly responsive to changes in energy balance (Huffman et al., 2022). At the end measurements in *Study I*, the participants were likely in a more positive energy balance than at the first measurements, and as a result, their 3-hydroxybutyrate levels were lower than at the first measurement. If RFO had been assessed in *Study I*, it would have likely declined, probably appearing as though menopause reduces the ability to use fatty acids at rest. However, this would have been a false conclusion because of the changes in energy balance. Second, in *Study IV*, the MHT group had numerically higher RFO compared with the POST group. However, this difference was no longer observed when adjusting for body composition variables and prior energy intake. Therefore, the explanation for the numerical group difference was that the LM- and FM-adjusted energy intake was approximately 250 kcal/d lower in the MHT group than in the POST group. Interested readers can compare FIGURE 15A with FIGURE 1 in the original publication (*Study IV*), where RFO is plotted using adjusted residuals, hence illustrating the issue.

Cardiorespiratory fitness was found to be the primary PFO determinant, which is in agreement with previous larger-scale cross-sectional studies (Chrzanowski-Smith, Edinburgh, Thomas, et al., 2021; G. Fletcher et al., 2017; M. C. Venables et al., 2005). *Study III* also demonstrated that PFO likely has a significant genetic component. Despite recruiting twin pairs based on potential physical activity discordance, the ICC estimates were similar to those of a previous twin study using RER as an outcome (Bouchard et al., 1989). In *Study IV*, no associations between energy intake or diet macronutrient distribution and PFO were found. However, an analysis of serum NEFA and glycerol concentrations before and after testing revealed that PFO was connected to the participant's metabolic state, as previously shown in male athletes (Frandsen et al., 2019).

Contrary to the hypothesis that menopause reduces fat oxidation capacity during exercise, PFO was strikingly similar between the menopause groups. Furthermore, no associations were found between PFO and serum concentrations of E2 or FSH. This contradicts previous research (Abildgaard et al., 2013) that suggested that fat oxidation during exercise is lower in postmenopausal women than in premenopausal women. However, there were notable differences between the study participants and their designs. First, Abildgaard et al. (2013) compared premenopausal and postmenopausal women, while most of the participants in the PRE/PERI group of *Study IV* were

perimenopausal. The menopausal transition may influence fat oxidation capacity already in its early stages, thus limiting the ability of *Study IV* to detect its effects. Second, the two studies used different testing protocols. Abildgaard et al. (2013) measured fat oxidation during a 45-minute cycling session at 45% of $\dot{V}O_{2PEAK}$ intensity, while *Study IV* used the graded FAT_{MAX} protocol. Most of the participants in *Study IV* reached their PFO early in the test, after around 4–12 minutes of cycling. E2 has been suggested to improve fat oxidation by increasing lipolysis at adipose tissue and intramyocellular levels (Oosthuyse et al., 2022); for example, the sex difference in lipolysis stimulation seems to increase with exercise duration (Hellström et al., 1996). Therefore, the testing protocol in *Study IV* may have been too short to detect any differences in lipid metabolism between the study groups. Additionally, previous research has found that in premenopausal women with normal but not overweight BMI, upper body dominant fat mass distribution is associated with lower fat oxidation capacity during exercise (Isacco et al., 2020). It is possible that the amount of FM could also moderate the effect of menopause on PFO since menopause redistributes FM to a more android pattern (Greendale et al., 2019; Juppi et al., 2022). Further investigation is warranted to determine whether menopause has a greater impact on PFO in women who are leaner or experience larger changes in body fat distribution during the menopausal transition.

The present thesis found some evidence that physical activity is positively associated with PFO, particularly among middle-aged women. This may be because the testing protocol used in *Study IV* was explicitly developed for PFO assessment. Most previous larger-scale cross-sectional studies have also observed an association between self-reported physical activity and PFO (G. Fletcher et al., 2017; M. C. Venables et al., 2005). In *Study IV*, a similar-sized association was also found when using accelerometer-measured physical activity as the explanatory variable. However, the positive effects of physical activity on PFO could not be confirmed by comparing physical activity discordant MZ cotwins. Those factors related to study design, participants, and PFO characteristics may explain the lack of any findings. The used testing protocol was not tailored to measure PFO, and deviations from the standard FAT_{MAX} protocol, such as testing participants in a postabsorptive state and using short 2-minute workloads, may have introduced errors into the measurements. However, it is worth noting that the load increases were small and that most participants reached steady-state conditions during the period where PFO was calculated. Additionally, research has shown that relatively high exercise volumes are necessary to see changes in PFO, even in individuals with low exercise levels at baseline (Nordby et al., 2015; Rosenkilde et al., 2010). In *Study III*, physical activity discordant cotwins had a mean AEE difference of 205 kcal/d for the past year, which may not have been sufficient to cause notable differences in PFO. Furthermore, it is important to note that the day-to-day variability of PFO was substantial (Chrzanowski-Smith et al., 2018, 2020) and higher than that of, for example, $\dot{V}O_{2PEAK}$ (Skinner et al., 1999). Therefore, larger sample sizes may be required to detect an effect of similar magnitude in PFO as in $\dot{V}O_{2PEAK}$.

6.4 Fat oxidation and blood glucose regulation

The secondary aim of the present thesis was to examine whether RFO and PFO can explain the variability in glycemic control and insulin sensitivity during a standard OGTT. The results did not support the hypothesis that RFO serves as a marker of a better ability to regulate blood glucose levels in individuals without known metabolic disorders. Additionally, the literature lacks biological evidence that can support this assumption. This finding is consistent with the results of Arad et al. (2022), who also did not find a relationship between RFO and insulin sensitivity, as measured by the Matsuda index. Furthermore, the study found that RFO was similar between metabolically healthy and unhealthy groups with normal weight and obesity, despite the metabolically unhealthy subgroups having lower insulin sensitivity. The study's strength lies in the effort made to ensure that all participants were in a similar metabolic state by providing them with all food for 9 days before measurements.

In *Study IV,* higher RFO in middle-aged women was even associated with poorer glycemic control, a finding consistent with previous research (Kunz et al., 2000). The likely explanation for this association is that the women who were more dependent on fat oxidation may have had temporarily reduced insulin sensitivity, impairing their ability to use glucose during an OGTT performed immediately after the RFO measurement. The direct relationship between RFO and glucose tolerance was supported by the lack of association attenuation when adjusting for various potential mediators. This finding is in line with previous research, which has shown that fasting and negative energy balance decrease both RER and insulin sensitivity (Jørgensen et al., 2015) and that longer fasting duration before an OGTT predicts higher 2-hour glucose levels (Clemmensen et al., 2020; Hulmán et al., 2013).

The present thesis neither supports the notion that a higher PFO indicates better glycemic control or insulin sensitivity. Although early studies (Robinson et al., 2015, 2016) suggested a positive correlation between PFO and insulin sensitivity, later studies, including the ones reported in the current thesis and by other researchers (Amaro-Gahete et al., 2021; Chrzanowski-Smith, Edinburgh, Thomas, et al., 2021), have been unable to confirm this relationship. One potential explanation for this discrepancy is the difference in exercise intensities at which PFO was reached in the cited studies. The young men studied by Robinson et al. (2015, 2016) reached PFO at much higher intensities (58% of $\dot{V}O_{2MAX}$) compared with the participants in the other studies (34–44% $\dot{V}O_{2PEAK}$). As pressure to use intramyocellular lipids increases with exercise intensity (Romijn et al., 1993), it is possible that the studies by Robinson et al. (2015, 2016) measured slightly different aspects of the fat oxidation pathway compared with other studies. It is worth noting that individuals with obesity and low cardiorespiratory fitness may have higher circulating levels of NEFA, which may explain why they have similar PFO to individuals with higher cardiorespiratory fitness but less FM (Lanzi et al., 2014). However, the study by Lanzi et al. (2014) found that

participants with obesity and low cardiorespiratory fitness had a lower ability to use fatty acids at higher relative exercise intensities, as indicated by left-sided fat oxidation curves. Therefore, to better understand the relationship between fat oxidation capacity and insulin sensitivity, it may be more beneficial to focus testing at higher relative exercise intensities, particularly given the potential link between insulin resistance and intramyocellular lipid handling (Roden & Shulman, 2019).

To understand the connections between fat oxidation during exercise and metabolic health while controlling for the influence of exercise intensity, the participants in the study by Arad et al. (2022) were tested for 45 minutes at both 75% and 100% of their gas exchange thresholds. The results confirmed that fat oxidation increases with exercise duration because fat oxidation rates were higher after 45 minutes than after 15 minutes of cycling at both intensities. A statistically significant correlation (r = 0.40, P = 0.026) between FFM-relative fat oxidation and the Matsuda index was observed only at 100% of the gas exchange threshold, indicating that fat oxidation capacity at higher exercise intensities may be a better indicator of insulin sensitivity. However, even this association was absent in individuals with obesity, highlighting the complexity of the relationship.

6.5 Strength and limitations

The main strengths of the present PhD thesis include a precise determination of menopause status using menstrual cycle and FSH concentration data and efforts to limit the confounding effect of age while investigating associations between menopause and study outcomes. In *Study I*, the follow-up was specifically designed to capture the period of the most dramatic changes in sex hormone levels around the final menstrual period, which can help minimize the effects of aging. Additionally, the original cohort was not a convenience sample but was randomly drawn from the Population Information System, increasing the generalizability of the findings. In *Study IV*, a sample of similar-aged women was recruited to produce a sample that would differ only by their menopause status, resulting in more similar-aged groups than typically seen in the literature. The MZ cotwin study design in *Study III* was unique in that it allowed for the investigation of the effect of physical activity while controlling for genotype and childhood environment. Furthermore, in *Study II*, we were able to partly control for these factors by studying mother–daughter pairs.

The main limitation of the current thesis is the small sample sizes in some studies. Despite an extensive search, only 10 MZ twin pairs where the brothers were discordant for physical activity were located in *Study III*. Additionally, recruitment for *Study IV* had to be halted prematurely in March 2020 because of the COVID-19 pandemic, leading to uneven participant distribution across groups and small numbers in the PRE/PERI and MHT groups. To compensate for these smaller-than-expected group sizes, data from four studies were pooled

in *Study II*, which may have introduced a potential confounding cohort effect on the results. However, a sensitivity analysis was conducted, and no clear effect was found.

Additionally, there are some methodological limitations to consider. First, the exercise protocol in *Study III* was not specifically designed to assess PFO. Second, there may have been limitations in assessing the effects of sex hormones. Here, E2 concentrations were analyzed using immunoassays, which have poor validity in measuring low E2 concentrations in postmenopause (Rosner et al., 2013). Furthermore, the menstrual cycle phase and oral contraception use were not considered in *Studies II* and *IV*. However, these limitations are unlikely to have affected the overall conclusions of the study because they are expected to introduce greater variation and make finding differences between age groups more difficult. Finally, MHT use was not standardized across studies, resulting in the inclusion of women who had used different formulations, dosages, and duration of MHT.

6.6 Future directions

The recent studies, including *Study I* and the publications by Auro et al. (2014) and Q. Wang et al. (2018), have provided a characterization of menopause-associated changes in the serum metabolome, ranging from population-level changes to those linked to sex hormone levels. However, further research is required to fully understand the underlying mechanisms and molecular pathways driving these changes. Additionally, although the changes in the serum metabolome have been identified, it remains unclear whether they have any clinically meaningful implications. As a result, it is crucial to conduct long-term follow-up studies following women into old age to investigate menopause-associated metabolite changes and their relationship with disease endpoints.

The literature on the associations between menopause and REE is limited because it has primarily relied on cross-sectional studies, which may not be adequate to fully understand the complex relationship between menopause and REE. Although REE measurements were not performed in *Study I*, the study's design provided an excellent template for future research endeavors. To gain a deeper understanding of the effects of menopause on REE, experimental evidence is also necessary. Quasi-experimental designs—such as comparing premenopausal women undergoing prophylactic salpingo-oophorectomy, perimenopausal women starting MHT, and postmenopausal women ceasing MHT use—to matched controls would provide insights into how changes in ovarian hormone concentrations impact metabolism. Additionally, in countries where financial compensation to research participants is allowed, ovarian hormone suppression is an established method to study the effects of menopause. To comprehensively understand how menopause affects energy expenditure, it is important to combine REE assessments with TEE and physical activity assessments. As the costs of DLW measurements decrease, this approach may

become more feasible in the future. To assess the potential reallocation of energy, studies should also measure body composition in detail, along with the activity of sympathetic nervous and inflammatory systems. Sophisticated statistical analysis methods could be used to analyze potential causal pathways.

The literature on the effects of menopause on fat oxidation during exercise is even more limited and inconclusive compared with REE. The previously suggested study designs would also fit this purpose. It is important to note that the use of the FAT_{MAX} protocol may not be the most effective method for evaluating the ability to use endogenous lipid stores during exercise. However, it should also be acknowledged that the critique of the FAT_{MAX} protocol is still speculative. An exciting research idea would be to compare the FAT_{MAX} protocol against longer-stage protocols in different menstrual cycle phases and menopause states. Additionally, it is important to note that substrate use measurements may be affected by significant intraindividual day-to-day variations; thus, duplicate measurements would improve the validity of such studies (Chrzanowski-Smith et al., 2020).

The constrained TEE model is still a hypothesis that requires further investigation. The limitations and knowledge gaps surrounding the model have been discussed (Halsey, 2021; Melanson, 2017). Although some ecological, observational, and experimental data support the model, long-term exercise interventions not focused on weight loss are needed for a more comprehensive understanding of the model. These studies should require DLW assessment to measure TEE. Metabolic chambers can be used to investigate the circadian variability of REE and mitochondrial respiration measurements from tissue samples to detect changes in mitochondrial efficiency.

7 MAIN FINDINGS

The key findings of the current PhD thesis are as follows:

1. At the serum metabolome level, menopause is associated with an increase in apoB-containing lipoproteins, as well as increases in VLDL and HDL triglyceride and LDL cholesterol concentrations. Additionally, menopause is associated with an increase in glycerol and leucine concentrations, a decrease in glutamine, citrate, and ketone concentrations, and a shift in the fatty acid profile from PUFA toward SFA. The hormonal changes associated with menopause may partially explain these changes in the serum metabolome. Furthermore, MHT is associated with protection from changes in LDL and HDL measures. These findings suggest that menopause may predispose women to cardiometabolic diseases.

2. The menopause status or serum levels of E2 and FSH are not associated with REE, RFO, or PFO. These results suggest that a slowing of basal metabolism or decline in the ability to use fatty acids cannot be considered as potential explanations for the changes in the serum metabolome or body composition associated with menopause.

3. Physical activity is not associated with REE or RFO. However, higher levels of self-reported and accelerometry-measured physical activity were found to be associated with higher PFO in middle-aged women. This suggests that exercise can be recommended for women who want to improve their ability to oxidize fat, regardless of the reason.

4. Neither higher RFO nor PFO indicate better glycemic control or insulin sensitivity. RFO increases with negative energy balance and may temporarily impair glucose tolerance. Therefore, researchers and clinicians should exercise caution in interpreting fat oxidation measurements as an indicator of an individual's metabolic health status.

YHTEENVETO (SUMMARY IN FINNISH)

Menopaussi johtuu munasarjojen munasolutuotannon loppumisesta, ja se todetaan 12 kuukautta viimeisten kuukautisten päättymisestä. Menopaussi muuttaa elimistön sukupuolihormonien pitoisuuksia: estradiolin pitoisuus laskee ja follikkeleita stimuloivan hormonin (FSH) nousee. Erityisesti estradiolin pitoisuuden laskun ajatellaan muuttavan elimistön aineenvaihduntaa. Tutkimusten mukaan menopaussilla on itsenäinen vaikutus rasvamassan lisääntymiseen erityisesti keskivartalon alueelle. Rasvamassan lisääntyminen kohottaa sydän- ja verenkiertoelimistön sekä aineenvaihdunnallisten sairauksien riskiä. Sairausriskin kohoaminen voi selittyä myös hormonimuutosten suorilla vaikutuksilla aineenvaihduntareittien toimintaan. Mekanismit menopaussin, kehonkoostumuksen ja sairausriskin välillä ovat pääosin selvittämättä.

Fyysinen aktiivisuus suojaa sydän- ja verenkiertoelimistön sekä aineenvaihdunnan sairauksilta. Fyysisen aktivisuuden vaikutusmekanismien tutkimista vaikeuttaa se, että samat geenit määrittävät sekä fyysistä aktiivisuutta että paremmin toimivaa energia-aineenvaihduntaa. Tästä syystä havainnoivat tutkimukset voivat yliarvioida fyysisen aktiivisuuden aineenvaihduntaa parantavia ja sairauksia ehkäiseviä vaikutuksia. Satunnaistetut kontrolloidut kokeet antavat tarkempaa tietoa syy-seuraussuhteista, mutta kokeet ovat tyypillisesti lyhytkestoisia. Tutkimusasetelmien heikkoudet voidaan ylittää tutkimalla identtisiä kaksosia, jotka eroavat toisistaan aikuisiän fyysisen aktiivisuuden suhteen. Asetelmalla pystytään kontrolloimaan sekä perimän että lapsuuden kasvuympäristön sekoittavat vaikutukset, jolloin fyysisen aktiivisuuden pitkäaikaisvaikutuksia voidaan tutkia luotettavasti.

Menopaussin aiheuttama hormonimuutos saattaa alentaa lepoenergiankulutusta ja heikentää kykyä käyttää rasvaa energianlähteenä. Tutkimusnäyttö aiheesta pohjautuu kuitenkin pitkälti eläinkokeisiin, eli tulosten yleistettävyys ihmisiin on kyseenalainen. Yllättäen myös fyysisen aktiivisuuden terveysvaikutusten on ajateltu selittyvän aineenvaihdunnan muutoksilla, jotka alentavat lepoenergiankulutusta. Teoria perustuu oletukseen, että päivittäinen kokonaisenergiankulutus on tarkkaan säädelty ilmiö esimerkiksi happoemästasapainon ja verensokerin tavoin. Jos fyysiseen aktiivisuuteen käytetty energiankulutus laskee, voi elimistö käyttää vapautuneen energian esimerkiksi rasvavarastojen kasvattamiseen, tulehdusprosesseihin ja voimakkaampiin stressireaktioihin. Fyysinen aktiivisuus voi pienentää sairausriskiä myös parantamalla rasva-aineenvaihdunnan toimintaa.

Tämän väitöskirjatutkimuksen ensisijaisena tavoitteena oli selvittää, ovatko menopaussi ja fyysinen aktiivisuus yhteydessä lepoenergiankulutukseen, rasvan käytön tehokkuuteen ja veren aineenvaihduntatuoteprofiilin muutoksiin. Rasvan käyttöä mitattiin levossa ja sen korkeinta mahdollista tehoa liikuntatestin aikana. Veren aineenvaihduntatuoteprofiilia voidaan käyttää koko elimistön aineenvaihdunnan tutkimiseen molekyylitasolla, koska kaikki kudokset käyttävät veren kuljettamia aineenvaihdunnan lähtöaineita ja vapauttavat vereen aineenvaihdunnan tuotteita.

Väitöskirjan toissijaisena tavoitteena oli selvittää, onko korkeampi rasvan käyttöteho levossa tai liikkuessa yhteydessä terveempään sokeriaineenvaihdun-

nan säätelyyn. Luurankolihassolujen herkkyys insuliinin vaikutuksille heikentyy, jos soluihin kertyy haitallisia rasva-aineita. Perimmäinen syy ilmiöön on liiallinen energiansaanti. Pitkälle edetessään tila edistää verensokerin kohoamista ja tyypin 2 diabeteksen puhkeamista. Tästä syystä korkeamman rasvan käyttökyvyn ajatellaan kertovan paremmasta insuliiniherkkyydestä ja terveemmästä sokeriaineenvaihdunnasta. Tieto perustuu kuitenkin vain muutamaan tutkimukseen.

Väitöskirja koostuu neljästä alkuperäistutkimuksesta. Ensimmäisessä tutkimuksessa selvitettiin menopaussin yhteyttä veren aineenvaihduntatuoteprofiilin muutoksiin käyttämällä ERMA-tutkimuksen aineistoa. Yhteensä 218 naista seurattiin menopaussin yli. Alkumittauksissa naiset olivat perimenopaussissa, eli heillä oli vielä epäsäännöllinen kuukautiskierto. Loppumittauksissa naisten kuukautiskierto oli päättynyt vähintään 6 kuukautta aiemmin. Tutkimuksen alussa tutkittavat olivat keskimäärin 51,7-vuotiaita (keskihajonta 1,9 vuotta) ja seurannan keston mediaani oli 14 kuukautta (kvartaaliväli 8–20 kuukautta). Tutkimuksen tavoitteena oli minimoida ikääntymisen sekoittava vaikutus tutkimalla aineenvaihduntatuoteprofiilin muutoksia ajanjaksolla, jolloin estradiolipitoisuuksien muutos on kaikkein voimakkain. Seurannan aikana 35 naista aloitti estradiolia sisältävän hormonikorvaushoidon, jolloin voitiin selvittää suojaako estradiolipitoisuuksien laskun ehkäisy aineenvaihduntatuoteprofiilin muutoksilta. Alku- ja loppumittausnäytteistä määritettiin 180 seerumin aineenvaihduntatuotetta ydinmagneettisella resonanssispektroskopialla sekä estradiolin ja FSH:n pitoisuudet immunomäärityksellä. Tilastoanalyyseissä huomioitiin ikä seurannan alussa, seurannan kesto, koulutustaso, tupakointistatus, alkoholinkäyttö, fyysisen aktiivisuuden taso ja ravitsemuksen laatu.

Väitöskirjan toisessa tutkimuksessa selvitettiin iän ja menopaussistatuksen yhteyttä lepoenergiankulutukseen. Tutkimusjoukon muodosti 120 naista (ikä 17–58 vuotta) EsmiRs-, Calex-, Physique- ja NO-REDS-tutkimuksista. Iän yhteyttä lepoenergiankulutukseen tutkittiin käyttämällä ikää jatkuvana muuttujana ja jakamalla tutkittivat ikäluokkiin. Ikäluokkajako 17–21-, 22–39- ja 40–59-vuotiaisiin perustui aiempaan tutkimustietoon mahdollisista energiankulutuksen käännekohdista elämänkaaren aikana. Aineisto sisälsi 16 äiti-tytärparia, joissa äiti oli keski-ikäinen ja tytär noin 20-vuotias. Tämä parittainen alaryhmäanalyysi mahdollisti perimän vaikutuksen osittaisen kontrolloinnin. Tutkimuksen 59 keski-ikäistä naista jaettiin edelleen menopaussiluokkiin: tutkittavista 19 ei ollut läpikäynyt menopaussia, 30 oli ohittanut menopaussin ja 10 oli ohittanut menopaussin mutta käytti hormonikorvaushoitoa. Heiltä mitattiin myös veren estradioli- ja FSH-pitoisuudet. Tilastolliset analyysit vakioitiin rasvattoman massan ja rasvamassan määrillä, ja niissä huomioitiin äiti-tytärparien sukulaisuussuhde.

Väitöskirjan kolmannessa osajulkaisussa tutkittiin fyysisen aktiivisuuden yhteyttä lepoenergiankulutukseen ja rasvan käyttökykyyn. Aineiston muodosti FITFATTWIN-tutkimuksen 23 identtistä iältään 32–37-vuotiasta mieskaksosparia, joista 10 parin veljekset erosivat toisistaan fyysiseltä aktiivisuudeltaan edeltävän kolmen vuoden ajalta. Tilastoanalyysit vakioitiin rasvattomalla massalla ja lepoenergiankulutuksen osalta myös rasvamassalla, ja niissä huomioitiin veljesten sukulaisuussuhde.

Väitöskirjan viimeinen tutkimus selvitti menopaussin ja sukupuolihormonien pitoisuuksien yhteyttä rasvan käytön tehokkuuteen. EsmiRs-tutkimus rekrytoi 42 naista (ikä 52–58), jotka jaettiin menopaussiluokkiin kuten väitöskirjan toisessa tutkimuksessa: 7 tutkittavaa ei ollut kokenut menopaussia, 26 oli ohittanut menopaussin ja 9 käytti hormonikorvaushoitoa. Tilastoanalyysit vakioitiin rasvattoman massan määrällä. Kahdessa viimeisessä väitöskirjan tutkimuksessa tutkittavat suorittivat sokerirasituskokeen. Veren sokeri- ja insuliinimittausten avulla määritettiin tutkittavien sokerinsieto ja insuliiniherkkyys.

Väitöskirjan ensimmäisessä osatyössä selvisi, että menopaussi oli yhteydessä valtimonkovettumatautia aiheuttavien LDL-partikkelien määrän lisääntymiseen. Muutoksia havaittiin myös systeemisissä merkkiaineissa, jotka eivät aiheuta sairauksia mutta kertovat aineenvaihdunnan tilasta. Erityisesti suurten VLDL- ja pienten HDL-partikkelien sekä niiden sisältämien triglyseridien määrä kasvoi. Myös glyserolin ja leusiinin pitoisuudet kohosivat ja ketoaineiden pitoisuudet laskivat. Veren rasvahappoprofiili muuttui siten, että tyydyttyneiden rasvahappojen osuus kasvoi ja monityydyttyneiden rasvahappojen osuus pieneni. Suurin osa muutoksista oli yhteydessä sukupuolihormonien pitoisuuksien muutokseen. Erityisesti systeemisten merkkiaineiden muutokset kertonevat myös positiivisesta energiatasapainosta ja insuliiniherkkyyden heikkenemisestä, joita menopaussi voi edistää. Hormonikorvaushoito suojasi LDL-partikkelien määrän nousulta ja kasvatti suurten HDL-partikkelien määrää ja HDL-kolesterolin pitoisuutta. Toisessa osatyössä havaittiin, että lepoenergiankulutus laski iän myötä. Keski-ikäisten naisten lepoenergiankulutus oli noin 100 kcal/vrk alhaisempi kuin nuorten naisten lepoenergiankulutus. Sama ero havaittiin, kun verrattiin keski-ikäisiä naisia heidän tyttäriinsä. Keski-ikäisten naisten joukossa menopaussistatus tai sukupuolihormonien pitoisuudet eivät kuitenkaan selittäneet lepoenergiankulutuksen eroja. Kolmannessa osatyössä fyysisen aktiivisuuden suhteen eroavien identtisten kaksosveljesten välillä ei havaittu eroja lepoenergiankulutuksessa eikä rasvan käytön muuttujissa. Neljännen osatyön keski-ikäisten naisten joukossa sekä itseraportoitu että kiihtyvyysanturimittauksella määritetty fyysinen aktiivisuus olivat yhteydessä korkeampaan rasvan käytön huipputehoon liikuntatestin aikana. Menopaussistatus tai sukupuolihormonien pitoisuudet eivät selittäneet rasvan käytön eroja. Korkeampi rasvan käytön teho levossa tai liikkuessa ei ollut yhteydessä parempaan sokerinsietoon eikä insuliiniherkkyyteen kolmannessa eikä neljännessä osatyössä.

Tämä väitöskirjatutkimus osoittaa, että menopaussi muuttaa naisten aineenvaihduntaa. Muutos veren aineenvaihduntatuoteprofiilissa johtuu osin suoraan sukupuolihormonipitoisuuksien muutoksesta ja kertoo kohonneesta aineenvaihduntasairauksien riskistä menopaussin jälkeen. Menopaussi ei tämän tutkimuksen perusteella alenna merkittävästi lepoenergiankulutusta tai heikennä kykyä käyttää rasvaa energianlähteenä. Fyysinen aktiivisuus saattaa tehostaa ainakin keski-ikäisten naisten kykyä käyttää rasvaa liikkuessa. Rasvan käyttökyvyn tehostuminen ei kuitenkaan suoraan paranna sokeriaineenvaihduntaterveyttä.

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ORIGINAL PAPERS

I

MENOPAUSE MODULATES THE CIRCULATING METABOLOME: EVIDENCE FROM A PROSPECTIVE COHORT STUDY

by

Karppinen, J.E., Törmäkangas, T., Kujala, U.M., Sipilä, S., Laukkanen, J., Aukee, P., Kovanen, V. & Laakkonen, E.K. 2022

European Journal of Preventive Cardiology vol 29 (10): 1448–1459

https://doi.org/10.1093/eurjpc/zwac060

ESC European Society of Cardiology

European Journal of Preventive Cardiology (2022) **00**, 1–12
https://doi.org/10.1093/eurjpc/zwac060

FULL RESEARCH PAPER
Gender-specific conditions

Menopause modulates the circulating metabolome: evidence from a prospective cohort study†

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Received 26 January 2022; revised 22 February 2022; accepted 17 March 2022

Aims We studied the changes in the circulating metabolome and their relation to the menopausal hormonal shift in 17β-oestradiol and follicle-stimulating hormone levels among women transitioning from perimenopause to early postmenopause.

Methods and results We analysed longitudinal data from 218 Finnish women, 35 of whom started menopausal hormone therapy during the study. The menopausal transition was monitored with menstrual diaries and serum hormone measurements. The median follow-up was 14 months (interquartile range: 8–20). Serum metabolites were quantified with targeted nuclear magnetic resonance metabolomics. The model results were adjusted for age, follow-up duration, education, lifestyle, and multiple comparisons. Menopause was associated with 85 metabolite measures. The concentration of apoB (0.17 standard deviation [SD], 99.5% confidence interval [CI] 0.03–0.31), very-low-density lipoprotein triglycerides (0.25 SD, CI 0.05–0.45) and particles (0.21 SD, CI 0.05–0.36), low-density lipoprotein (LDL) cholesterol (0.17 SD, CI 0.01–0.34) and particles (0.17 SD, CI 0.03–0.31), high-density lipoprotein (HDL) triglycerides (0.24 SD, CI 0.02–0.46), glycerol (0.32 SD, CI 0.07–0.58) and leucine increased (0.25 SD, CI 0.02–0.49). Citrate (−0.36 SD, CI −0.57 to −0.14) and 3-hydroxybutyrate concentrations decreased (−0.46 SD, CI −0.75 to −0.17). Most metabolite changes were associated with the menopausal hormonal shift. This explained 11% and 9% of the LDL cholesterol and particle concentration increase, respectively. Menopausal hormone therapy was associated with increased medium-to-large HDL particle count and decreased small-to-medium LDL particle and glycine concentration.

Conclusions Menopause is associated with proatherogenic circulating metabolome alterations. Female sex hormones levels are connected to the alterations, highlighting their impact on women's cardiovascular health.

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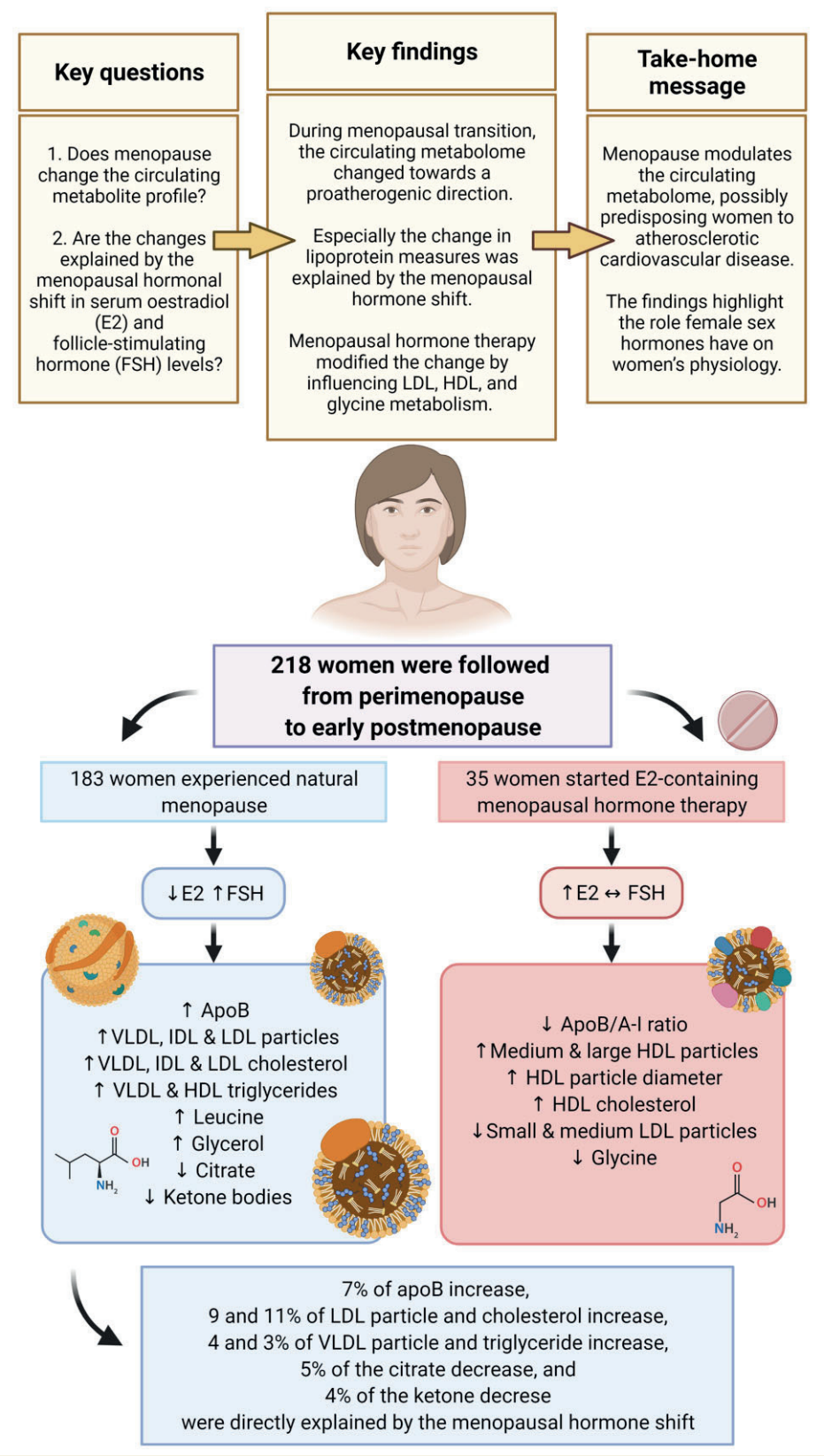
Graphical Abstract



Keywords Menopause • Hormone replacement therapy • Cardiovascular diseases • Metabolomics • Oestradiol

Introduction

Most women face menopause at the age of 48–52 years,[1] resulting from the cessation of ovarian follicular activity and diagnosed 12 months after the final menstrual period.[2] The characteristic menopausal hormonal shift consists of a decline in 17β-oestradiol (E2) and a concomitant increase in follicle-stimulating hormone (FSH) levels.[1] Oestrogen-containing menopausal hormone therapy (MHT) alleviates menopausal symptoms and restores systemic E2 without lowering FSH to premenopausal levels.[3]

Menopause is thought to predispose women to atherosclerotic cardiovascular disease (ACVD) since they develop obstructive coronary artery disease 7–10 years later than men, and this risk rises after menopause.[4] Also, premature menopause is associated with an increased ACVD risk.[5] The causality between these phenomena is difficult to prove, as the menopause-driven metabolic changes may predispose women to present ACVD at an older age, making it difficult to distinguish their effects from other ageing-related changes.[6] Moreover, as it is generally not possible to predict the timing of menopause, establishing appropriate longitudinal study settings is challenging. Perimenopause (the menopausal transition phase) also differs in timing and duration among individuals.[1] Therefore, individual information on menopause progression benefits the investigation of menopausal effects.

Research on clinical biomarkers supports the relationship between menopause and ACVD risk. In longitudinal studies, menopause is associated with increases in circulating triglyceride and low-density lipoprotein (LDL) cholesterol levels. However, the effect of menopause on high-density lipoprotein (HDL) cholesterol concentration and its direction is controversial.[7–10] The biomarker changes may result directly from the menopausal hormonal shift or indirectly via increased adiposity.[11] The role of female sex hormones is strengthened by studies on MHT in postmenopausal women. Oestrogen-only MHT lowers LDL cholesterol and increases HDL cholesterol concentrations accompanied by a rise in triglyceride levels when administered orally.[12] In combination with MHT, the effects on HDL cholesterol are modulated by selected progestogen.[12] MHT may also improve blood glucose regulation,[13] reflecting broad systemic effects on metabolism.

Compared with clinical methods, metabolomics offer a wider lens to investigate menopausal effects on the circulating metabolome. Two population-level nuclear magnetic resonance (NMR) metabolomics studies on menopause have been conducted. The cross-sectional study by Auro *et al.*[14] examined associations between age, sex, and menopause and the circulating metabolome in 26 065 Finnish and Estonian individuals, including 10 083 women. Later, Wang *et al.*[15] investigated cross-sectional associations in the UK among 3312 women, with 1492 longitudinal samples taken 2.5 years apart. Their results were similar; menopause was associated with a proatherogenic shift in lipoprotein measurements and non-lipid metabolites, such as amino acids.[14,15] Both studies relied on self-reported menopausal status and did not associate the findings to the female sex hormone levels.

Therefore, here, we investigated whether the menopause-related hormonal shift modulates the circulating metabolome in a longitudinal design, where the follow-up of the menopausal transition progression was individualized and monitored with repeated FSH level measurements. The premise was that menopause has an identifiable metabolomic fingerprint resulting from the shift of female sex hormone levels.

Methods

Study design and participants

This study used data from the Estrogenic Regulation of Muscle Apoptosis (ERMA) prospective cohort study[16] and was approved by the ethics committee of the Central Finland Health Care District (KSSHP Dnro 8U/2014). The study complied with the Declaration of Helsinki, and participants gave informed consent.

The recruitment was carried out from 2014 to 2015. The sample was randomly drawn from the Population Information System. An invitation and a prequestionnaire were sent to 6878 women aged 47–55 living in the Jyväskylä area, of whom 3229 (47%) responded. Based on the prequestionnaire data, 1627 women were invited to menopausal status determination. Exclusion criteria were self-reported body mass index >35 kg/m^2 and medical conditions or use of medication affecting the ovaries, the hormone or inflammatory profile, or daily functioning. The menopausal status of 1393 women was determined, and 1158 women were called to the Health and Sports Laboratory of the University of Jyväskylä for physiological and psychological measurements.

The ERMA study aimed to create a cohort for the investigation of menopausal effects with a minimum influence of ageing. Therefore, 381 perimenopausal women were invited to participate in the longitudinal Core-ERMA study (*Figure 1*). The first measurements were performed during 2015 and 2016. Women kept a menstrual diary during follow-up and visited the laboratory for the measurement of FSH levels every 3 or 6 months until early postmenopause. The follow-up was scheduled according to the participant's menopausal transition progression. The final last measurements were performed in December 2018.

Of the 381 participants, the follow-up was completed by 234 women. One participant died, and 26 dropped out. Three women reported inconsistent MHT use and 117 women did not reach postmenopause or their menopausal status was uncertain at the last measurement, leading to exclusion. From this study, we excluded 12 women using lipid or glucose-lowering medication and 4 women diagnosed with cancer. Therefore, the metabolomics analyses were performed for 218 participants, with 35 (15%) starting MHT during follow-up.

Hormone and metabolites profile

Blood samples were collected between 7 and 10 a.m. after overnight fasting and processed for serum collection with a standard procedure. The samples were aliquoted and stored at –80°C until analysis. E2 and FSH levels were measured with IMMULITE 2000 XPi (Siemens Healthcare Diagnostics, UK). Metabolites were analysed with a targeted proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy platform (Nightingale Health Ltd., Helsinki, Finland; biomarker quantification version 2020).[17] The technical details of the method have been reported previously.[18] The platform quantifies 250 metabolite measures and 180 were selected as outcomes. We left the 70 lipoprotein lipid ratios out from the analyses due to the limited sample size and as they mostly provide overlapping information with absolute lipid concentrations.

Menopausal status and menopausal hormone therapy use

The 2011 Stages of Reproductive Aging Workshop (STRAW+10) guidelines were used to determine the menopausal status.[2] An exception was that the participants kept a menstrual diary for at least 3 months before the first blood sampling. Perimenopausal women were required to have

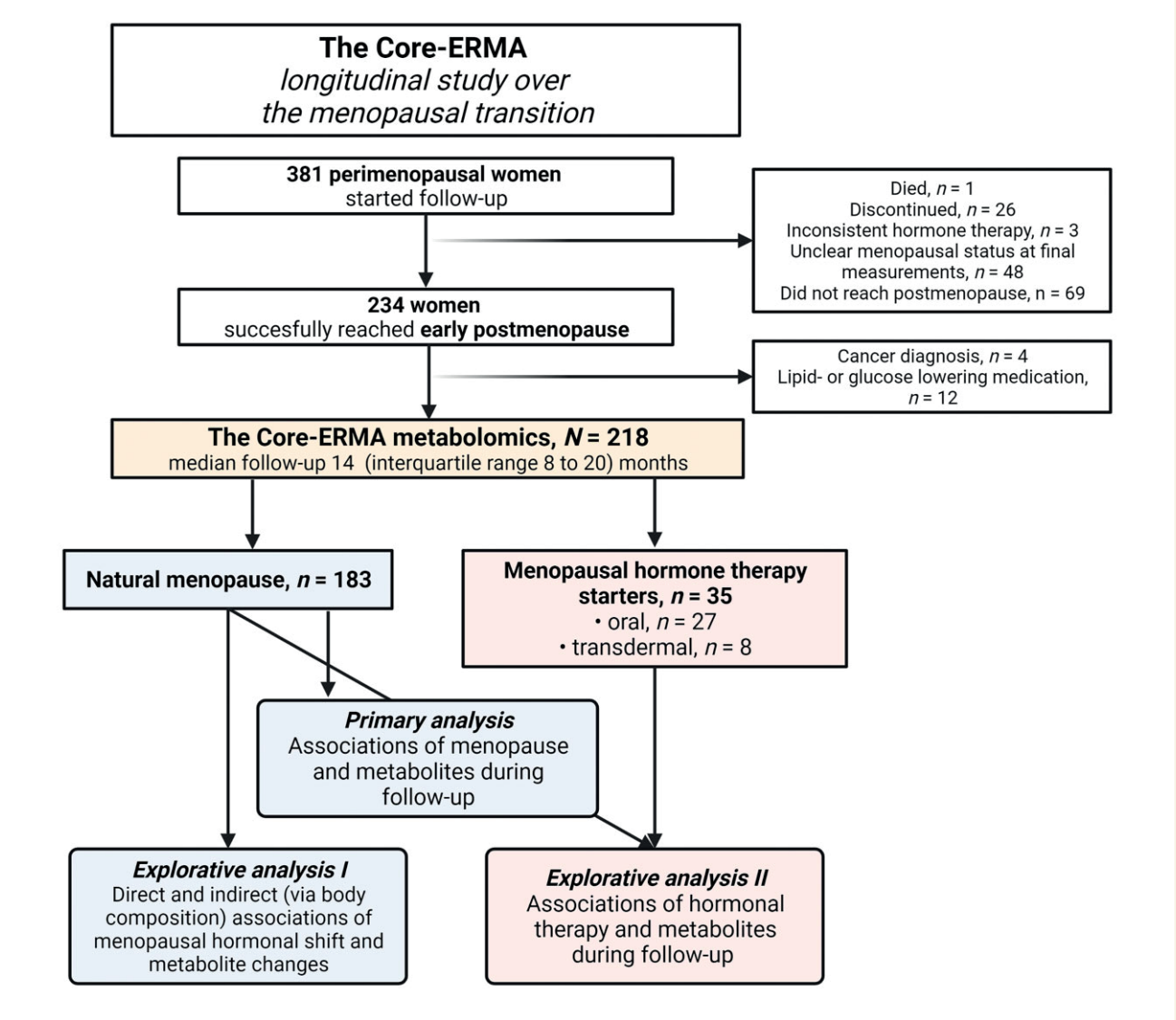


Figure 1 Study flowchart and statistical analysis approach.

irregular or no menstrual bleeding and FSH levels of 17–30 IU/L. During follow-up, women were determined postmenopausal after two consecutive FSH measurements of >30 IU/L and 6 months of amenorrhoea. The criterion differs from the guidelines where postmenopause begins after 12 months of amenorrhoea due to practical limitations. We decided to compromise the length of the follow-up period to maximize the number of women with valid end measurements inside a limited project funding period. The median amenorrhoea duration was 8.1 months (interquartile range: 6.5–10.4). However, the absence of menses was <6 months in 28 participants. To account for the protocol deviation, we performed a sensitivity analysis without these participants. The postmenopausal status of 25 participants was determined solely based on FSH levels because of prior hysterectomy ($n = 11$) or ambiguity in menstrual diary reporting ($n = 14$). The FSH levels were measured again at the last measurement.

MHT was queried at each follow-up meeting, and MHT participants were invited to the last measurement 6 months after the initiation of the treatment to allow the medication to exert its effects. One woman reported starting MHT 17 days before and another participant 2 days before the last measurement. We only classified the former as an MHT user because her 2-day use of low dose transdermal E2 was unlikely to affect the studied outcomes substantially. Her metabolite levels did not significantly differ from the other participants' levels. Of the 35 MHT starters, 27 used oral products containing either E2-only ($n = 7$), E2 in combination with dydrogesterone ($n = 16$) or norethisterone acetate ($n = 4$). Concerning the E2-only MHT starters, five were users of levonorgestrel-releasing intrauterine devices, and two did not need progestogen because of past hysterectomy. The rest of the sample ($n = 8$) used transdermal MHT, seven of whom used E2-gel with a levonorgestrel-releasing intrauterine device and one E2 and norethisterone acetate patches. In 2020, 12% of Finnish women aged ≥45 years used MHT.[19] Therefore, the ratio of MHT users in our sample (15%) reflects frequency of MHT use in the Finnish general population.

Education level, lifestyle factors, and body composition

Supplementary material online, *Method Supplement* includes a detailed description of the study covariates. Briefly, the education level (primary,

secondary, or tertiary) and lifestyle factors were recorded with structured questionnaires. Due to the low number of participants with primary education, primary and secondary were combined in the statistical analyses. Smoking status was classified as 'never', 'quitter', or 'current smoker'. As the number of current smokers was low, dummy variables were created to describe whether the participant had ever smoked and smoked currently. Alcohol use was calculated as portions per week. Physical activity was calculated as metabolic equivalent hours per day (MET-h/d) by assessing the duration, frequency, and intensity of leisure-time physical activity and the time spent on active commuting.[20] Use of common foods in Finnish food culture were recorded with a 45-item food-frequency questionnaire and the diet quality was measured with an 11-element diet quality sum score (DQS) adapted from a validated tool,[21] where a higher score reflects a healthier diet. Height was measured with a stadiometer at the first measurement. Body mass and body fat percentage were measured with InBody720 (Biospace, Seoul, Korea).

Statistical analyses

The statistical analyses are described in detail in the Supplementary material online, *Method Supplement*. Questionnaire-based data were missing from one participant at the first and two participants at the last measurement. We completed the missing values by inspecting participants' answers from other visits or mean imputation. Metabolite data were nearly complete, and we did not impute the rare missing values. R version 4.0.0 or newer was used for statistical analyses unless stated otherwise.

The primary results are the associations between menopause and metabolite measurements in 183 women experiencing natural menopause, i.e. who did not start MHT during follow-up (*Figure 1*). Analyses were performed using linear mixed-effect models with random intercept after metabolite Box-Cox transformation and standardization with respect to the first measurements. The metabolites were treated as outcomes and the menopausal status as exposure. Two adjusted models were built in addition to a crude model. The first adjusted model (the main study results) included the covariates of age at the first measurement, follow-up duration, education level, and lifestyle factors. In the second adjusted model, the body fat percentage (potential mediator between menopause and metabolites) was included as a covariate. The Keff-Šidák correction was used to account for multiple testing. We also performed a sensitivity analysis where we excluded the participants whose amenorrhoea duration was less than 6 months before the last measurements.

Two exploratory analyses were performed. First, the direct and indirect associations (via body fat percentage change) between the menopausal hormonal shift and metabolite changes in the women experiencing natural menopause ($n = 183$) were investigated using latent change score modelling. Both E2 and FSH were included in the model simultaneously because this combination better characterizes an individual's sex hormone profile as E2 levels fluctuate during perimenopause. The used model (Supplementary material online, *Method Supplement Figure S1*) can be seen as an extension of the paired *t*-test, where change is controlled for metabolite concentrations at the first measurement. The calculated effect sizes are interpreted similarly to squared semi-partial correlations and indicate how much of the metabolite change is explained by the menopausal hormonal shift. Mplus version 7.4 was used to estimate the model parameters. False discovery rate adjustment was used to correct for multiple testing. The association of MHT initiation during follow-up with the metabolite measurements was studied in the whole sample ($N = 218$, $n_{\text{MHT starters}} = 35$) using menopausal status and MHT interaction as the exposure. The model structures and multiple testing corrections were performed as in the primary analysis.

Results

Participant characteristics can be found in *Table 1*. The mean age of participants was 51.7 (SD = 1.9) at the first measurement. Hypertension was the most commonly diagnosed condition in the cohort. Participants did not use lipid-lowering agents, even though more than half had elevated LDL cholesterol levels (>3 mmol/L). MHT starters were younger than non-users at the first measurement. Moreover, they had lower FSH levels and systolic/diastolic blood pressure. As for the rest of the variables, the groups showed similar characteristics at the beginning of the study.

The median follow-up duration was 14 months (interquartile range: 8–20), ranging from 4 months to 3.5 years. Expectedly, E2 decreased and FSH increased in women not using MHT during follow-up. Among MHT starters, E2 increased without an apparent change in FSH levels. As for lifestyle factors, alcohol use decreased in both groups and body fat percentage increased by 1% for the total sample with a similar trend in both groups.

Menopause and metabolite associations

In Supplementary material online, *Result Supplement Table S1*, the absolute metabolite concentrations during the study and the unstandardized metabolite change scores of the 183 women experiencing natural menopause are shown. Menopause was associated with a statistically significant change in 85 metabolite measures (Supplementary material online, *Result Supplement Table S3*; the key findings are summarized in *Figure 2*). The apolipoprotein B (apoB)-containing particle count increased by 0.17 SD (99.95% confidence interval [CI] 0.03–0.31), resulting from the increased very-low-density lipoprotein (VLDL; 0.21 SD, CI 0.05–0.36) and LDL particle counts (0.17 SD, CI 0.03–0.31). The VLDL increase favoured the increase of VLDL particle size (0.22 SD, CI 0.03–0.41). The apolipoprotein A-I (apoA-I) and total HDL particle counts did not change markedly. However, it is uncertain whether small HDL subclass is affected by menopause (0.19 SD, CI −0.01 to 0.40). From the lipid measurements, cholesterol concentrations in all apoB-containing lipoprotein classes increased from 0.17 to 0.20 SD. The increase in VLDL triglyceride (0.25 SD, CI 0.05–0.45) and HDL triglyceride concentrations (0.24 SD, CI 0.02–0.46) was even more pronounced. Total serum fatty acid concentration did not change but the fatty acid profile shifted from polyunsaturated to saturated direction. As for the other metabolites, citrate concentration decreased by −0.36 SD (CI −0.57 to −0.14) and glycerol concentration increased by 0.32 SD (CI 0.07–0.58). Glutamine concentration decreased by −0.46 SD (CI −0.72 to −0.20) and leucine concentration increased by 0.25 SD (CI 0.02–0.49). Acetoacetate and 3-hydroxybutyrate levels decreased by −0.44 SD (CI −0.72 to −0.15) and −0.46 (CI −0.75 to −0.17), respectively. When body fat percentage was included as a covariate, the association sizes of lipoprotein, lipid, glycerol, and amino acid concentrations were roughly 0.03–0.05 SD smaller (Supplementary material online, *Result Supplement Table S3*). The adjustment had a negligible effect on the associations of menopause with citrate and ketone levels.

The results were very similar in the sensitivity analysis where the 28 participants with <6 months of amenorrhoea were excluded; however, almost all found associations became slightly more robust (Supplementary material online, *Result Supplement Table S6*).

Table 1 Characteristics of the participants at the first and last measurements

	Natural menopause ($n = 183$)		MHT starters ($n = 35$)	
	Perimenopause	**Postmenopause**	**Perimenopause**	**Postmenopause**
Age (years)	51.9 (1.9)	53.1 (1.9)	50.6 (1.8)	52.0 (1.9)
Follow-up (days)	–	397 (243–603)	–	464 (334–707)
HT use (days)	–	–	–	220 (191–242)
Sex hormones				
E2 (nmol/L)	0.25 (0.17–0.41)	0.16 (0.11–0.27)	0.25 (0.18–0.42)	0.28 (0.20–0.61)
FSH (IU/L)	30.0 (23.4–48.5)	69.8 (46.8–89.7)	23.0 (16.5–36.1)	36.1 (18.4–60.0)
Education				
Primary	5 (3%)	5 (3%)	1 (3%)	1 (3%)
Secondary	100 (55%)	100 (55%)	15 (43%)	15 (43%)
Tertiary	78 (42%)	78 (43%)	19 (54%)	19 (54%)
Body composition				
Height (cm)	165.1 (5.7)	–	166.3 (5.5)	–
Body mass (kg)	69.5 (11.3)	70.2 (11.6)	70.4 (10.2)	70.5 (10.8)
Body mass index categories				
Healthy weight (<25 kg/m^2)	92 (50%)	85 (46%)	20 (57%)	19 (54%)
Overweight (25–29.9 kg/m^2)	66 (36%)	71 (39%)	10 (29%)	12 (34%)
Obesity (≥30 kg/m^2)	25 (14%)	27 (15%)	5 (14%)	4 (11%)
Per cent body fat (%)	30.9 (8.3)	32.1 (8.0)	30.5 (7.1)	31.4 (6.3)
Smoking				
Never	122 (67%)	122 (67%)	26 (74%)	26 (74%)
Quitter	49 (27%)	50 (28%)	6 (17%)	5 (14%)
Smoker	12 (7%)	11 (6%)	3 (9%)	4 (11%)
Alcohol use (portions/week)	3.0 (1.0–5.0)	2.5 (1.0–5.0)	4.5 (2.5–5.8)	2.5 (1.0–4.9)
Diet quality score (0–11)	5.7 (2.3)	5.5 (2.1)	5.5 (2.4)	5.6 (2.4)
Physical activity (MET-h/d)	3.6 (1.7–4.8)	3.8 (2.3–5.6)	4.5 (1.8–7.5)	4.8 (2.5–7.5)
Blood pressure				
Diastolic (mmHg)	85 (10)	82 (10)	80 (9)	77 (11)
Systolic (mmHg)	134 (19)	131 (18)	126 (16)	121 (14)
Diagnosed hypertension	24 (13%)	29 (16%)	4 (11%)	4 (11%)
ATC1 class C medication	25 (14%)	34 (19%)	5 (14%)	6 (17%)
Dyslipidaemia and blood glucose				
LDL cholesterol >3 mmol/L[a]	99 (54%)	119 (65%)	22 (62%)	20 (57%)
HDL cholesterol <1.2 mmol/L	5 (3%)	4 (2%)	1 (3%)	0 (0%)
Triglycerides				
Optimal (<1.2 mmol/L)	123 (67%)	107 (58%)	24 (69%)	24 (69%)
Elevated (>1.7 mmol/L)	22 (12%)	27 (15%)	1 (3%)	5 (14%)
Fasting glucose >6 mmol/L	8 (4%)	12 (7%)	2 (6%)	3 (9%)

Data as means (standard deviation), medians (interquartile range), or counts (%).
[a]Calculated with Friedewald formula.
E2, 17β-oestradiol; FSH, follicle-stimulating hormone; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MET, metabolic equivalent of task; MHT, menopausal hormone therapy.

Notably, the association between menopause and small HDL particle count was statistically significant (0.24 SD, CI 0.01–0.47).

Menopausal hormonal shift and metabolite changes

The menopausal hormonal shift directly explained the change in 64 of the 85 metabolites identified as menopause-responsive in the primary analysis, with effect sizes ranging from 2.1 to 11.2% (Supplementary material online, *Result Supplement Table S4*). Based on the size of the standardized regression estimates of the two hormones included in the model, the decline of E2 levels had more impact on the results than the increased FSH levels. The hormonal shift was not associated with indirect metabolite changes since the shift was not associated with body fat percentage change.

The key findings are summarized in *Figure 3*. The menopausal hormonal shift directly explained the concentration increase in apoB

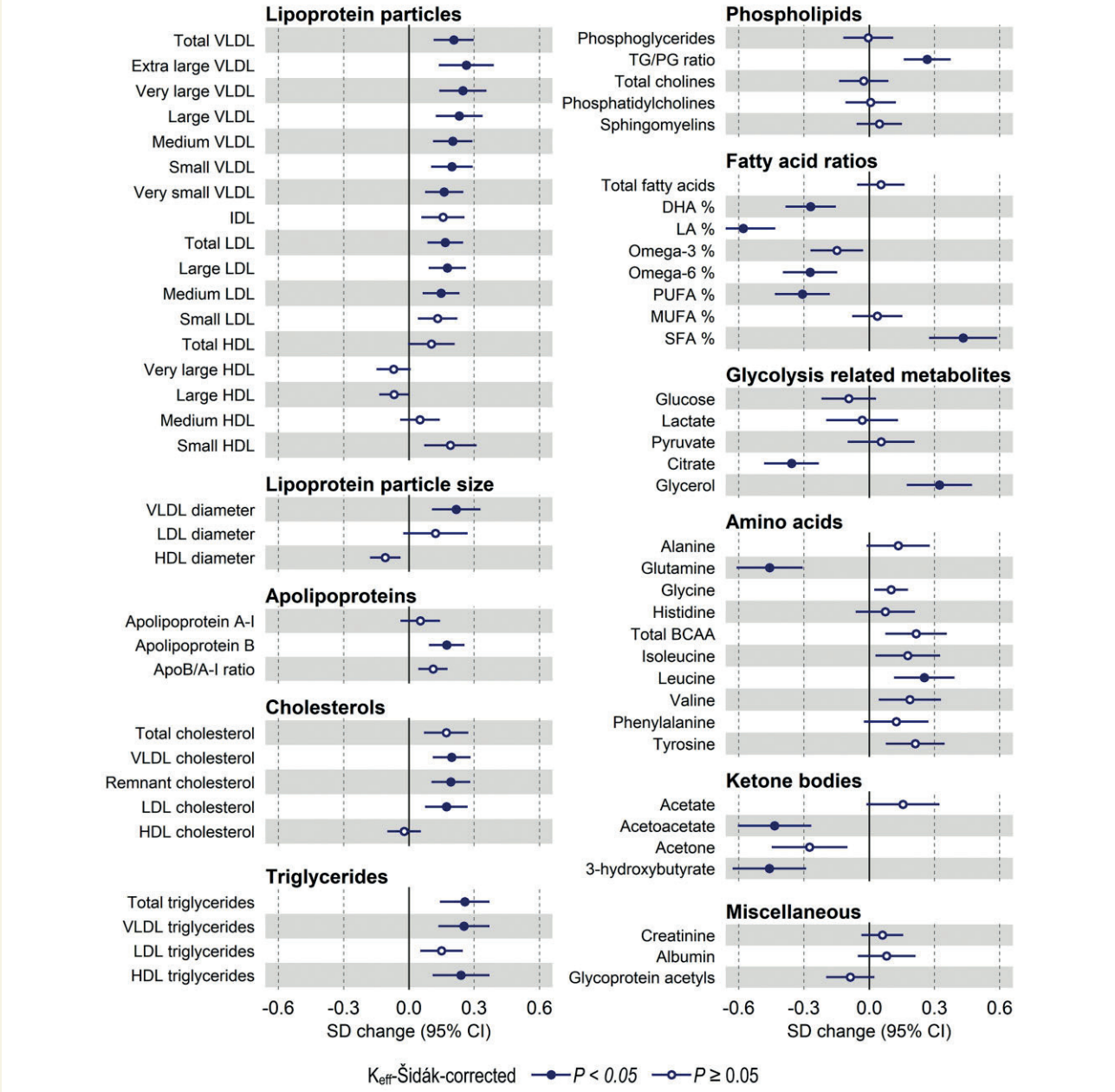


Figure 2 The associations of the menopausal transition and key metabolite measurements in women experiencing natural menopause ($n = 183$) adjusted for age at the first measurement, follow-up duration, education level, smoking status, alcohol use, physical activity, and diet quality. The figure describes the standardized change (expressed as standard deviation [SD] units) in the metabolite levels during follow-up with respect to the first measurement values, allowing the comparison between metabolite measures with different units and concentrations. Apo, apolipoprotein; BCAA, branched-chain amino acids; CI, confidence interval; DHA, docosahexaenoic acid; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LA, linoleic acid; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG/PG, triglyceride/phosphoglyceride; VLDL, very-low-density lipoprotein.

(7.4%), LDL particles (8.5%), and LDL cholesterol (10.6%). The effect sizes concerning the total VLDL particle count (4.0%) and triglyceride levels (2.9%) were more modest. Even though the association of menopause and the increased small HDL particle count was inconclusive in the primary analysis, the exploratory analysis strongly linked them with an effect size of 10.9%.

From the results concerning the association of non-lipoprotein and lipid with menopause in the primary analysis, a direct association with the hormonal shift was confirmed for glutamine (2.4%), citrate (5.0%), 3-hydroxybutyrate (4.4%), and acetoacetate (3.4%) concentrations. The largest effect size of the hormonal shift was found for tyrosine concentration (7.8%), although menopause and tyrosine

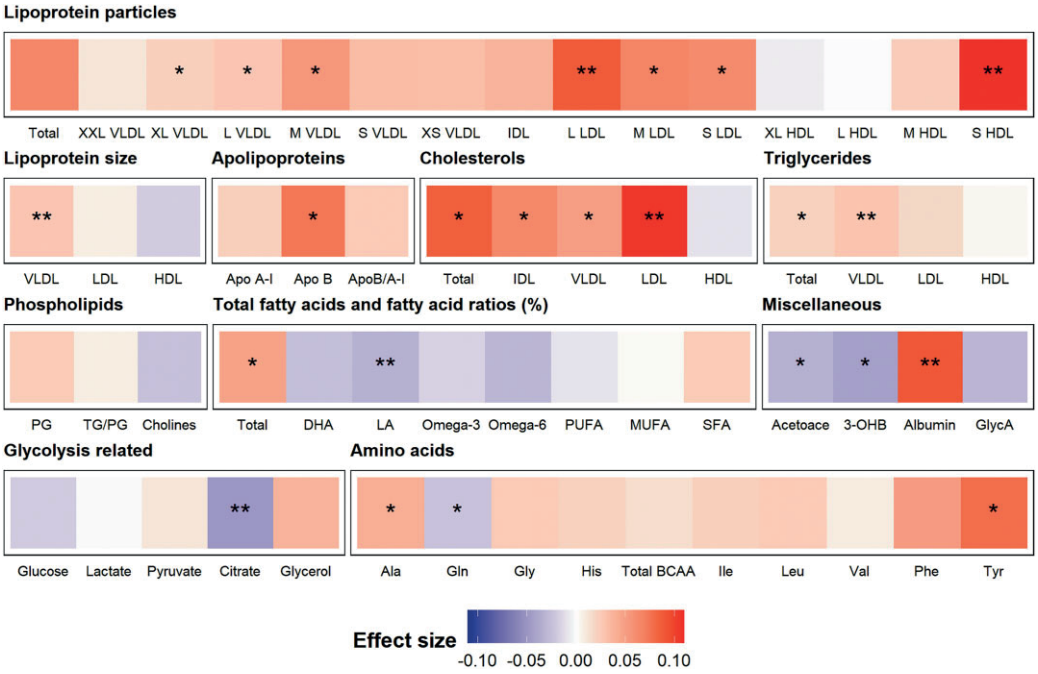


Figure 3 The associations of menopausal hormonal change (the decline in oestradiol and increase in follicle-stimulating hormone levels) with key metabolite changes. The colours and their intensity show the association direction and the effect size. The models were adjusted for age at the first measurement, follow-up duration, education level, smoking status, alcohol use, physical activity, and diet quality. False discovery corrected *P*-values: * ≤0.05; ** ≤0.01. 3-OHB, 3-hydroxybutyrate; Ala, alanine; Apo, apolipoprotein; BCAA, branched-chain amino acids; DHA, docosahexaenoic acid; HDL, high-density lipoprotein; Gln, glutamine; Gly, glycine; GlycA, glycoprotein acetyls; His, histidine; IDL, intermediate-density lipoprotein; Ile, isoleucine; LA, linoleic acid; LDL, low-density lipoprotein; Leu, leucine; MUFA, monounsaturated fatty acids; Val, valine; PG, phosphoglycerides; Phe, phenylalanine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG/PG, triglyceride/phosphoglyceride ratio; Tyr, tyrosine; VLDL, very-low-density lipoprotein.

level association did not remain statistically significant in the primary analysis after multiple comparison corrections.

Menopausal hormone therapy and metabolite changes

In Supplementary material online, *Result Supplement Table S2*, the absolute metabolite concentrations for MHT starters during the study including the unstandardized change scores are presented. MHT initiation during follow-up was characterized by a decreased apoB/apoA-I ratio (Supplementary material online, *Result Supplement Table S5*; the key statistically significant findings are summarized in *Table 2*), resulting from the increased particle count in medium and large HDL subclasses and the decreased small and medium LDL subclass particle counts. VLDL was less affected by MHT than LDL and HDL. As for non-lipid metabolites, we found an inverse association between MHT and glycine concentration. Adjusting for body fat percentage did not influence these associations.

Discussion

This study investigated the associations of menopause and circulating metabolome in 183 women transitioning from perimenopause to early postmenopause. The study also explored whether the menopausal hormonal shift explains the observed changes and whether MHT initiated during the follow-up ($n = 35$) was associated with metabolite changes. The results showed that menopause-induced hormonal shift is associated with a proatherogenic metabolomic fingerprint. MHT specifically influences LDL, HDL, and glycine metabolism. These findings broadly agree with earlier metabolomics studies on menopause,[14,15] and now connect the previous and present observations to the female sex hormone levels.

Menopause modulates lipoprotein and lipid metabolism towards a proatherogenic profile

apoB-containing lipoproteins cause ACVD,[22] with LDL being the primary disease driver.[23] Studies using both clinical methods and metabolomics have identified LDL as one of the most menopause-responsive biomarkers.[8,9,14,15] In agreement with this, we report increased apoB and LDL cholesterol and particle concentration, 7–11% of which are directly explained by the menopausal hormonal shift. The underlying mechanism is probably the well-established oestrogen-mediated LDL receptor modulation,[24,25] influencing LDL clearance from the circulation.[26] However, similar to Auro *et al.*[14] but contrary to Wang *et al.*,[15] menopause did not alter LDL size distribution in our study. MHT was associated with decreased particle counts in medium and small LDL subclasses, providing further evidence that female sex hormones regulate LDL

Table 2 Menopause and menopausal hormone therapy interactions with key metabolite measurements

	Crude model			Adjusted model[a]		
Metabolite	**Interaction**	**99.95% CI**	***P***	**Interaction**	**99.95% CI**	***P***
apoB/apoA-I ratio	−0.54	−0.86 to −0.21	<0.001	−0.55	−0.88 to −0.22	<0.001
apoA-I	0.51	0.08 to 0.93	0.005	0.55	0.13 to 0.97	0.001
Glycine	−0.46	−0.80 to −0.11	0.001	−0.46	−0.80 to −0.12	0.001
Medium HDL particles	0.54	0.10 to 0.97	0.003	0.58	0.15 to 1.01	0.001
Large HDL particles	0.35	0.06 to 0.64	0.004	0.36	0.07 to 0.66	0.002
HDL cholesterol	0.36	0.02 to 0.70	0.028	0.39	0.05 to 0.74	0.008
HDL diameter	0.35	0.05 to 0.65	0.007	0.35	0.04 to 0.74	0.009
Small LDL particles	−0.41	−0.76 to −0.05	0.005	−0.44	−0.83 to −0.05	0.011
LDL particles	0.31	−0.76 to −0.05	0.010	−0.39	−0.75 to −0.03	0.020
Medium LDL particles	−0.39	−0.74 to −0.03	0.017	−0.37	−0.77 to −0.01	0.039

[a]Age at the first measurement, follow-up duration, education level, smoking status, alcohol use, physical activity, and diet quality.
apo, apolipoprotein; CI, confidence interval; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
The confidence intervals and *P*-values were Keff-Šidák-corrected for multiple comparisons.

metabolism. These observations support the clinical guidelines to initiate MHT early into menopause as this timing offers the greatest cardioprotective benefits.[4]

Triglyceride-rich lipoproteins also contribute to the ACVD risk.[27] Menopause was associated with an increased VLDL particle count, favouring larger particles possibly in the range of VLDL1 size. Moreover, VLDL triglyceride and cholesterol concentrations increased. The observed pattern was similar to the results of Auro *et al.*[14] but different from Wang *et al.*[15] Our findings also agree with a recent study[28] on the sex differences in plasma metabolites at multiple life stages that identified VLDL triglycerides as the most menopause-responsive lipid measurement.

The menopause and VLDL associations observed in this study were robust against body fat percentage adjustment. Still, only ~4% of the VLDL changes were directly explained by the menopausal hormonal shift. Therefore, the potential causal pathway between menopause and VLDL increase probably entails mediators like insulin resistance, as overproduction of VLDL particles and hypertriglyceridaemia are hallmarks of chronically elevated insulin levels.[27] VLDL was less affected by MHT than the LDL variables, possibly resulting from the effect on the liver caused by the use of oral products (the majority of MHT starters in this study).[26]

Unlike apoB-containing lipoproteins, total HDL particle and cholesterol concentrations are associated with reduced ACVD risk in observational studies, but their causal role is debatable.[29] Consistent with previous metabolomic studies,[14,15] the apoA-I count and the HDL particle or cholesterol concentrations did not decrease during follow-up. However, in our previous study,[7] using data obtained with clinical immunoassay from the same serum samples, HDL cholesterol levels increased. HDL cholesterol increase was also observed during a 4-year follow-up in premenopausal women transitioning into postmenopause.[10] This discrepancy could result from the different measurement methods.

However, based on the present metabolomics analyses, the effects of menopause on HDL metabolism do not favour cardiovascular health. First, menopause was associated with increased HDL triglyceride concentration that, contrary to HDL cholesterol levels, is associated with a higher risk of coronary heart disease.[30] Furthermore, the menopausal hormonal shift was associated with a particle count increase in the small HDL subclass, whereas MHT was associated with a particle count increase in medium and large HDL subclasses. HDL subclasses relate differently to ACVD risk as only larger particles are protective.[31] Moreover, HDL size distribution is linked to insulin resistance as smaller particles accumulate when glucose tolerance worsens.[32] Our results agree with the longitudinal The Study of Women's Health Across the Nation (SWAN) study, where menopause was associated with an increase in the small HDL particle and HDL triglyceride concentrations.[33] In the future, examining menopause-associated structural and functional HDL changes will provide more insight into the phenomenon.

Menopause is associated with a metabolic signature indicative of deteriorating insulin sensitivity

Our findings from other metabolite classes link menopause to a broader deterioration of cardiovascular and metabolic health. Blood glucose levels did not increase during follow-up. However, glucose homeostasis is tightly regulated, and insulin sensitivity changes may influence other metabolites more rapidly. For example, menopause was associated with increased glycerol and decreased 3-hydroxybutyrate and acetoacetate concentrations. Glycerol levels are inversely associated with insulin sensitivity and predict type 2 diabetes.[34] Conversely, ketone levels are positively associated with insulin sensitivity in non-diabetic subjects[35] like our participants. The menopause-associated decrease in ketone production fits into the picture with the observed VLDL triglyceride increase, indicating that fatty acids are rather esterified into triglycerides than used for oxidation in the liver after menopause.

Moreover, we noticed that the serum fatty acid profile changed from polyunsaturated to saturated, relating to elevated type 2 diabetes risk.[36] One explanation for this profile change is increased *de*

novo lipogenesis.[37] Insulin resistance increases hepatic fatty acid synthesis, upscaling VLDL production.[38] Altered diet is another explanation. Based on the diet assessment, participants' dietary habits were stable during the follow-up. However, this method provides only a crude estimation of the overall diet quality and does not capture possible changes in energy intake.

Furthermore, menopause was associated with amino acid changes, as previously reported.[14,15,28] Leucine concentration increased during follow-up, and the menopausal hormonal shift was associated with increased tyrosine levels. Elevated aromatic amino acids and branched-chained amino acid concentrations follow insulin resistance[37,39,40] and predict type 2 diabetes risk.[41] Higher levels of branched-chained amino acids were associated with higher ACVD risk in women.[42] Conversely, higher glycine and glutamine concentrations predict a decreased risk of type 2 diabetes.[37,41] In line with our other findings, glutamine concentration decreased during follow-up, contradicting previous observations.[14,15] Even though ovariectomy in rats did not alter skeletal muscle glutamine synthetase expression or activity,[43] the glutamine concentration decrease may be due to the reduced branched-chained amino acid catabolism because the primary nitrogen acceptor, α-ketoglutarate, is initially converted to glutamate and, then, partially to glutamine.[44] We did not find a statistically significant association between menopause and glycine levels. However, MHT was associated with decreased glycine concentration, similar to previous mass spectrometry studies using randomized controlled trial samples.[45,46] Therefore, all MHT effects may not favour cardiovascular health.

Menopause-associated changes in the circulating metabolome are linked to bone health but not increased inflammation

Oestrogen receptors exist throughout the body, with menopause causing broad systemic effects on multiple organ systems.[47] The decreased citrate concentration during follow-up is a notable finding that links the circulating metabolome possibly to the skeletal system, where about 90% of body citrate resides.[48] In mice, ovariectomy led to bone mineral density loss and decreased bone and plasma citrate levels.[49] This decrease may result from reduced citrate synthesis due to osteoblast loss and increased citrate consumption by osteoclasts and lipid synthesis.[50] As menopause relates to a bone mass decrease,[51] serum citrate concentration could be a novel imaging-free biomarker of bone health in the population. An alternative explanation for the citrate association is that the observed change results from the potential deterioration of insulin sensitivity, as citrate levels were associated with insulin resistance among Finnish bariatric surgery patients.[52] MHT did not influence citrate levels in this study.

Concerning the rest of the quantified metabolites, we found no or inconsistent evidence on their relationship with menopause. Contrary to Wang *et al.*,[15] we did not observe a positive association between menopause or the menopausal hormonal shift and the inflammation marker GlycA. Moreover, we did not observe an inverse association between MHT and GlycA levels as Auro *et al.* did.[14] Our findings indicate that at least the short observation time around the final menstrual period (the median follow-up was 14 months) do not reveal a sharp increase in systemic inflammation. Although menopause was previously associated with increased albumin concentration,[15] and MHT users had lower albumin levels than non-users,[14] neither menopause nor MHT was significantly associated with albumin concentration in this study. Nevertheless, the menopausal hormonal shift was still related to increased albumin levels.

Strengths and limitations

The key strengths of this study are the longitudinal design and the detailed menopausal transition monitoring by repeated hormone measurements. Moreover, we focused on changes occurring around the final menstrual period. Hence, we individualized the follow-up time for each woman to ensure they were at the same early postmenopausal state at the last measurement instead of using a standard follow-up time for all the participants.

The main study limitation is the relatively small sample size for a metabolomics study, probably leading to the detection of the most notable associations only. Additionally, the NMR metabolomics platform focuses on lipoprotein and lipid measurements and does not capture all ACVD-relevant metabolites, such as clotting factors. In the exploratory analysis, we also divided participants into MHT non-starters and starters *post hoc*. We recommend caution when interpreting the findings as women who start MHT may differ from women not requiring medical assistance with menopausal symptoms. Moreover, the whole-body fat percentage was considered the adiposity variable for indirect associations between menopausal hormonal shift and metabolite changes. The hormonal shift did not associate with the body fat percentage change and, therefore, the indirect link with metabolite changes was discarded. In this aspect, visceral fat mass quantification might have been more informative. Our study is also vulnerable to reverse causality, residual confounding, and selection bias. The metabolite levels may have changed already before menopause. Moreover, our statistical approach cannot fully control the influence of measured and unmeasured confounders. Last, our sample of homogeneous and generally healthy Finnish women is unlikely fully representative of the study population, and generalizability of our findings is somewhat limited.

In conclusion, menopause is associated with a proatherogenic circulating metabolome change that relates to the induced hormonal shift and is partially modified by MHT. These findings highlight the impact of female sex hormones on women's cardiovascular health.

Supplementary material

Supplementary material is available at *European Journal of Preventive Cardiology*.

Acknowledgements

We thank the ERMA women for their participation and the Faculty of Sport and Health Sciences staff for their contribution to the data collection. Structured graphical abstract and *Figure 1* were created with BioRender.com.

Funding

This work was supported by the Academy of Finland [275323 to V.K. and 309504, 314181 and 335249 to E.K.L.].

Conflict of interest: None declared.

Authors' Contributions

J.E.K., T.T., U.M.K., and E.K.L. contributed to the conception and design of the work. U.M.K., S.S., P.A., V.K., and E.K.L. contributed to the conception of the ERMA cohort. J.L. offered medical expertise and guidance. J.E.K. drafted the manuscript. All other authors critically revised the manuscript. All approved the final version of the manuscript.

Data availability

The data underlying this article cannot be shared publicly for the privacy of the participants. The data will be shared upon request to the corresponding author.

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II

AGE BUT NOT MENOPAUSAL STATUS IS LINKED TO LOWER RESTING ENERGY EXPENDITURE

by

Karppinen, J.E., Wiklund, P., Ihalainen, J.K., Juppi, H-K, Isola, V., Hyvärinen, M., Ahokas, E.K., Kujala, U.M., Laukkanen, J., Hulmi, J.J., Ahtiainen, J.P., Cheng, S. & Laakkonen, E.K.

Manuscript submitted for publication.

Preprint available from https://doi.org/10.1101/2022.12.16.520683

Age but not menopausal status is linked to lower resting energy expenditure

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Keywords: resting energy expenditure, menopause, estrogen, hormone replacement therapy

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Supported by the Academy of Finland grants 309504, 314181, 335249 (to EKL) and 135038 (to SC) and by the Ministry of Education and Culture of Finland grants OKM/54/627/2006, OKM/77/627/2008 (to SC) and OKM/10/626/2021 (to JKI). The manuscript preparation was supported by the Academy of Finland grant 330281 (to EKL). The contents of this article are solely the responsibility of the authors.

Author disclosures: The authors report no conflicts of interest.

ABSTRACT

Context

It remains uncertain whether aging before late adulthood and menopause are associated with fat-free mass and fat mass–adjusted resting energy expenditure (REE_{adj}).

Objectives

We investigated whether REE_{adj} differs between middle-aged and younger women and between middle-aged women with different menopausal statuses. We repeated the age group comparison between middle-aged mothers and their daughters to partially control for genotype. We also explored whether serum estradiol and follicle-stimulating hormone concentrations explain REE_{adj} in midlife.

Methods

We divided 120 women, including 16 mother–daughter pairs, into age groups; group I (n=26) consisted of participants aged 17–21, group II (n=35) of those aged 22–38 and group III (n=59) of those aged 41–58 years. The women in group III were further categorized as pre- or perimenopausal (n=19), postmenopausal (n=30) or postmenopausal hormone therapy users (n=10). REE was assessed using indirect calorimetry, body composition using dual-energy X-ray absorptiometry and hormones using immunoassays.

Results

The REE_{adj} of group I was 126 kcal/d (95% CI: 93–160) higher than that of group III, and the REE_{adj} of group II was 88 kcal/d (95% CI: 49–127) higher. Furthermore, daughters had a 100 kcal/d (95% CI: 63–138 kcal/d) higher REE_{adj} than their middle-aged mothers (all P<0.001). In group III, REE_{adj} was not lower in postmenopausal women and did not vary by sex hormone concentrations.

Conclusions

We demonstrated that REE_{adj} declines with age in women before late adulthood, also when controlling partially for genetic background, and that menopause may not contribute to this decline.

Introduction

Energy expenditure is often assumed to begin declining in early to middle adulthood, but Pontzer et al. (1) challenged this assumption by showing that fat-free mass (FFM) and fat mass (FM)-adjusted total energy expenditure (TEE_{adj}) were stable between the ages of 20 and 63. However, they found that similarly adjusted resting energy expenditure (REE_{adj}) stabilizes at adult levels at age 18 and declines from age 46 onwards, although the limited numbers of middle-aged participants with a measured REE prevented the authors from making definitive inferences about the onset of REE_{adj} decline, leading to a conclusion that age does not affect energy expenditure in adults before the age of 60 (1). Nevertheless, previous studies are consistent with an earlier turning point for REE_{adj} (2–4), and we therefore sought to assess whether REE_{adj} declines before late adulthood. Our dataset also included mother–daughter dyads, some of which had TEE measured with doubly labeled water, enabling us to partly control the analyses for genetic background and to explore whether increasing age showed similar associations with TEE_{adj} as it does with REE_{adj}.

Like aging, menopause is widely believed to reduce REE_{adj}, and the topic has broad interest because many women gain FM during the menopausal transition (5,6) and associate the change in body composition with slowing metabolism. During the menopausal transition, ovarian follicular activity ceases, causing a striking shift in women's sex hormone

profile. The decline in systemic estradiol (E2) concentration in particular is thought to decrease REE_{adj}, potentially via both central (7) and peripheral (8) mechanisms, while the increase in follicle-stimulating hormone (FSH) secretion may also play a role (9,10). Menopausal hormone therapy (MHT) can restore E2 and decrease FSH levels to some extent, which should reverse the potential menopause-associated decline in REE_{adj}. However, whether menopause truly decreases REE_{adj} is still uncertain because longitudinal studies following women over the menopausal transition (11,12), cross-sectional studies comparing women with different menopausal states (13–16) and MHT interventions (15,17–19) have been inconclusive. Therefore, in addition to investigating whether REE_{adj} differs between young and middle-aged women, we also assessed whether REE_{adj} differs between middle-aged women with different menopause statuses. We restricted the menopause analysis to middle-aged participants to limit the confounding effects of age. We also explored whether serum E2 and FSH concentrations explain REE_{adj} in midlife.

Materials and Methods

Participants

The participants were 120 women who had taken part in one of four studies performed at the Faculty of Sport and Health Sciences of the University of Jyväskylä (**Figure 1**). They were required to be healthy and not taking medications that could affect metabolism, although hormonal contraception and MHT use were allowed.

The Calex study (data collection 2008–2011) investigated whether lifestyle factors influence muscle and adipose tissues (20). The current study used data from 17 middle-aged and 21 younger women with measured REE. This dataset included 16 mother–daughter pairs; ten pairs also had their TEEs measured. The data has been partly used in an

earlier validation study (21). The EsmiRs study (Estrogen and microRNAs as Modulators of Women's Metabolism, 2019–2020) examined resting and exercise metabolism in middle-aged women (22), and we included all 42 participants with measured REEs from that study. The Physique study (23) investigated the effects of competition weight loss in normal-weight participants; here, we used the baseline data from 23 young women collected before their weight loss. Finally, the Athletic Performance and Nutrition study (NO RED-S, 2021, Ihalainen et al. unpublished) studied the health of winter sports athletes; from that study, we used baseline data from 17 young women, measured after the transition season when their training load was the lowest and under ten weekly hours for all participants.

Studies were conducted according to the Declaration of Helsinki and approved by the Ethics Committee of the Central Finland Health Care District (Calex; memo 22/8/2008 and 5/2009, EsmiRs; 9U/2018, Physique; 19U/2018) or the Ethics Committee of the University of Jyväskylä (NO RED-S 514/13.00.04.00/2021). Participants gave informed consent.

Figure 1

Age categorization

We used age as a continuous variable and categorized the participants into three age groups, based on previous findings. REE_{adj} plateaued at age 18.0 (95% CI: 16.8–19.2) and started to decline at age 46.5 (95% CI: 40.6–52.4), while TEE_{adj} plateaued at 20.5 (95% CI: 19.8–21.2) and started to decline at 63.0 years (95% CI: 60.1–65.9) (1). We therefore assigned participants aged 18~21 years to group I, 22~39 years to group II and 40~60 years to group III.

Hormonal and menopausal status

Participants in groups I and II were either naturally menstruating women at different menstrual cycle phases or hormonal contraception users. Whether REE varies slightly according to menstrual cycle phase is still questionable (24). In group I, 12 participants reported using combination oral contraceptives, one participant used a hormonal ring, and detailed information on contraceptive use was not available for five participants. In group II, eight participants used combination oral contraceptives, three used progestin-only oral contraception, and three used a hormonal intrauterine device. Based on primarily cross-sectional evidence, there appears to be no clear association between hormonal contraceptive use and REE (25).

We determined the menopausal status of group III women with the Stages of Reproductive Aging Workshop (STRAW) + 10 guidelines (26) using menstrual and serum FSH data: 11 were premenopausal (PRE), eight were perimenopausal (PERI), 30 were postmenopausal (POST) and ten were postmenopausal MHT users (MHT). We combined the PRE and PERI women into a PRE/PERI group to represent women with meaningful ovarian E2 production but performed a sensitivity analysis without the PERI women because E2 levels decline in perimenopause. One PRE/PERI woman used a hormonal intrauterine device. In the MHT group, seven participants used oral E2 in combination with dydrogesterone (n = 5) or norethisterone acetate (n = 2). Two participants used an E2 patch containing also norethisterone acetate or combined with oral dydrogesterone, and one participant used an E2-only patch. All participants had used MHT for at least four months, with most having used it for years. Details concerning menopausal status determination and MHT preparations used by the women are in **Supplemental Methods**.

Sex hormones

For sex hormone assessment, the serum was separated from fasting venous blood samples according to standard procedures and stored at −80°C. E2 and FSH concentrations were measured for group III participants using enzyme-amplified chemiluminescence immunoassays (IMMULITE 2000 XPi, Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The analytical sensitivity for the E2 kit (Catalog # L2KE22, RRID: AB_2936944) is 0.055 nmol/l with an accurate reportable range of 0.073–7.342 nmol/l. The coefficient of variation in our lab using control samples has been 15%. We compared the used immunoassay method with liquid chromatography-mass spectrometry (HUSLAB, Helsinki University Hospital, Helsinki, Finland) and found a good correlation in all test samples (n = 166, r = 0.91). However, when the comparison was restricted to samples with E2 concentrations less than 0.1 nmol/l, as determined by with liquid chromatography-mass spectrometry, the correlation between methods was lower (n = 76, r = 0.42). The analytical sensitivity of the FSH kit (Catalog # L2KFS2, RRID: AB_2756389) is 0.1 IU/l, and the coefficient of variation in our lab has been 5%.

Body composition

Body composition was assessed with dual-energy X-ray absorptiometry (DXA; DXA Prodigy, GE Lunar Corp., Madison, WI, USA). We calculated the appendicular lean mass index (ALMI) by scaling appendicular lean mass (kg) to height (m) squared to estimate the level of muscularity among participants.

Resting and total energy expenditure

REE was measured in all studies using the same Vmax Encore 92 metabolic cart (Sensormedics, Yorba Linda, CA, USA) and ventilated hood in the same thermoneutral laboratory; the cart was calibrated accordingly before each measurement. The REE assessment details are in **Supplemental Methods**. Measurements were performed in the morning after overnight fasting, with resting periods of 0–30 min and measurement periods of 15–30 min. We excluded at least the first 5 min of measurement data; for all participants, we located a steady-state period of at least 5 min during which the coefficient of variation was 10% or less for $\dot{V}O_2$ and $\dot{V}CO_2$, and we calculated REE with the modified Weir equation (27). We made REE comparable between different-sized participants using residuals—the differences between measured and predicted values—from a linear regression model generated using the data of the study sample (**Supplemental Table 1**):

$$\text{REE (kcal/d)} = 413.4 + 20.2 \times \text{FFM (kg)} + 1.7 \times \text{FM (kg)}$$

Herein, we refer to residual REE as REE_{adj}. We also built three alternative explanatory models (**Supplemental Table 1**): the first included age as a covariate with FFM and FM; the second added ALMI to account for differences in muscularity (28); and in the third, we added the study data collection period as a covariate to examine potential biases introduced by including data from two different time periods.

To assess TEE in the Calex study, the overnight fasting participants gave a urine sample and ingested a doubly labeled water dose of 1 g per kg of body mass (21). A second urine sample was collected after 4–6 h, and a third 14 days later. The samples were analyzed in triplicate using mass spectrometry (Metabolic Solutions Inc., Merrimack, NH, USA) at the University of Alabama. TEE was calculated as in Schoeller et al. (29).

Statistical analyses

We performed the statistical analyses using R 4.2.1 (30). The analytic code is available without restriction at https://doi.org/10.5281/zenodo.7684731. We report descriptive statistics as means with standard deviations or as medians with first and third quartiles, but we did not test group differences in order to preserve statistical power. We verified the model's assumptions before accepting the results and used an alpha level of 0.05 for statistical significance.

We estimated the association between age and REE_{adj} and compared the measured REE and REE_{adj} between the age groups with linear mixed-effect models using the *nlme* package (31), with family identification as a random effect. We also performed a sensitivity analysis using FFM, FM and ALMI-adjusted REE residuals as the outcome. For the mother–daughter pairs, we first compared the REE_{adj} and then TEE using the measured TEE as the outcome and FFM and FM as covariates. We estimated intraclass correlation coefficients using the *psych* package (32) with one-way random-effects models—intraclass correlation compares within- and between-pair variations, thereby expressing how strongly the mothers and daughters resemble each other.

Last, we used linear regression to compare the measured REE and REE_{adj} between the menopause groups using the POST group as the reference. Given that body composition parameters may explain REE differently among women of different ages, we performed supporting analyses using measured REE as the outcome, menopause status or sex hormone concentrations as the explanatory variable, and FFM, FM and age as covariates in separate regression models.

Results

Participant characteristics

Table 1 shows participant characteristics and energy expenditures across age and menopause groups, and **Supplemental Table 2** shows the same in the mother–daughter pairs. Based on the descriptive statistics, group II women had higher FFM and lower FM. In group III, sex hormone concentrations varied between the menopause groups, as expected. As groups I and II included naturally menstruating women in different menstrual cycle phases and hormonal contraceptive users, we did not compare hormone levels between age groups.

Table 1

Age and energy expenditure

FFM and FM explained 47% of the REE variance, while the inclusion of age increased the adjusted R^2 to 68%. **Figure 2** illustrates how age impacts REE estimation by presenting the relationships between the predicted and measured REE values. Neither the ALMI nor information on the study data collection period improved the explanatory value. **Supplemental Table 1** presents the full results.

Figure 2

Age was inversely associated with REE_{adj} (B = -3.9; 95% CI: -4.8 to -3.1; $P <$ 0.001). Group I had 140 kcal/d (95% CI: 82–199) higher measured REE and 126 kcal/d (95% CI: 93–160) higher REE_{adj} (**Figure 3A**) than group III, while group II had 187 kcal/d (95% CI: 133–240) higher measured REE and 88 kcal/d (95% CI: 49–127) higher REE_{adj} ($P <$ 0.001 for all). The group differences in the FFM, FM and ALMI-adjusted REE were slightly smaller (**Supplemental Table 3**).

In the 16 mother–daughter pairs, the daughters had 100 kcal/d (95% CI: 63–138; $P < 0.001$) higher REE_{adj} than their mothers (**Figure 3C**). In the ten pairs with REE and TEE measurements, the daughters had 85 kcal/d (95% CI: 45–125; $P = 0.003$) higher REE_{adj} than their mothers, but there was no significant difference in TEE_{adj} ($B = 26$ kcal/d; 95% CI: -128–180; $P = 0.75$, **Figure 3D**). Intraclass correlation coefficients were 0.05 (95% CI: -0.43–0.52; $P = 0.42$) for REE_{adj}, 0.83 (95% CI: 0.49–0.96; $P < 0.001$) for TEE and 0.92 (95% CI: 0.71–0.98; $P < 0.001$) for TEE_{adj} (**Supplemental Figure 1**).

Figure 3

Resting energy expenditure in midlife

Compared with the POST group, the measured REE was not significantly different in either the PRE/PERI ($B = 46$ kcal/d; 95% CI: -26–119; $P = 0.21$) or MHT groups ($B = -27$ kcal/d; 95% CI: -118–63; $P = 0.55$); neither was REE_{adj} (PRE/PERI; $B = 7$ kcal/d; 95% CI: -42–55; $P = 0.78$; MHT: $B = -31$ kcal/d; 95% CI: -91–30; $P = 0.31$, **Figure 3B**). The exclusion of PERI women did not alter the results (data not shown). In the specific models generated for the middle-aged subsample that controlled for FFM, FM and age, the MHT group had a lower REE than the POST group (**Table 2**). Furthermore, neither E2 nor FSH showed a statistically significant association with REE.

Table 2

Discussion

Aging through adulthood and menopause are thought to slow basal metabolism, potentially predisposing women to obesity. This study demonstrates that increasing age is associated with

a decline in REE_{adj} among young and middle-aged women, also after partly controlling for genetic background. However, menopause did not contribute to the age-associated decline in REE_{adj}. Furthermore, E2 or FSH concentrations were not related to REE_{adj} in middle-aged women.

The decline in age-associated REE_{adj} aligns with previous cross-sectional studies by Pontzer et al. (1), Geisler et al. (3) and Siervo et al. (4), in which the decline began in women at 46.5, 35.2 and 47 years of age, respectively. The turning point for REE_{adj} therefore may occur before 60 years of age (1), but whether the phenomenon represents an actual slowing of cellular metabolism remains unclear. Low metabolic rate organs contribute more to FFM as we age (33), but aging also causes tissue quality changes; for example, the brain grey–white matter ratio (34) and skeletal muscle density (35) decline from young to middle adulthood, meaning that each kilogram of the brain or muscle tissue has fewer metabolically active cells as aging proceeds. Current body composition assessment methods, like DXA, cannot detect such changes (33), so FFM adjustments that assume FFM composition and quality are constant may overestimate the age-associated decline in $REE_{adj.}$ Indeed, Roubenoff et al. (36) found no association between age and FFM-adjusted REE when assessing FFM using total body potassium analysis, which directly estimates cell mass. Therefore, the direct influence of age on REE_{adj} may be less impactful than initially observed, but it may enhance REE estimation **(**as shown in **Figure 2B**) by enabling adjustments for age-related changes in body composition within the model. If slowing cellular metabolism contributes to the observed decline in REE_{adj}, it may result from altered systemic hormone and cytokine stimulation (33), with intrinsic changes in hormone responsiveness, protein synthesis, maintenance of membrane potentials and mitochondrial function (37).

We also compared the TEE_{adj} of middle-aged mothers and their daughters and found that they were similar, despite differences in their REE_{adj}, indicating that TEE is highly

heritable (38). This also suggests that the possible age-associated decline in REE_{adj} may have a negligible effect on TEE before late adulthood, as also reported by Pontzer et al. (1).

Our findings concur with previous research that menopausal status and sex hormone levels do not robustly determine REE_{adj} during midlife. Although ovarian hormone suppression studies in premenopausal women have shown some yet not fully convincing evidence of a REE_{adj} decline (39,40), MHT interventions show no clear effect on REE in postmenopause (15,17,18). In the present study, the MHT users had even lower REE than postmenopausal women who did not use MHT after adjusting for FFM, FM and age. However, given that the MHT group was the smallest in our study, this difference is unlikely to be attributed to MHT use *per se*. Observational evidence also indicates that menopause has a minimal impact on REE_{adj}. For instance, Lovejoy et al. (11) found that sleeping energy expenditure and TEE_{adj} decreased in women transitioning from premenopause to postmenopause during a four-year follow-up, but the changes were no different from participants who remained premenopausal, which suggests that the decreases were related to aging, not menopause. Furthermore, the menopausal transition was not associated with REE in the longitudinal MONET study (12), and cross-sectional studies have shown no association between menopausal status and measured REE (14,16), FFM-adjusted REE (13,15) or FFM-adjusted TEE (41), while a study that did show a higher REE in MHT users than non-users (14) failed to adjust the analyses for differing tissue masses. Finally, Pontzer et al. (1) found no differences in REE_{adj} and TEE_{adj} trajectories between middle-aged women and men, indicating that sex specific changes in energy expenditure are not observed during midlife.

The lack of a clear association between menopause and REE_{adj} is unexpected because the cessation of reproductive functions and altered hormonal profile should decrease basal metabolism. There are at least three potential explanations; first, with the limitations of body composition assessment and indirect calorimetry methods (33), the energy expenditure

311 of female reproductive processes may be so small relative to other functions contributing to
312 REE that its loss is difficult to detect. The second explanation is that the effects of menopause
313 cannot be disentangled from the effects of aging, especially because aging progresses
314 differently between individuals, although this is insufficient to explain why MHT
315 interventions do not increase REE (15,17,19). The third, more speculative explanation is that
316 women reallocate energy during menopause from reproduction to other purposes; Hazda
317 hunter-gatherer women, for example, increase the time spent on gathering resources for their
318 offspring (42), potentially reallocating the freed energy to movement and maintenance of the
319 locomotor system (43). Women in industrialized nations live differently and may therefore
320 use the energy to build energy reserves and bodily defense mechanisms, reallocating the freed
321 energy inside the REE component (43). Increased FM, especially to the trunk, may further
322 promote metabolic deterioration (44), inflammation (45) and sympathetic nervous system
323 activity (46), thereby further elevating the REE (47–50). Such trade-offs could explain why
324 women's REE_{adj} does not decline and their cardiometabolic risk profile worsens after
325 menopause in industrialized societies (51).

326 Finally, it should be mentioned that a decrease in absolute REE following
327 skeletal muscle loss could also contribute to menopause-associated FM accumulation if
328 women do not match the drop by reducing energy intake. We previously reported that a peri-
329 to postmenopausal transition during a mean follow-up of 14 months resulted in a 0.2 kg lean
330 mass loss, likely from skeletal muscle (52); but, assuming that the mass-specific metabolic
331 rate of skeletal muscle is 13 kcal/d at rest (53), the loss would reduce REE by 2.6 kcal/d,
332 which cannot explain the 0.8 kg increase in FM (6), especially as the tissue changes happen
333 gradually.

334 The main limitations of this study are its cross-sectional and secondary nature.
335 We pooled existing studies to generate a sufficiently large sample and cannot entirely exclude

a clustering effect. Considering the impact of sex hormones, we did not control for the use of hormonal products or menstrual cycle phases. Furthermore, serum E2 concentrations were analyzed with immunoassays, whose accuracy is limited for the low E2 levels seen post-menopause. While we could not adjust the results for differences in physical activity levels due to the lack of a uniform assessment method, we can assume that participants in group II had the highest physical activity levels. Long-term physical activity is hypothesized to lower REE (54); however, this response has not been clearly identified (55). Even if physical activity would reduce REE, group II still had higher REE than group III, suggesting that physical activity differences are unlikely to impact the robustness of our conclusions.

In conclusion, REE adjusted for DXA-measured FFM and FM declines in women from young to middle adulthood, likely due to aging rather than menopause, but whether falling cellular metabolic rates contribute is unclear. Current evidence does not support the inference that menopause reduces REE. Longitudinal data from middle-aged women with differing sex hormone trajectories are needed to reconcile whether menopause truly affects REE in a meaningful way.

Acknowledgments

We thank the participants for their time and effort and the staff of the Faculty of Sport and Health Sciences of the University of Jyväskylä.

The authors' responsibilities were as follows—JEK conducted EsmiRs study measurements, gave guidance for the Physique and NO RED-S studies, analyzed data, and wrote the manuscript. EKL led and designed the EsmiRs study with the help of JEK, H-KJ, MH, UMK, and JL. PW conducted Calex study measurements and gave guidance for the EsmiRs study. SC led and designed the Calex study with help from PW. JPA and JH led and designed the

Physique study with the help of VI, who also participated in data collection. JKI led, designed, and conducted data in the NO RED-S study with the help of EKA. All authors revised the manuscript and approved its final version.

Data availability

Datasets generated during and/or analyzed during the current study are not publicly available but are available from the principal investigators of each study (Calex: CS and EKL, EsmiRs [doi.10.17011/jyx/dataset/83491]: EKL, Physique: JPA, and NO RED-S: JKI) on reasonable request.

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546 **TABLE 1.** Participant characteristics and energy expenditures according to age and menopause groups

	Age groups (*N* = 120)			Menopause groups (*n* = 59)		
	I	**II**	**III**	**PRE/PERI**	**MHT**	**POST**
Variable	***n* = 26**	***n* = 35**	***n* = 59**	***n* = 19**	***n* = 10**	***n* = 30**
Age, y	19.8 (1.1)	28.2 (4.3)	53.8 (3.5)	50.7 (4.3)	55.1 (1.7)	55.3 (1.9)
Sex hormone concentrations						
E2, nmol/l				0.29 (0.18–0.55)	0.29 (0.17–0.38)	0.09 (0.06–0.12)
FSH, IU/l				8 (7–25)	38 (34–61)	80 (71–102)
Anthropometrics						
Height, cm	165.6 (5.6)	165.3 (5.8)	166.4 (5.0)	168.1 (4.6)	166.5 (5.6)	165.3 (4.8)
Body mass, kg	65.0 (10.5)	64.5 (7.9)	68.5 (9.1)	69.1 (9.0)	71.3 (12.3)	67.1 (7.9)
BMI, kg/m^2	23.7 (3.5)	23.6 (2.0)	24.7 (3.1)	24.5 (3.2)	25.7 (4.2)	24.5 (2.7)
Fat-free mass, kg	44.2 (5.0)	49.8 (5.3)	44.2 (4.2)	45.6 (4.1)	43.4 (4.2)	43.6 (4.1)
Fat mass, kg	20.9 (8.2)	14.7 (5.8)	24.2 (7.3)	23.6 (7.8)	27.9 (8.4)	23.4 (6.4)
Appendicular lean mass, kg	18.5 (2.6)	21.8 (2.7)	17.9 (1.9)	18.4 (2.0)	17.6 (1.9)	17.6 (1.9)
Appendicular lean mass index	6.7 (0.7)	8.0 (0.8)	6.5 (0.6)	6.5 (0.5)	6.4 (0.6)	6.4 (0.6)
Body fat percentage, %	31 (8)	23 (7)	36 (7)	35 (8)	40 (5)	36 (6)
Resting energy expenditure						
Measured, kcal/d	1,430 (110)	1,474 (146)	1,286 (124)	1,323 (105)	1,249 (143)	1,276 (128)
Predicted$_{\text{FFM \& FM}}$, kcal/d	1,339 (104)	1,442 (107)	1,345 (87)	1,372 (84)	1,335 (98)	1,332 (85)
Predicted$_{\text{FFM, FM \& age}}$, kcal/d	1,440 (103)	1,467 (94)	1,286 (92)	1,322 (86)	1,283 (115)	1,264 (84)
Total energy expenditure	***n* = 10**		***n* = 10**			
Measured, kcal/d	2,162 (310)		2,148 (236)			

Data as means (SD) or medians (IQR), $^{\#}$ *n* = 19

E2, estradiol; FSH, follicle-stimulating hormone; MHT, menopausal hormone therapy; PRE/PERI, pre- or perimenopause; POST, postmenopause

547

548 **TABLE 2.** The associations between body composition, age, menopausal status, serum sex hormone concentrations, and resting energy
549 expenditure in midlife (n = 59)

	Mass and age		**Menopause status**		**E2**		**FSH**	
	B	*P*-value	*B*	*P*-value	*B*	*P*-value	*B*	*P*-value
Intercept	850.1	< 0.001	976.3	< 0.001	838.6	< 0.001	881.4	< 0.001
Fat-free mass, kg	17.2	< 0.001	17.2	< 0.001	17.2	< 0.001	17.4	< 0.001
Fat mass, kg	6.6	< 0.001	7.3	< 0.001	6.7	< 0.001	6.8	< 0.001
Age, y	-9.0	0.001	-11.2	< 0.001	-8.9	0.005	-10.1	0.001
Menopause status								
PRE/PERI			-39.7	0.11				
MHT			-58.4	0.025				
E2, nmol/l					5.2	0.89		
FSH, IU/l							0.2	0.43
R^2/adjusted R^2	0.70/0.68		0.73/0.71		0.70/0.68		0.70/0.68	

E2, estradiol; FSH, follicle-stimulating hormone; MHT, menopausal hormone therapy; PRE/PERI, pre- or perimenopause; POST, postmenopause

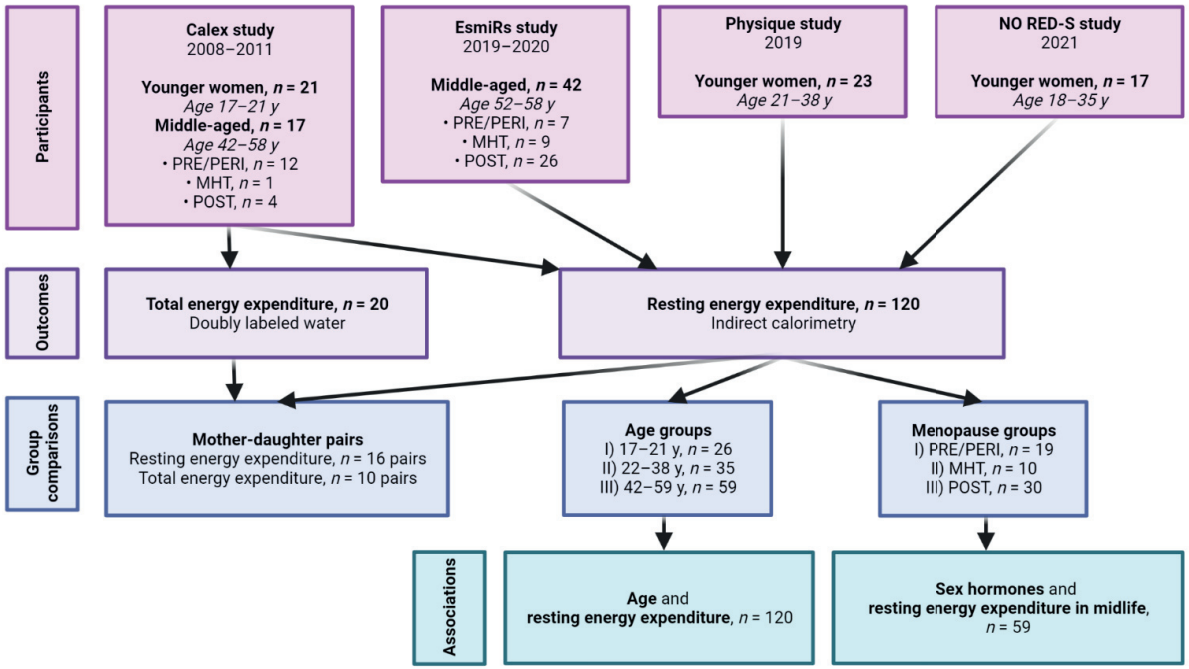


FIGURE 1. Description of the study participants, outcomes, and statistical approaches.

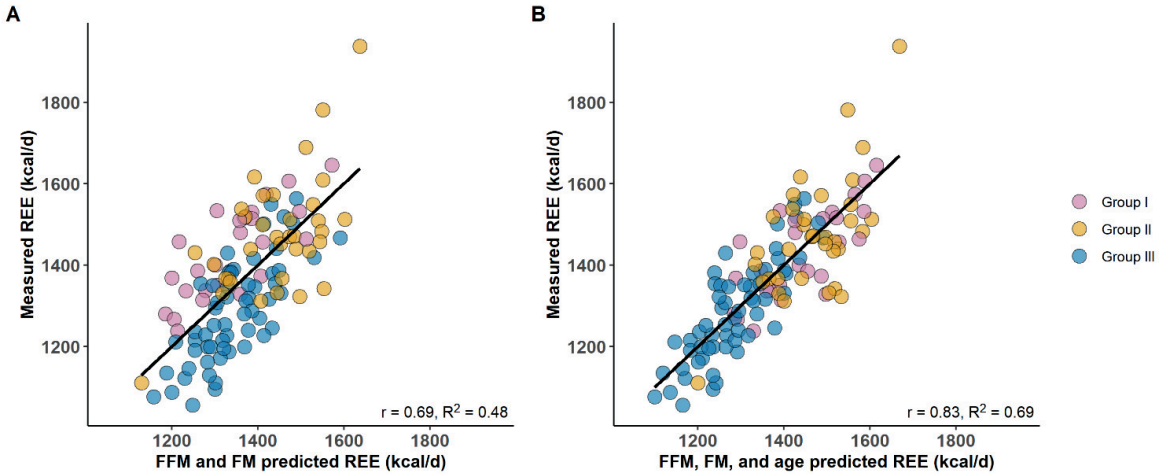
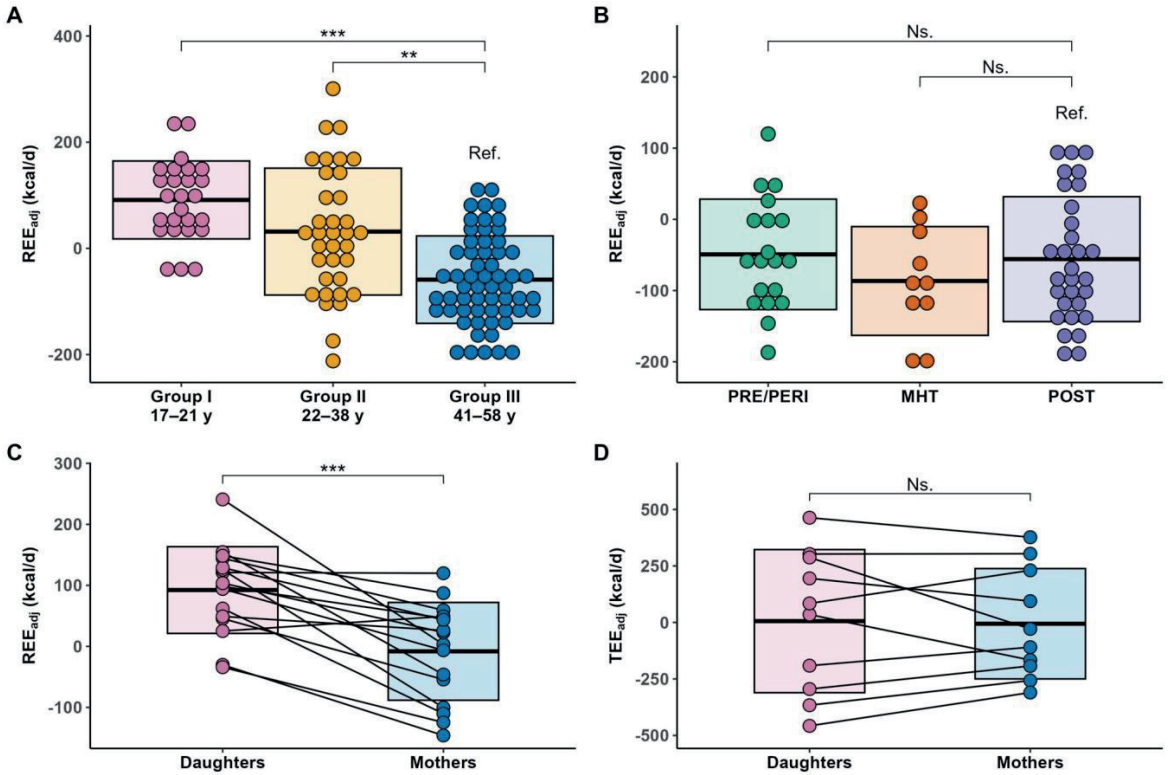


FIGURE 2. The relationship between measured REE and predicted REE among the 120 participants. (A) REE predicted with fat-free mass (FFM) and fat mass (FM), and (B) also with age.



564

565 **FIGURE 3.** The association of age and fat-free mass and fat mass–adjusted resting energy expenditure (REE_{adj}) and total energy expenditure
566 (TEE_{adj}). (A) REE_{adj} across age groups, (B) REE_{adj} across menopause groups, (C) REE_{adj} in 16 mother–daughter pairs, (D) TEE_{adj} in 10 mother–
567 daughter pairs. The boxplots show the mean and SD of each group.

Supplemental Information

Menopausal status determination

The determination of menopausal status followed the Stages of Reproductive Aging Workshop (STRAW) + 10 guidelines, relying on menstrual cycle data collected through structured questionnaires and follicle-stimulating hormone (FSH) concentration measurements. The general method has been previously reported by Kovanen et al. (1).

In the Calex study, participants were first asked about their current menstrual status, then provided information about their menstrual history over the past year, including changes in cycle duration and time since the last period. They also reported any prior hysterectomies/oophorectomies and the use of hormonal products. FSH concentrations were measured from single fasting serum samples.

In the EsmiRs study, participants self-reported their menopausal status using given information about menopause-related menstrual cycle changes. They also reported the time since their last period, the regularity of their cycles, and any prior hysterectomies/oophorectomies or use of hormonal products. FSH concentrations were measured on three separate days and the data collected on the same day as the REE measurement was used for statistical analysis.

The determination of menopausal status was as follows:

1. **Premenopausal (PRE, *n* = 10):** Participants reported no changes in menstrual cycles, and FSH concentrations ranged from 2.9 to 14.6 IU/l. One participant had a prior hysterectomy and her menopause status was determined based on FSH measurement. One woman used a hormonal intrauterine device.

2. **Perimenopausal (PERI, *n* = 9):** Participants reported menstrual irregularities, and FSH concentrations ranged from 7.4 to 114.0 IU/l. The participant with the lowest FSH concentration had notably higher levels at other EsmiRs measurement points. The participant with the highest FSH concentration reported having 7 to 9 periods in the past year and her last period was 35 to 59 days ago. One woman did not report menstrual cycle information and one participant had a prior hysterectomy, and their statuses were determined based on FSH measurements.

3. **Postmenopausal (POST, *n* = 30):** Participants reported not menstruating anymore or that it had been at least 6 months since their last period and had high FSH concentrations ranging from 27.1 to 137 IU/l. One EsmiRs study participant had an FSH concentration of less than 30 IU/l but had notably higher levels at other measurement points. Another participant did not have menstrual cycle information, and another had a prior hysterectomy, with their statuses determined using FSH measurements.

4. **Postmenopausal menopausal hormone therapy users (MHT, *n* = 10):** Nine women in the group used combination MHT and one used E2-only MHT. Seven participants took oral MHT with one of the following combinations: 1-mg estradiol hemihydrate and 5-mg dydrogesterone (*n* = 5, Femoston-Conti), 1-mg estradiol valerate and 0.5-mg norethisterone acetate (*n* = 1, Cliovelle), or 2-mg estradiol hemihydrate and 1-mg norethisterone acetate (*n* = 1, Kliogest). Two participants used transdermal patches containing either 3.2-mg estradiol hemihydrate and 11.2-mg norethisterone acetate (*n* = 1, Evorel Conti) or 0.585-mg estradiol hemihydrate and 5-mg oral dydrogesterone (*n* = 1, Estradot and Terolut). One participant used transdermal patch containing 0.39-mg estradiol hemihydrate (Estradot).

Assessment of resting energy expenditure

Calex. Participants were instructed to avoid strenuous physical activity and to refrain from consuming fatty and protein-rich foods the night before. They arrived at the laboratory by car after an overnight fast and rested for 30 minutes before a 30-minute measurement period. The first 10 minutes of $\dot{V}O_2$ and $\dot{V}CO_2$ data were excluded, and the longest steady-state segment (the coefficients of variation [CV] ≤ 10% for $\dot{V}O_2$ and $\dot{V}CO_2$) was used to calculate resting energy expenditure (REE).

EsmiRs. Participants were instructed to refrain from moderate-to-vigorous physical activity for two days before measurements, limit physical activity on the measurement day, and arrive at the laboratory by car. They were also instructed to avoid alcohol for 48 hours and caffeine for 12 hours before measurements and to have a light evening meal. Measurements were performed after an overnight fast, following the same protocol as in the Calex study. REE calculations were also done using the same method as previously described. Three women were excluded from a previous study on fat oxidation (2) due to higher RER values (0.91–0.94), as the calculation of substrate use is extremely sensitive even to minor hyperventilation. However, they were included in the present study because the calculation of REE is less sensitive to such effects.

Physique. Participants were instructed to abstain from exercise for 24 hours before measurements and to avoid physical activity on the morning of the measurement. They were also advised to avoid alcohol and caffeine for 12 hours prior and to fast overnight. They rested while the metabolic cart was being prepared, followed by a 20-minute measurement period, with the first 5 minutes of data excluded from analyses. The 5-minute steady state period (CV ≤ 10% for $\dot{V}O_2$ and $\dot{V}CO_2$) with the lowest CV for $\dot{V}O_2$ and $\dot{V}CO_2$ was used to calculate REE.

NO RED-S. Participants were instructed to avoid exercising on the previous day and to refrain from alcohol use 12 hours before measurements. After an overnight fast, they arrived at the laboratory and rested for 30 minutes before a measurement period of 15-30 minutes, varying slightly between individuals depending on how quickly they reached a steady state. The first 5 minutes of data were excluded, and the longest period with $\dot{V}O_2$ and $\dot{V}CO_2$ CVs ≤ 10% was used to calculate REE.

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Supplemental Tables

Supplemental Table 1. Results of resting energy expenditure explanatory models

	Model 1		**Model 2**		**Model 3**		**Model 4**	
	B (95% CI)	*P-value*	*B (95% CI)*	*P-value*	*B (95% CI)*	*P-value*	*B (95% CI)*	*P-value*
Intercept	413.4 (221.9 – 604.9)	**<0.001**	658.5 (500.8 – 816.2)	**<0.001**	647.0 (480.0 – 814.1)	**<0.001**	630.1 (458.0 – 802.3)	**<0.001**
Fat-free mass, kg	20.2 (16.3 – 24.0)	**<0.001**	17.7 (14.6 – 20.7)	**<0.001**	16.8 (11.8 – 21.8)	**<0.001**	18.1 (14.9 – 21.3)	**<0.001**
Fat mass, kg	1.7 (-0.9 to 4.2)	0.195	4.9 (2.8 – 7.0)	**<0.001**	5.0 (2.9 – 7.1)	**<0.001**	4.6 (2.5 – 6.8)	**<0.001**
Age, y			-5.0 (-6.2 to -3.9)	**<0.001**	-5.0 (-6.2 to -3.8)	**<0.001**	-4.9 (-6.1 to -3.6)	**<0.001**
ALMI					6.6 (-24.1 to 37.3)	0.671		
Data collection period (Calex study = 1; EsmiRs, Physique and NO RED-S studies = 0)							16.0 (-22.6 to 54.7)	0.413
R^2 / R^2 adjusted	0.477 / 0.468		0.690 / 0.682		0.691 / 0.680		0.692 / 0.681	

ALMI, appendicular lean mass index

Supplemental Table 2. Characteristics and energy expenditures in the mother–daughter pairs

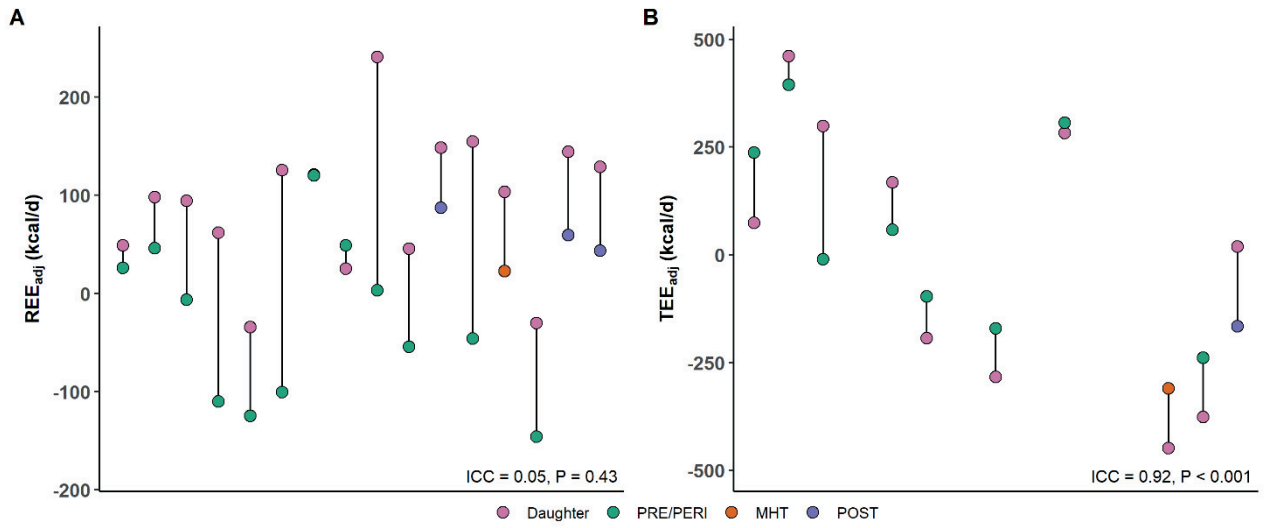
	Resting energy expenditure		Total energy expenditure	
Variable	**Mothers** ***n*** **= 16**	**Daughters** ***n*** **= 16**	**Mothers** ***n*** **= 10**	**Daughters** ***n*** **= 10**
Age, y	49.4 (3.6)	19.5 (1.0)	48.7 (4.0)	19.3 (1.0)
Anthropometrics				
Height, cm	167.6 (5.2)	165.0 (6.2)	168.4 (4.9)	165.2 (7.0)
Body mass, kg	70.5 (11.4)	65.3 (11.5)	73.8 (10.7)	65.4 (9.3)
BMI, kg/m^2	25.2 (4.4)	24.0 (3.8)	26.2 (4.5)	23.9 (2.5)
Fat-free mass, kg	45.8 (4.4)	42.9 (3.7)	46.7 (4.5)	42.5 (3.8)
Fat mass, kg	24.7 (10.1)	22.5 (9.3)	27.1 (10.2)	22.8 (7.1)
Appendicular lean mass, kg	18.5 (2.2)	17.9 (2.3)	18.9 (2.3)	17.6 (2.6)
Appendicular lean mass index	6.6 (0.6)	6.6 (0.7)	6.6 (0.6)	6.4 (0.7)
Body fat percentage, %	35 (10)	33 (9)	37 (9)	34 (7)
Resting energy expenditure				
Measured, kcal/d	1,371 (107)	1,408 (101)	1,377 (111)	1,371 (84)
$Predicted_{FFM\ \&\ FM}$, kcal/d	1,379 (92)	1,315 (83)	1,400 (92)	1,309 (83)
$Predicted_{FFM,\ FM\ \&\ age}$, kcal/d	1,339 (96)	1,427 (95)	1,369 (84)	1,369 (84)
Total energy expenditure				
Measured, kcal/d			2,148 (236)	2,162 (310)

Data as means (SD)

Supplemental Table 3. Adjusted resting energy expenditure (REE) in age groups I and II compared with group III. In the primary analysis, the outcome was REE residuals adjusted for fat-free mass (FFM) and fat mass (FM). The REE residuals were also adjusted for appendicular lean mass index (ALMI) in the sensitivity analysis.

	$REE_{FFM, FM}$		$REE_{FFM, FM, ALMI}$	
	B (95% CI)	*P*-value	*B* (95% CI)	*P*-value
Intercept	-56.8 (-80.3 to -33.3)	<0.001	-46.5 (-70.2 to -22.7)	<0.001
Group I	126.5 (93.1 – 159.9)	<0.001	118.4 (82.5 – 154.4)	<0.001
Group II	88.4 (49.2 – 127.5)	<0.001	60.1 (20.8 – 99.3)	0.003

Supplemental Figure



Supplemental Figure 1. The similarity of mothers and daughters in fat-free mass and fat mass–adjusted **A)** resting energy expenditure (REE_{adj}) and **B)** total energy expenditure (TEE_{adj}). The intraclass correlation coefficient (ICC) describes how strongly the family members resemble each other. The pairs are ordered by the mother's age, from the youngest to the oldest. The colors show the mother's menopausal status.

III

FAT OXIDATION AT REST AND DURING EXERCISE IN MALE MONOZYGOTIC TWINS

by

Karppinen, J.E., Rottensteiner, M., Wiklund, P., Hämäläinen, K., Laakkonen, E.K., Kaprio, J., Kainulainen, H. & Kujala U.M. 2019

European Journal of Applied Physiology vol 119 (11–12): 2711–2722

https://doi.org/10.1007/s00421-019-04247-x

European Journal of Applied Physiology
https://doi.org/10.1007/s00421-019-04247-x

ORIGINAL ARTICLE

Fat oxidation at rest and during exercise in male monozygotic twins

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Received: 29 January 2019 / Accepted: 24 October 2019

Abstract

Purpose We aimed to investigate if hereditary factors, leisure-time physical activity (LTPA) and metabolic health interact with resting fat oxidation (RFO) and peak fat oxidation (PFO) during ergometer cycling.

Methods We recruited 23 male monozygotic twin pairs (aged 32–37 years) and determined their RFO and PFO with indirect calorimetry for 21 and 19 twin pairs and for 43 and 41 twin individuals, respectively. Using physical activity interviews and the Baecke questionnaire, we identified 10 twin pairs as LTPA discordant for the past 3 years. Of the twin pairs, 8 pairs participated in both RFO and PFO measurements, and 2 pairs participated in either of the measurements. We quantified the participants' metabolic health with a 2-h oral glucose tolerance test.

Results Fat oxidation within co-twins was correlated at rest [intraclass correlation coefficient (ICC) = 0.54, 95% confidence interval (CI) 0.15–0.78] and during exercise (ICC = 0.67, 95% CI 0.33–0.86). The LTPA-discordant pairs had no pairwise differences in RFO or PFO. In the twin individual-based analysis, PFO was positively correlated with the past 12-month LTPA ($r = 0.26$, $p = 0.034$) and the Baecke score ($r = 0.40$, $p = 0.022$) and negatively correlated with the area under the curve of insulin ($r = -0.42$, $p = 0.015$) and glucose ($r = -0.31$, $p = 0.050$) during the oral glucose tolerance test.

Conclusions Hereditary factors were more important than LTPA for determining fat oxidation at rest and during exercise. Additionally, PFO, but not RFO, was associated with better metabolic health.

Keywords Twins · Exercise · Lipid metabolism · Oral glucose tolerance

Communicated by Philip D Chilibeck.

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Abbreviations

AUC	Area under the curve
DXA	Dual-energy x-ray absorptiometry
ICC	Intraclass correlation coefficient
LBM	Lean body mass
LTPA	Leisure-time physical activity
MET	Metabolic equivalent of task
MZ	Monozygotic
OGTT	Oral glucose tolerance test
PFO	Peak fat oxidation
REE	Resting energy expenditure
RER	Respiratory exchange ratio
RFO	Resting fat oxidation
VCO_2	Volume of carbon dioxide
VO_2	Volume of oxygen
VO_{2peak}	Peak oxygen uptake

Published online: 31 October 2019

Introduction

Fat oxidation rates at rest (Goedecke et al. 2000; Robinson et al. 2016) and during exercise (Venables et al. 2005; Randell et al. 2016; Fletcher et al. 2017) vary among individuals. The key determinants of resting fat oxidation (RFO) are not precisely identified in the scientific literature. During exercise, the main determinant of substrate use is exercise intensity (Romijn et al. 1993). The peak fat oxidation (PFO) rate is usually achieved at moderate exercise intensities (~40–60% of maximal oxygen uptake) (Venables et al. 2005; Randell et al. 2016; Fletcher et al. 2017), and the rate can be regarded as the highest systemic capacity to oxidise fat. The exercise intensity, where PFO is reached, is called FAT_{MAX} (Achten et al. 2002). As reviewed by Maunder et al. (2018), the most important determinants of PFO are training status and testing modality, biological sex, as well as habitual and acute nutrition. Large cross-sectional studies have accounted for only 34–47% of the variance in PFO (Venables et al. 2005; Randell et al. 2016; Fletcher et al. 2017). Thus, a large part of the inter-individual variability in PFO remains unexplained. Genetic differences likely play an important role because several physical and performance traits, including maximal oxygen uptake (Bouchard et al. 1998), lean body mass (LBM), muscle strength (Arden and Spector 1997) and skeletal muscle fiber-type proportion (Simoneau and Bouchard 1995) have significant genetic components. Studies investigating the respiratory exchange ratio (RER) at rest and during exercise have demonstrated that the relative use of fatty acids in both conditions show familial resemblance (Bouchard et al. 1989; Toubro et al. 1998). However, to our knowledge, no researcher has studied the absolute fat oxidation rates at rest and during exercise among monozygotic (MZ) twins.

Previous observational studies (Venables et al. 2005; Randell et al. 2016; Fletcher et al. 2017) have highlighted the influence of modifiable lifestyle factors, such as physical activity, on the capacity to oxidise fats. As genes also affect physical activity participation (Stubbe et al. 2006; Mustelin et al. 2012; Aaltonen et al. 2013), observational studies possibly overestimate the influence of physical activity. Experimental studies can provide evidence on the cause-and-effect relationship; however, long-term exercise training trials investigating fat oxidation are rare because they are expensive and arduous to perform. An option to counteract the shortcomings and the difficulties of both study designs is to compare the fat oxidation capacity of MZ co-twins who are discordant in long-term physical activity. This study design controls for genetic predisposition and mostly for the impact of the childhood environment. Therefore, the possible difference between co-twins likely results from different physical activity habits.

Besides investigating the determinants of fat oxidation capacity, researchers have been interested in understanding whether fat oxidation capacity interacts with metabolic health. This seems plausible as efficient utilization of fatty acids could protect from e.g. insulin resistance (Phielix et al. 2012). Indeed, some studies have found an association between systemic fat oxidation and better metabolic health status (Hall et al. 2010; Rosenkilde et al. 2010; Robinson et al. 2015). However, obesity-related increase in fatty acid availability has also been linked to higher fat oxidation levels (Perseghin et al. 2002; Hodson et al. 2010; Ara et al. 2011; Dandanell et al. 2017a). Thus, it remains debated whether higher fat oxidation capacity is beneficial to metabolic health and more research is needed.

In this study, our goal was to investigate the influence of internal (genetics) and external (physical activity) factors on fat oxidation at rest and during exercise. Additionally, we aimed to examine the association between fat oxidation capacity and oral glucose tolerance test (OGTT)-induced metabolic response.

Materials and methods

Participants and study design

This study is part of the FITFATTWIN study, whose purpose was to identify possible pairwise differences in health and fitness parameters between male MZ co-twins (aged 32–37 years). The recruitment process was previously reported in detail (Rottensteiner et al. 2015). In short, the studied MZ twin pairs were identified from the longitudinal FinnTwin16 cohort, which follows Finnish twins born from October 1974 to December 1979. The co-twins from 202 male MZ pairs provided data on their physical activities in an online survey, which formed the fifth wave of the FinnTwin16 study data collection. This data was used to identify co-twins who were potentially discordant in leisure-time physical activity (LTPA). From the whole population, 39 twin pairs met the initial selection criteria and were selected to participate in a telephone interview, consisting of questions about their physical activities and health habits. Based on the interview, 20 twin pairs were invited to participate in the study; of these, 17 twin pairs accepted the invitation. Additionally, 6 twin pairs who were identified as concordant in LTPA were recruited from the FinnTwin16 cohort. These pairs were selected to represent varying physical activity levels, from sedentary to athletic. Thus, a total of 23 twin pairs participated in the laboratory measurements performed on 2 consecutive days. The complete timetable of the measurements was reported earlier as supplementary material in Rottensteiner et al. (2015).

Of the 23 twin pairs, 19 pairs participated in the exercise test and 22 pairs participated in the resting metabolism measurement (18 pairs took part in both measurements). One twin individual's resting metabolism measurement was excluded due to hyperventilation. Thus, the analyses of genetic influence on PFO and RFO were conducted among 19 and 21 twin pairs, respectively. In total, PFO and RFO were determined for 41 and 43 twin individuals, respectively, and the twin individual-based analyses were conducted in these groups. One twin pair declined to participate in the OGTT, and the analyses between PFO or RFO and OGTT variables were performed in groups of 39 and 41 twin individuals, respectively.

Based on detailed LTPA interviews and a questionnaire (see the next subsection), 10 of the 23 twin pairs were identified as LTPA-discordant for the past 3 years. The determination of discordance was thoroughly explained by Rottensteiner et al. (2015). Of the 10 LTPA-discordant twin pairs, 8 pairs participated in both metabolism measurements, and 2 pairs took part in one of the measurements. Therefore, a pairwise comparison on the effect of LTPA on PFO or RFO was performed between 9 twin pairs, respectively.

Leisure-time physical activity (LTPA)

The LTPA level was determined with two separate interviews and the Baecke questionnaire. A brief retrospective interview (Waller et al. 2008; Leskinen et al. 2009; Rottensteiner et al. 2015), including structured questions on the LTPA's average frequency, duration, and intensity, as well as the average frequency and duration of commuting, was used to estimate the total LTPA volume at 1-year intervals over the past 6 years. The LTPA volume was calculated by multiplying the activity's monthly frequency, minute duration and metabolic equivalent of task (MET)-intensity and commuting physical activity was calculated by multiplying the standard 4-MET intensity with the daily commuting minute duration and the weekly frequency (5 times a week). The total LTPA volume was expressed as the sum score of the daily MET-hours, and the mean LTPA (MET-h/day) over the past 3 years (3-year LTMET index) was used to describe each participant's activity level.

A more thorough interview was used to estimate the past 12-month LTMET index. The interview was based on the Kuopio Ischemic Heart Disease Risk Factor Study Questionnaire (Lakka and Salonen 1997), with additional physical activities. The participants were asked about the number of times per month (and the average duration) they participated in 20 different types of physical activities or other physical activities specified by each respondent. The participants were also asked to classify the intensity of each activity based on a 4-level scale. Like the past 3-year LTMET index, the 12-month LTMET index was calculated as MET-h/day. The participants also completed a 16-item Baecke questionnaire, which measured their recent work, sports and LTPA (Baecke et al. 1982). The total sum score was used for the twin individual-based analysis.

Peak oxygen uptake (VO_{2peak}) and peak fat oxidation (PFO)

A graded incremental exercise test with a gas-exchange analysis was performed on the first day of the laboratory visit. The participants were instructed to avoid vigorous exercise and alcohol use 48 h and avoid eating 2 h prior to testing. The exercise test was performed with an electrically braked bicycle ergometer (Ergoselect 200, Ergoline GmbH, Germany). The testing began with a 2-min stage at 20 W, followed by a 2-min stage at 25 W. Next, the work rate increased by 25 W every 2 min until volitional exhaustion. The breath-by-breath gas exchange was recorded with a Vmax Encore 29 metabolic cart (Sensormedics, Yorba Linda, CA, USA), which was calibrated according to the manufacturer's instructions before each measurement. The volume of oxygen (VO_2) inspired and the volume of carbon dioxide (VCO_2) expired were averaged at 30-s intervals for the whole test duration. The VO_{2peak} was determined as the average of the two highest consecutive VO_2-measurements. Fat oxidation was calculated for each exercise stage from the last 30-s period with Frayn's (1983) equation, assuming that the urinary nitrogen excretion was negligible. The highest calculated fat oxidation rate was selected as the PFO and the corresponding exercise intensity as the FAT_{MAX} (%VO_{2peak}). Each participant's heart rate and cardiac function were monitored continuously with a 12-lead electrocardiography system (CardioSoft v.5.02 GE Medical System Corina, GE Medical System Inc., USA). The rating of perceived exertion (RPE) was determined at the end of each stage with the Borg (6–20) scale (Borg 1982). The exercise test was classified as maximal if the RPE was 19–20/20 or the RER was > 1.1 at the end of the test. The exercise test protocol was submaximal for 4 subjects. Among the participants tested with the submaximal protocol, their fat oxidation rates declined before their last performed exercise stage. Thus, their PFO results were included in the study, and their VO_{2peak} was extrapolated based on the submaximal results.

Body composition

Each participant's body composition was measured in the morning of the second measurement day, following overnight fasting. For the body mass and height measurements, the participants were barefoot and wore light outfits. Their body mass and height were respectively measured using an electronic scale with a 0.1-kg accuracy and a stadiometer with a 0.5-cm accuracy. Their total mass, LBM, fat mass and

body fat percentage were measured with dual-energy x-ray absorptiometry (DXA) (DXA Prodigy, GE Lunar Corp., Madison, WI, USA).

Resting metabolism

Each participant's resting metabolism was measured (after the DXA measurement) in a dimly light room. Similar to the exercise test, the same Vmax Encore 29 metabolic cart was used and calibrated accordingly. First, the participants rested 10 min in a supine position. Then, their gas exchange was recorded for 16 min using the ventilated canopy method, and their VO_2 and VCO_2 were averaged at 1-min intervals. First 5 min measurement data were excluded. Resting metabolism variables were calculated from a steady-state measurement period (VO_2 and VCO_2 coefficient of variation $\leq 10\%$ between minutes). The average steady-state duration was 9.2 ± 2.7 min. The resting energy expenditure (REE) was calculated with the modified Weir equation (Weir 1949; Mansell and Macdonald 1990), and Frayn's (1983) equation was used to calculate the RFO. A protein correction factor of 0.11 mg/kg/min was applied to take into account the nitrogen exertion (Flatt et al. 1985; Hall et al. 2010; Robinson et al. 2016).

Metabolic health

A standard 2-h OGTT followed the resting metabolism measurement. After the collection of their fasted blood samples, the participants ingested a 75-g glucose solution (GlucosePro, Comed LLC, Tampere, Finland). Next, their blood samples were collected at 30-min, 1-h and 2-h intervals post-ingestion. All blood samples were collected from each participant's antecubital vein when he was in a supine position. The plasma glucose concentration was analysed with Konelab 20 XT (Thermo Fisher Scientific, Vantaa, Finland) and the serum insulin concentration was analysed using IMMULITE® 1000 (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The Matsuda index was determined based on the equation: 10,000/square root of [(fasting glucose × fasting insulin) × (mean glucose × mean insulin during OGTT)] (Matsuda and DeFrozo 1991). Additionally, the area under the curve (AUC) was calculated for insulin and glucose with the trapezoidal method.

Ethical approval

Good clinical and scientific practices and guidelines, as well as the Declaration of Helsinki, were followed while conducting the study. The study was approved by the Ethics Committee of the Central Finland Health Care District (Dnro 4U/2011). All participants provided their written informed consent before the laboratory measurements.

Statistical analysis

Statistical analysis was carried out with IBM SPSS Statistics 24.0 and Stata 15.0. A one-way random model was used to calculate the intraclass correlation coefficients (ICCs) between the MZ co-twins. An ICC compares within-pair variation with between-pair variation and thus explains how similar the co-twins are when compared with the other pairs. Pairwise correlations and differences were analysed with Pearson correlation coefficient and paired-sample *t* test, respectively. Twin individual-based correlations were analysed with simple linear regression, and the within-pair dependency was taken into account (Williams 2000) with the clustering option of Stata. In all regression analyses, RFO or PFO was treated as the dependent variable. All the variables or the regression analysis residuals were determined normally distributed with the Shapiro–Wilk test or with the visual inspection of the histograms and the normality plots. The *p* value 0.05 was selected to represent statistical significance. For clarity, RFO or PFO without a unit symbol is used in the text when the statistical significance persists both when using absolute or LBM relative values in the analysis.

Results

Participant characteristics

Table 1 presents the participant characteristics. Overall, the study population consisted of healthy men (aged 32–37 years) with varying physical activity, body composition and cardiorespiratory fitness levels.

Hereditary factors and metabolism at rest and during exercise

The calculated ICCs of the resting metabolism variables and PFO showed significant resemblance between co-twins (Table 2). We also categorised the co-twins as more active or less active based on their 12-month LTMET index to calculate pairwise correlations (Figs. 1 and 2). This division did not lead to significant mean differences between the groups in RFO (0.001 g/min, $p = 0.68$) or PFO (0.02 g/min, $p = 0.47$).

LTPA and metabolism at rest and during exercise

Table 3 presents the results of the pairwise comparison between the LTPA-discordant co-twins ($n = 9$–10). Figure 3 illustrates individual RFO and PFO results and

Table 1 Characteristics of the participants ($n=46$)

	Mean ± SD	Minimum	Maximum
Age	34.5 ± 1.5	32	37
Body composition			
Height (cm)	178 ± 7	157	190
Body mass (kg)	76.7 ± 9.8	51.5	96.2
BMI (kg/m^2)	24.1 ± 2.7	19.8	33.6
Lean body mass (kg)	56.8 ± 7.0	40.4	73.2
Fat mass (kg)	16.7 ± 6.7	5.2	31.6
Body fat percentage (%)	21.4 ± 6.7	7.6	36.0
Physical activity			
3-year-LTMET index (MET-h/day)	4.7 ± 4.6	0.2	18.3
12-month-LTMET index (MET-h/day)	4.2 ± 4.6	0.1	27.7
Baecke questionnaire (score)	8.3 ± 1.3	5.6	12.1
Cardiorespiratory fitness[a]			
VO_{2peak} (l/min)	3.2 ± 0.6	2.3	4.6
VO_{2peak} (ml/kg/min)	41 ± 9	29	66
VO_{2peak} (ml/kg LBM/min)	55 ± 8	40	75
Resting metabolism[b]			
REE (kcal/d)	1 685 ± 190	1 297	2 074
RER	0.82 ± 0.04	75	89
RFO (g/min)	0.06 ± 0.02	0.02	0.09
RFO (mg/kg LBM/min)	1.0 ± 0.3	0.5	1.6
Fat oxidation during exercise[a]			
PFO (g/min)	0.39 ± 0.14	0.13	0.81
PFO (mg/kg LBM/min)	6.8 ± 2.2	2.4	13.7
FAT_{MAX} (%VO_{2peak})	40 ± 10	26	72
Metabolic health			
Fasting glucose (mmol/l)	5.5 ± 0.5	4.7	6.6
Fasting insulin (IU/ml)	3.9 ± 3.2	0.2	14.6
2-h OGTT glucose (mmol/l)[c]	5.1 ± 1.1	3.1	7.6
Matsuda index[c]	18.4 ± 17.8	2.3	64.6
Insulin AUC (IU/ml/h)[c]	66.1 ± 38.4	17.6	186.8
Glucose AUC (mmol/l/h)[c]	11.7 ± 2.4	8.2	19.4

AUC area under the curve, *BMI* body mass index, *LBM* lean body mass, *MET* metabolic equivalent of task, *OGTT* oral glucose tolerance test, *PFO* peak fat oxidation, *REE* resting energy expenditure, *RER* respiratory exchange ratio, *RFO* resting fat oxidation, *VO_{2peak}* peak oxygen uptake

[a] $n=41$

[b] $n=43$

[c] $n=44$ participants

within-pair relationships. As reported earlier (Rottensteiner et al. 2015), long-term LTPA-discordant co-twins had different body fat percentage and cardiorespiratory fitness levels. However, there were no differences in REE, RER at rest or RFO between active and inactive co-twins. On average, the active co-twins tended to have higher PFO rates and lower FAT_{MAX} when compared with the inactive co-twins, but the differences were not statistically significant. In the twin individual-based analysis (Table 4), only PFO (g/min) was positively correlated with the 12-month LTMET index ($r=0.26$, $p=0.034$), the Baecke score ($r=0.40$, $p=0.022$) and VO_{2peak} (l/min) ($r=0.51$, $p=0.028$).

Fat oxidation at rest and during exercise and metabolic health

RFO or PFO were not correlated with fasting glucose, fasting insulin or the Matsuda index in the twin individual-based analysis (Table 4). PFO (g/min) negatively correlated with the AUC of insulin ($r=-0.42$, $p=0.015$) and the AUC of glucose ($r=-0.31$, $p=0.050$). In contrast, RFO positively

Table 2 The intraclass correlation coefficients (ICCs) between MZ co-twins

Variable	ICC	95% CI	*p* value
Resting metabolism[a]			
REE (kcal/day)	0.58	(0.21 to 0.80)	0.002
RER	0.51	(0.12 to 0.77)	0.007
RFO (g/min)	0.54	(0.15 to 0.78)	0.004
RFO (mg/kg LBM/min)	0.57	(0.20 to 0.80)	0.003
Fat oxidation during exercise[b]			
PFO (g/min)	0.67	(0.33 to 0.86)	< 0.001
PFO (mg/kg LBM/min)	0.59	(0.21 to 0.82)	0.002
FAT_{MAX} ($\%VO_{2peak}$)	0.51	(0.09 to 0.77)	0.010

PFO peak fat oxidation, *REE* resting energy expenditure, *RER* respiratory exchange ratio, *RFO* resting fat oxidation

[a] $n = 21$ MZ twin pairs

[b] $n = 19$ MZ twin pairs

correlated ($r = 0.31$, $p = 0.019$) with glucose AUC when expressed relative to LBM.

Discussion

For the first time, our study data showed that fat oxidation rates at rest and during exercise were similar between MZ co-twins, even though the study group was enriched with pairs who had discordant LTPA habits. The co-twins also exhibited similar FAT_{MAX} values and thus tended to reach PFO at the same absolute exercise intensities. Although we were unable to confirm the effect of long-term LTPA on fat oxidation capacity in our small sub-population of long-term LTPA-discordant MZ co-twins, PFO (g/min) was associated with LTPA in the twin individual-based analysis. We also observed that PFO (g/min), but not RFO, was associated with a favourable response to glucose loading.

This study's major finding is that hereditary factors influence fat oxidation capacity. The finding supports those of Toubro et al. (1998) and Bouchard et al. (1989), who reported that RER at rest and during low-intensity cycling showed significant familial resemblance. In a study involving male MZ twin pairs (Bouchard et al. 1989), the ICCs of RER ranged from 0.63 to 0.54 during cycling at low intensities (50 W and 100 W, respectively). As the researchers also investigated the substrate use of dizygotic twins, they were able to control their analysis for the common environmental effect. Their calculated heritability estimates ranged from 0.40 to 0.62. However, as RER only describes the relative use of energy substrates, this study broadens the concept by showing that absolute fat oxidation rates behave accordingly and supports the earlier suggestion that genes play a role in determining fat oxidation capacity during exercise (Jeukendrup and Wallis 2005; Randell et al. 2016). This assumption seems evident, as the large cross-sectional studies investigating fat oxidation during exercise have been able to describe only partly the observed inter-individual variability in PFO (Venables et al. 2005; Randell et al. 2016; Fletcher et al. 2017).

We identified a subpopulation of MZ twin pairs, where the co-twins differed in their past 3-year LTPA. As reported earlier (Rottensteiner et al. 2015, 2016; Tarkka et al. 2016; Hautasaari et al. 2017), the LTPA discordance created diet-independent differences between active and inactive

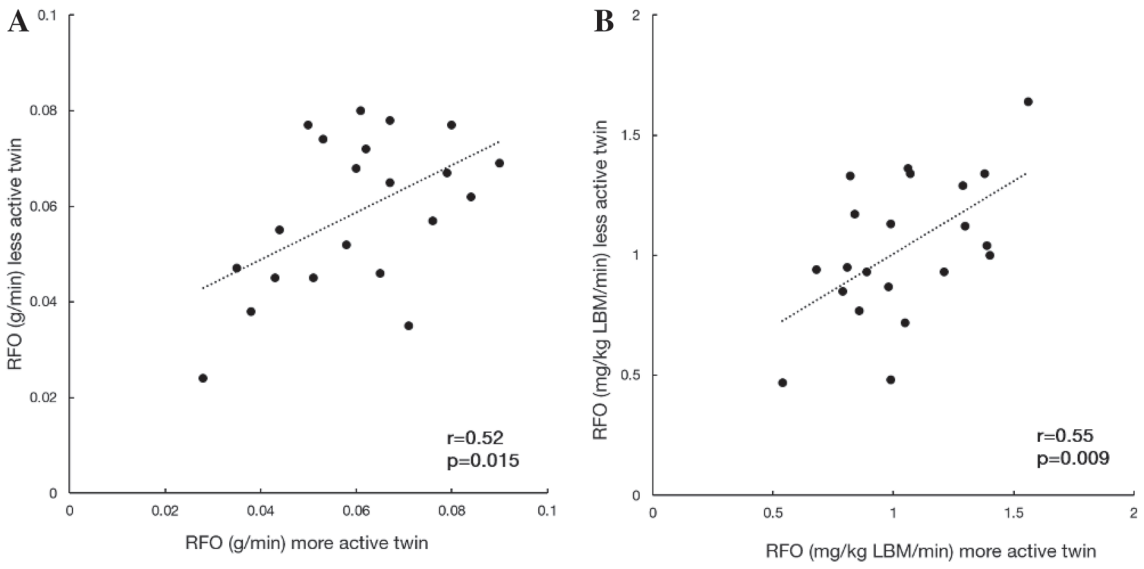

Fig. 1 Pairwise correlations of **a** absolute and **b** lean body mass (LBM) relative resting fat oxidation (RFO) in 21 MZ twin pairs

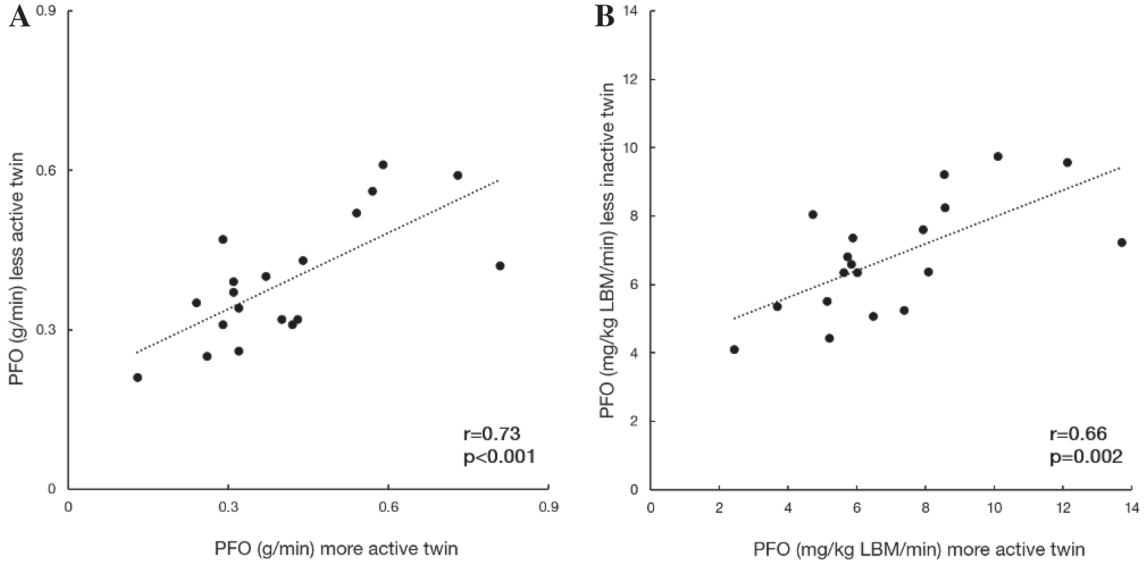

Fig. 2 Pairwise correlations of **a** absolute and **b** lean body mass (LBM) relative peak fat oxidation (PFO) during exercise in 19 MZ twin pairs

Table 3 Characteristics of the long-term-discordant MZ twin pairs

Variable	Active ($n=10$)	Inactive ($n=10$)	Mean difference (95% CI)	p value
Physical activity*				
3-year-LTMET index (MET-h/day)	5.0 ± 2.7	1.7 ± 1.3	3.3 (1.9 to 4.8)	0.001
12-month-LTMET index (MET-h/day)	3.9 ± 1.2	1.2 ± 0.9	2.8 (2.0 to 3.5)	< 0.001
Body composition*				
Lean body mass (kg)	56.9 ± 4.8	55.5 ± 6.1	1.4 (−0.3 to 3.0)	0.094
Fat mass (kg)	16.0 ± 4.5	19.2 ± 6.6	−3.3 (−6.7 to 0.2)	0.059
Body fat percentage (%)	20.7 ± 4.0	24.0 ± 4.6	−3.3 (−6.2 to −0.4)	0.029
Cardiorespiratory fitness[a]				
VO_{2peak} (l/min)	3.3 ± 0.3	2.9 ± 0.5	0.4 (0.2 to 0.6)	0.001
VO_{2peak} (ml/kg LBM/min)	58 ± 5	52 ± 5	7 (3 to 10)	0.001
Metabolism at rest[b]				
REE (kcal/day)	1735 ± 187	1675 ± 191	59 (−48 to 166)	0.24
RER	0.82 ± 0.03	0.80 ± 0.03	0.02 (−0.01 to 0.04)	0.16
RFO (g/min)	0.06 ± 0.02	0.06 ± 0.02	0.00 (−0.01 to 0.01)	0.52
RFO (mg/kg LBM/min)	1.0 ± 0.3	1.1 ± 0.3	−0.1 (−0.3 to 0.1)	0.35
Fat oxidation during exercise[a]				
PFO (g/min)	0.46 ± 0.20	0.38 ± 0.12	0.08 (−0.02 to 0.18)	0.11
PFO (mg/kg LBM/min)	8.0 ± 3.1	6.9 ± 1.8	1.1 (−0.7 to 2.9)	0.18
FAT_{MAX} (%VO_{2peak})	40 ± 9	43 ± 8	−4 (−8 to 1)	0.077

LBM lean body mass, *MET* metabolic equivalent of task, *OGTT* oral glucose tolerance test, *PFO* peak fat oxidation, *REE* resting energy expenditure, RER: respiratory exchange ratio, *RFO* resting fat oxidation, VO_{2peak} peak oxygen uptake

*Data reported earlier (Rottensteiner et al. 2015)

[a] $n=9$ MZ twin pairs

[b] $n=9$ MZ twin pairs, one pair is different than in the exercise test-based variables

co-twins in cardiorespiratory fitness, intra-abdominal adiposity, glucose homeostasis, and brain morphology and function. In this study, we found no differences between the co-twins in their systemic energy metabolism at rest or during exercise. In the twin individual-based analysis, only PFO (g/min) was associated with LTPA. In previous

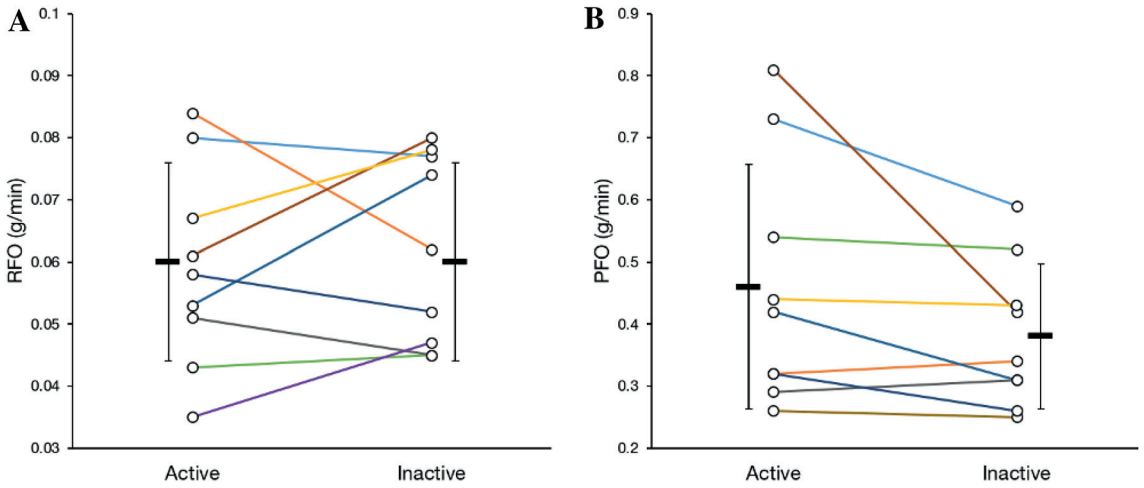

Fig. 3 **a** Resting fat oxidation (RFO) and **b** peak fat oxidation (PFO) during exercise in the leisure-time physical activity discordant MZ twin pairs ($n=9$, 8 pairs successfully participated in both measurements). Figures include group means and standard deviations. Colours represent the same twin pairs in both charts. Note the different scale in the *y*-axis

observational studies, PFO was associated with self-reported physical activity (Venables et al. 2005; Fletcher et al. 2017), and trained subjects (Nordby et al. 2006) or athletes (Dandanell et al. 2018) exhibited superior PFO compared with controls. However, it is highly likely that physical activity participation and fat oxidation capacity have shared genetic factors, and the relationship noted in observational studies is partly genetically mediated. In experimental studies, endurance-training interventions commonly increased PFO, at least in untrained populations (reviewed by Maunder et al. 2018). Earlier mechanistic evidence from our laboratory also supports the role of physical activity as a modulator of PFO. In same-sex twin pairs, an over 30-year long physical activity discordance led to significant differences in myocellular gene expression related to oxidative phosphorylation and lipid metabolism (Leskinen et al. 2010). The effects of physical activity on RFO have been investigated less, with mixed results. A modest increase in fat oxidation rates at rest has been reported in some (Barwell et al. 2009; Whyte et al. 2010) but not in all (Scharhag-Rosenberger et al. 2010) trials. When the current scientific evidence is taken together with our results, physical activity seems to be able to influence PFO, while its effect on RFO is questionable.

In the twin individual-based analysis, we observed that PFO (g/min) was associated with a favourable response to glucose loading. The observed inverse association between PFO (g/min) and insulin concentration during the OGTT was especially convincing. However, we found no association between PFO and the Matsuda index, our main surrogate of insulin sensitivity. As explained in the methods section, the Matsuda index is influenced by fasting values, which were not associated with PFO in our study. Previously, Robinson et al. (2015) showed that PFO was inversely associated with a fasting-based QUICKI index. As Robinson et al. (2015) had a larger sample size ($n=53$) and measured the PFO in the fasting state, they were more able to find the associations between the PFO and the fasting-based values, which generally vary less among healthy individuals when compared with the responses to glucose loading. Here, we show that probably an even more noticeable inverse association exists between PFO (g/min) and the insulin response to the OGTT. However, it should be mentioned that PFO does not always seem to be associated with a healthier metabolic phenotype because an obesity-related increase in fatty acid availability has also been linked to higher PFO (Ara et al. 2011; Dandanell et al. 2017a).

In contrary to PFO, RFO was not associated with a healthy metabolic response to the OGTT. Previous studies have noted mixed findings. Rosenkilde et al. (2010) reported that in a population of overweight but otherwise healthy men, the group with low RER at rest had higher PFO and a healthier metabolic profile when compared with the group with high RER. However, there were no differences in fasting glucose or insulin levels between the groups. Some case–control studies (Perseghin et al. 2002; Hodson et al. 2010) have shown an elevated RFO in obese subjects when compared with their lean counterparts. An elevated RFO could potentially function as a protective mechanism against insulin resistance (Perseghing et al. 2002) and liver fat accumulation (Hodson et al. 2010) when lipid availability increases. Overall, further research is needed to clarify the interaction between systemic fat oxidation and metabolic health.

Our study has both strengths and limitations. A key strength was our ability to measure RFO and PFO in 21 and 19 MZ twin pairs, respectively. This enabled us to investigate

Table 4 Results of the twin individual-based analysis

	PFO (g/min)	PFO (mg/kg LBM/min)	RFO (g/min)	RFO (mg/kg LBM/min)
Body composition				
BMI (kg/m^2)	$r=0.12$ $p=0.53$	$r=0.054$ $p=0.76$	$r=0.16$ $p=0.25$	$r=0.079$ $p=0.58$
Lean body mass (kg)	$r=0.41$ $p=0.007$		$r=0.33$ $p=0.12$	
Fat mass (kg)	$r=-0.13$ $p=0.57$	$r=-0.091$ $p=0.66$	$r=0.08$ $p=0.63$	$r=0.10$ $p=0.48$
Self-reported leisure time physical activity				
12-month LTMET-index (MET-h/day)	$r=0.26$ $p=0.034$	$r=0.10$ $p=0.41$	$r=0.14$ $p=0.23$	$r=-0.08$ $p=0.46$
3-year-LTMET-index (MET-h/day)	$r=0.30$ $p=0.081$	$r=0.20$ $p=0.26$	$r=0.22$ $p=0.13$	$r=0.05$ $p=0.32$
Baecke questionnaire (score)	$r=0.40$ $p=0.022$	$r=0.25$ $p=0.13$	$r=0.002$ $r=0.98$	$r=-0.15$ $p=0.24$
Cardiorespiratory fitness				
VO_{2peak} (l/min)	$r=0.51$ $p=0.028$		$r=0.21$ $p=0.30$	
VO_{2peak} (ml/kg LBM/min)		$r=0.36$ $p=0.085$		$r=-0.006$ $p=0.97$
Fat oxidation during exercise				
PFO (g/min)			$r=0.30$ $p=0.14$	
PFO (mg/kg LBM/min)				$r=0.29$ $p=0.12$
Glucose homeostasis and insulin sensitivity				
Fasting glucose (mmol/l)	$r=-0.11$ $p=0.42$	$r=-0.16$ $p=0.26$	$r=0.084$ $p=0.60$	$r=0.05$ $p=0.80$
Fasting insulin (µU/l)	$r=-0.17$ $p=0.33$	$r=-0.13$ $p=0.48$	$r=$-0.10 $p=0.59$	$r=-0.079$ $p=0.63$
Matsuda index	$r=0.10$ $p=0.60$	$r=0.026$ $p=0.91$	$r=0.18$ $p=0.29$	$r=0.067$ $p=0.67$
Insulin AUC (µU/l/h)	$r=-0.42$ $p=0.015$	$r=-0.35$ $p=0.055$	$r=-0.04$ $p=0.85$	$r=0.12$ $p=0.58$
Glucose AUC (mmol/l/h)	$r=-0.31$ $p=0.050$	$r=-0.27$ $p=0.11$	$r=0.21$ $p=0.082$	$r=0.31$ $p=0.019$

AUC area under the curve, *BMI* body mass index, *LBM* lean body mass, *MET* metabolic equivalent of task, *OGTT* oral glucose tolerance test, *PFO* peak fat oxidation, *REE* resting energy expenditure, *RER* respiratory exchange ratio, *RFO* resting fat oxidation, VO_{2peak} peak oxygen uptake

the influence of hereditary factors on RFO and PFO in a reasonably sized study group. The calculated ICCs represent the upper bound of heritability, as differences between MZ twins are due to non-genetic factors. However, as MZ twin pairs share also many aspects of their development and environment, the actual heritability of the trait may be lower. A more precise estimation of heritability would require several kinds of relatives (for quantitative trait modeling) or very large study population (for measurement of all genetic variation by whole genome sequencing). Despite our systematic nationwide search, we could only recruit 10 MZ twin pairs, where the co-twins were long-term LTPA-discordant, which weakened our study's power to find significant pairwise differences. Furthermore, the participants' MZ twin status made it more difficult to find significant twin individual-based correlations because clustering was necessary to take into account the within-pair dependency. Additionally, since our study included only males, the results cannot be generalised to females.

Another strength of our study was our protocol's inclusion of an OGTT; we did not depend on using fasting-based values. This enabled us to conduct a more in-depth examination of the possible associations between fat oxidation and metabolic health. However, our study protocol was not optimal for PFO determination, which should be considered when interpreting the results. Nutrition intake

the day before (Støa et al. 2016) and on the same day (Achten and Jeukendrup 2003; Edinburgh et al. 2018) will alter substrate use. In this study, we did not control for the nutrition intake before the exercise test. For example, this could partially explain why we did not find any association between RFO and PFO, as previously shown by Robinson et al. (2016). Moreover, we used 2-min exercise stages during PFO testing. The 2-min stages might be too short to reach a steady-state, especially for the subjects with lower cardiorespiratory fitness (Dandanell et al. 2017b; Chrzanowski-Smith et al. 2018). To assess whether the stage duration excessively affected the results, we compared VO_2 and VCO_2 between intervals 90–105 s and 105–120 s of the PFO-stage. There were no systematic differences in VO_2 or VCO_2 between the intervals. Mean coefficients of variation were $4 \pm 4\%$ and $4 \pm 4\%$ for VO_2 and VCO_2, respectively. Coefficient of variation of VO_2, VCO_2 or both exceeded 10% in 3 out of 41 participants. Removing these participants from the analyses did not materially change the results. Therefore, the influence of the stage duration was considered acceptable. Also, the measured PFO (g/min) results were associated with the most important determinants described in the literature, and as expected, correlated between the MZ co-twins. Thus, the measurements seemed to reflect the PFO of our study participants.

In conclusion, we show that fat oxidation rates at rest and during exercise are similar between MZ co-twins. Our results support the suggestion that hereditary factors influence fat oxidation capacity. The internal factors likely set the baseline for fat oxidation capacity that the external factors can modulate. In our study, the role of physical activity seemed smaller, especially concerning RFO. Furthermore, we observed that only higher capacity to utilize fatty acids during exercise associated with better metabolic health.

Acknowledgements Open access funding provided by University of Jyväskylä (JYU).

Author contributions JEK, MR, PW and UMK conceived and designed research. MR, PW, KH and UMK conducted experiments. JK was responsible for the creation and maintenance of the base cohort from which the study sample was recruited. JEK analysed data and drafted the manuscript. All authors contributed to the interpretation of data and critical revision of the manuscript. All authors read and approved the final version of the manuscript.

Funding The FITFATTWIN study was supported by the Finnish Ministry of Education and Culture (OKM/56/626/2013 to UMK), META-PREDICT (within the European Union Seventh Framework Programme, HEALTH-F2-2012–277936 to UMK). Data collection for the FT16 study was supported by the National Institute of Alcohol Abuse and Alcoholism (grants AA-12502, AA-00145, and AA-09203 to RJ Rose) and the Academy of Finland (grants 100499, 205585, 118555, 141054, 264146, 308248 and 312073 to JK).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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IV

ASSOCIATIONS OF RESTING AND PEAK FAT OXIDATION WITH SEX HORMONE PROFILE AND BLOOD GLUCOSE CONTROL IN MIDDLE-AGED WOMEN

by

Karppinen, J.E., Juppi, H.K., Hintikka, J., Wiklund, P., Haapala, E.A., Hyvärinen, M., Tammelin, T.H., Aukee, P., Kujala, U.M., Laukkanen, J. & Laakkonen, E.K. 2022

Nutrition, Metabolism and Cardiovascular Diseases vol 32 (9): 2157–2167

https://doi.org/10.1016/j.numecd.2022.06.001

Nutrition, Metabolism & Cardiovascular Diseases (2022) **32**, 2157–2167

Available online at www.sciencedirect.com

Nutrition, Metabolism & Cardiovascular Diseases

journal homepage: www.elsevier.com/locate/nmcd

Associations of resting and peak fat oxidation with sex hormone profile and blood glucose control in middle-aged women

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Received 8 February 2022; received in revised form 31 May 2022; accepted 1 June 2022
Handling Editor: A. Siani
Available online 8 June 2022

KEYWORDS
Fat oxidation;
Menopause;
Estradiol;
Glucose tolerance;
Insulin sensitivity

Abstract *Background and aims:* Menopause may reduce fat oxidation. We investigated whether sex hormone profile explains resting fat oxidation (RFO) or peak fat oxidation (PFO) during incremental cycling in middle-aged women. Secondarily, we studied associations of RFO and PFO with glucose regulation.
Method and results: We measured RFO and PFO of 42 women (age 52–58 years) with indirect calorimetry. Seven participants were pre- or perimenopausal, 26 were postmenopausal, and nine were postmenopausal hormone therapy users. Serum estradiol (E2), follicle-stimulating hormone, progesterone, and testosterone levels were quantified with immunoassays. Insulin sensitivity (Matsuda index) and glucose tolerance (area under the curve) were determined by glucose tolerance testing. Body composition was assessed with dual-energy X-ray absorptiometry; physical activity with self-report and accelerometry; and diet, with food diaries. Menopausal status or sex hormone levels were not associated with the fat oxidation outcomes. RFO determinants were fat mass ($\beta = 0.44$, $P = 0.006$) and preceding energy intake ($\beta = -0.40$, $P = 0.019$). Cardiorespiratory fitness ($\beta = 0.59$, $P = 0.002$), lean mass ($\beta = 0.49$, $P = 0.002$) and physical activity (self-reported $\beta = 0.37$, $P = 0.020$; accelerometer-measured $\beta = 0.35$, $P = 0.024$) explained PFO. RFO and PFO were not related to insulin sensitivity. Higher RFO was associated with poorer glucose tolerance ($\beta = 0.52$, $P = 0.002$).
Conclusion: Among studied middle-aged women, sex hormone profile did not explain RFO or PFO, and higher fat oxidation capacity did not indicate better glucose control.

Abbreviations: AUC, area under the curve; COVID-19, coronavirus disease; E2, estradiol; FSH, follicle-stimulating hormone; HT, hormone therapy; NEFA, non-esterified fatty acids; OGTT, oral glucose tolerance test; PFO, peak fat oxidation; POST, postmenopause; PRE/PERI, pre- or perimenopause; RER, respiratory exchange ratio; RFO, resting fat oxidation.

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https://doi.org/10.1016/j.numecd.2022.06.001

1. Introduction

Menopause follows the loss of ovarian follicular activity and leads to circulating 17β-estradiol (E2) decline with concomitant follicle-stimulating hormone (FSH) increase [1]. The E2 deficiency has been thought to reduce fat oxidation capacity [2,3]. Premenopausal women depend more on fat oxidation than men during exercise [4–7] but not necessarily at rest [8]. Whether the sex-related difference is lost [9,10] or not [11] after menopause is uncertain. However, E2 supplementation increases fat oxidation in men [12,13], and estrogen receptor α regulates myocellular fatty acid metabolism [14], supporting the role of E2 as a fat oxidation capacity determinant.

Only a few human studies have directly investigated whether fat oxidation responds to menopause. Lovejoy et al. [15] found that 24-h fat oxidation decreased in women who transitioned from premenopause to postmenopause during a 4-year follow-up. However, they could not show that the decrease differed from women who were still premenopausal at the time of the end measurements. In contrast, Albildgaard et al. [16] reported premenopausal women to use fatty acids more than postmenopausal participants while cycling for 45 min at 50% of the $\dot{V}O_{2MAX}$ intensity and the FSH levels to negatively correlate with fat oxidation rate. Notably, the relative exercise intensity at which individuals reach their peak fat oxidation (PFO) rates varies [4–7]. Using incremental exercise testing may therefore improve the assessment of fat oxidation capacity compared with single-stage testing [17]. To our knowledge, this approach has not been used to study associations between sex hormone profile and fat oxidation in middle-aged women.

The E2 levels can be raised with menopausal hormone therapy (HT). Nevertheless, fat oxidation did not differ between HT users and non-users during a 30-min treadmill test performed at 80% of $\dot{V}O_{2MAX}$ intensity [18]. The effects of HT on resting fat oxidation (RFO) have been suggested to depend on the administration route. Dos Reis et al. [19] reported lower RFO in women taking oral conjugated estrogens than in transdermal E2 users, perhaps because of the liver first-pass effect. However, randomized controlled crossover trials could not confirm this observation when conjugated estrogens were compared with transdermal E2 [20] or placebo [21] treatments. Moreover, whether administered orally or transdermally, E2-therapy did not affect RFO in hypogonadal girls with Turner syndrome [22].

Poor fat oxidation capacity may induce lipid intermediate accumulation and impaired insulin signaling in oxidative tissues [23]. Menopause is associated with disturbances in blood glucose regulation [24]. Therefore, investigating the relationship between fat oxidation and glucose control is especially relevant in middle-aged women. Both RFO [25] and PFO [26] have been associated with insulin sensitivity in other populations. However, the findings are not universal [7,27,28], and the studies have relied on fasting-based outcomes.

Aging is a confounder in menopause-related studies. Therefore, our primary aim was to investigate whether sex hormone profile is related to fat oxidation in women of similar ages but varying menopausal states. Our premise was that circulating E2 would be positively associated and FSH would be negatively associated with fat oxidation, especially during exercise. We also presumed that women with higher E2 levels, due to endogenous production or HT use, would exhibit higher fat oxidation than postmenopausal women. Our secondary aim was to investigate whether RFO and PFO are associated with blood glucose control during glucose challenge. We expected higher PFO to be associated with lower insulin release.

2. Methods

A detailed description of the study methodology is in **the Supplement**. The study was approved by the ethics committee of the Central Finland Health Care District (KSSHP Dnro 9U/2018) and complied with the Declaration of Helsinki. The participants provided informed consent.

2.1. Study population

We recruited participants from the ERMA (The Estrogenic Regulation of Muscle Apoptosis) cohort [29] during its fourth-year follow-up, the EsmiRs (Estrogen and microRNAs as Modulators of Women's Metabolism) study. In total, 304 women participated in the basic EsmiRs measurements at the Health and Sports Laboratory of the University of Jyväskylä between January 2019 and March 2020. The measurements ended prematurely because of the coronavirus disease (COVID-19) pandemic.

For this EsmiRs Metabolism substudy, we recruited women who were either premenopausal or perimenopausal (PRE/PERI), postmenopausal (POST), or postmenopausal HT users (HT). The exclusion criteria were: 1) body mass index <18 or >30 kg/m^2, 2) oophorectomy or hysterectomy, 3) disease or medication use affecting metabolism, 4) hormonal contraception, and 5) regular smoking. A study nurse checked the participants' eligibility and group allocation during the basic EsmiRs measurements. We expected the PRE/PERI and HT groups to have higher systemic E2 levels and, therefore, higher PFO than the POST group. Using the data of Abildgaard et al. [16], we performed an *a priori* power calculation showing that 12 participants per group would be needed to detect a between-group difference in lean mass adjusted PFO with an alpha level of 0.05 and 80% power.

This substudy included two laboratory visits (Supplementary Fig. 1). At the first visit, the focus was on resting metabolism and glucose tolerance, and at the second visit, on exercise metabolism. The median (interquartile range) duration between the visits was 2 weeks (1–3 weeks).

2.2. Participants' hormonal status and final group assignments

Our sample was 13 PRE/PERI, 20 POST, and nine HT participants when the recruitment prematurely ended. In

the final menopausal status determination, the participants' self-reported menstrual data were supplemented with FSH measurements to ensure correct status assignment defined by the Stages of Reproductive Aging Workshop +10 guidelines [30]. During the process, we needed to reclassify six PRE/PERI women to be postmenopausal owing to their high FSH levels. Of the remaining seven PRE/PERI women, two were premenopausal, three were early perimenopausal, and two were late perimenopausal. Four POST women had extreme mean E2 levels ranging from 0.27 to 0.54 nmol/l, more than four times over the third quartile of 0.09 nmol/l. As we aimed to study the influence of E2 deficiency, we excluded these participants from the group comparisons. Of the HT users, seven used oral and two transdermal HT containing E2 and progestogen (see the Supplement for details). We considered combining women using different HT administration routes acceptable as oral E2 did not seem to limit RFO in a previous randomised controlled trial [22] or our sample (Supplementary Fig. 2).

We did not fully reach our recruitment goal owing to the COVID-19 pandemic. Therefore, we also performed an explorative analysis to test whether pooling the PRE/PERI ($n = 7$) and HT groups ($n = 9$) into a larger HIGH E2 group ($n = 16$) for comparison against the E2 deficient postmenopausal women (LOW E2 group, $n = 22$) would change our inferences from the primary analyses.

2.3. Diet and physical activity

We instructed the participants to avoid lifestyle changes during the study, abstain from exercise and alcohol for 48 h and caffeine for 12 h, and eat the last meal between 8:00 and 10:00 p.m. before the measurements. The participants drank 100 ml and 150 ml of water after waking up and after the body composition measurements, respectively. They minimised activities in the morning on the day of the measurements and arrived at the laboratory by car. The participants declared that they had followed the instructions.

Leisure-time physical activity was assessed with a structured questionnaire [31] and with a seven-day ActiGraph accelerometer (Pensacola, Florida, USA) wear between the basic EsmiRs and first substudy measurements [32,33]. The participants kept food diaries for 2 days and matched their diet 24 h before the substudy measurements. The intraclass correlation coefficients for energy intake and food quotient, the metabolisable respiratory quotient of the diet reflecting its macronutrient distribution, were 0.71 and 0.92, respectively ($P < 0.001$ for both).

2.4. Body composition

At the basic EsmiRs measurements, body composition was assessed using dual-energy X-ray absorptiometry (DXA Prodigy, GE Lunar Corp., Madison, WI, USA). Body composition was also assessed with InBody720 (Biospace, Seoul, Korea) at each visit.

2.5. Resting metabolism

Indirect calorimetry data were collected with a Vmax Encore 92 metabolic cart (Sensormedics, Yorba Linda, CA, USA). First, the participants rested for 30 min. Gas exchange was then measured for 30 min with the canopy method. The data collected during the first 10 min were discarded. Steady-state periods were determined as segments at which the coefficients of variation between the subsequent minutes and during the segments were ≤10% for $\dot{V}O_2$ and $\dot{V}CO_2$, and ≤5% for respiratory exchange ratio (RER). The median (interquartile range) steady-state duration was 20 min (13–20 min). RFO was calculated with the Frayn [34] equation, assuming that the urinary nitrogen excretion was negligible. The measurement of RFO was unreliable in three participants (PRE/PERI, $n = 2$; POST, $n = 1$) on the basis of high RER (≥0.91) values [35].

2.6. Exercise metabolism

The exercise testing was performed with an Ergoselect 200 bicycle ergometer (Ergoline GmbH, Germany) with a cycling cadence of 70 ± 5 rpm. The protocol included the PFO and $\dot{V}O_{2PEAK}$ phases. Venous blood samples were drawn before and after the test.

The PFO phase started at 20 W. The workload was increased by 20 W every 4 min until RER reached 1.0 or the seventh test stage. Two participants completed the PFO phase without reaching a RER of 1.0., but their fat oxidation had started to decline before the last stage. Gas exchange was measured breath by breath and recorded as rolling 30-s averages. Fat oxidation for the last minute of each stage was calculated with the Frayn equation [34]. PFO was determined as the highest calculated fat oxidation rate; and FAT_{MAX} ($\%\dot{V}O_{2PEAK}$), as the exercise intensity at which PFO was reached.

The $\dot{V}O_{2PEAK}$ phase directly followed the PFO phase. The phase started at 100 W, and the workload was increased by 1 W/3 s. Gas exchange was recorded as 10-s rolling averages. We determined the $\dot{V}O_{2PEAK}$ as the highest $\dot{V}O_2$ average during a 30-s period and W_{MAX} as the highest workload reached. The exercise test ended with a 5-min cooldown at 50 W. The gas exchange data were unreliable in two HT participants because of metabolic cart failure or mask-wearing difficulties. One PRE/PERI participant could not continue to the $\dot{V}O_{2PEAK}$ phase due to fatigue resulting from low energy diet.

2.7. Blood glucose control and biomarkers

An oral glucose tolerance test (OGTT) followed the resting metabolism measurement. Venepuncture samples were drawn before and 30, 60, 90, and 120 min after ingestion of a 75-g glucose solution (GlucosePro 250 ml, Comed Oy, Ylöjärvi, Finland). The plasma glucose levels were analyzed with Konelab 20 XT (Thermo Fisher Scientific, Vantaa, Finland); and the serum insulin levels, with IMMULITE 2000 (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). We determined insulin sensitivity with the

Matsuda index and glucose tolerance by calculating the total glucose area under the curve (AUC). We also calculated fasting-based homeostatic model assessment for insulin resistance (HOMA-IR) index and AUC for insulin.

For the quantification of other biomarkers, serum was stored at −80 °C. The levels of sex hormones (E2, FSH, progesterone, and testosterone) and sex hormone-binding globulin were analyzed with IMMULITE 2000. Non-esterified fatty acids (NEFA) levels were analyzed with Indiko (Thermo Fisher Scientific, Vantaa, Finland). Glycerol and 3-hydroxybutyrate levels were measured using nuclear magnetic resonance spectroscopy (Nightingale Health Ltd., Helsinki, Finland) [36].

2.8. Statistical analyses

We used the R statistical environment (version 4.0.5) for analyses. The code is available at https://osf.io/afmu7. We performed analyses using linear regression, analysis of variance, or analysis of covariance, confirmed the model assumptions before accepting the results, and used an alpha level of 0.05 for statistical significance (detailed description in the Supplement). The main models included lean mass as a covariate to control the analyses for the oxidative tissue mass. We refrained from using ratio scaling as it misrepresents the relationship between the numerator and denominator [37,38].

First, we identified the RFO and PFO determinants by using the fat oxidation measures as the outcomes and the variable of interest as the exposures. We also constructed multivariable explanatory models on the basis of previous research. Next, we tested the associations between sex hormone levels and fat oxidation measures and whether the sex hormones improved the explanatory models. We then compared RFO and PFO between the menopausal groups. We also performed an explorative analysis by comparing the HIGH E2 and LOW E2 groups.

Lastly, we studied the associations between fat oxidation and blood glucose regulation by using OGTT measures as the outcomes and RFO or PFO as the exposure. The OGTT measures were log-transformed to improve residual normality. We also performed sensitivity analyses by excluding participants with incomplete OGTT data or including NEFA levels as a covariate. NEFA levels were used as a proxy for participants' energy balance and metabolic state.

Table 1 Participant characteristics in the whole study sample and in the pre- or perimenopausal (PRE/PERI), postmenopausal (POST), and postmenopausal hormone therapy user (HT) groups. Four POST women were excluded from group comparisons for having high estradiol levels.

	Full sample (N = 42)	PRE/PERI (n = 7)	POST (n = 22)	HT (n = 9)
Age, years	55.3 (1.6)	54.9 (1.5)	55.6 (1.6)	55.4 (1.4)
Sex hormones, mean of two measurements				
E2, nmol/l	0.11 (0.06–0.25)	0.18 (0.15–0.22)	0.06 (0.05–0.09)	0.27 (0.25–0.42)
FSH, IU/l	66.0 (37.4)	20.8 (14.2)	89.2 (31.5)	49.8 (25.7)
Progesterone, nmol/l	0.35 (0.23–0.57)	3.27 (1.23–7.55)	0.31 (0.17–0.39)	0.35 (0.26–0.39)
Testosterone, nmol/l	0.54 (0.30)	0.61 (0.29)	0.47 (0.29)	0.65 (0.33)
SHBG, nmol/l	75.2 (33.1)	72.3 (14.4)	69.0 (29.2)	93.4 (47.4)
Body composition				
Height, cm	166.0 (4.9)	166.7 (4.6)	165.8 (5.0)	166.6 (6.0)
Body mass, kg	67.8 (8.1)	69.9 (8.9)	67.2 (7.7)	68.9 (10.1)
BMI, kg/m^2	24.6 (2.5)	25.1 (2.8)	24.4 (2.4)	24.8 (3.2)
Lean mass, kg	41.2 (3.8)	42.4 (3.0)	40.9 (3.6)	40.2 (3.7)
Fat mass, kg	24.1 (6.1)	24.8 (6.4)	23.8 (6.1)	26.2 (6.6)
Percent body fat, %	36.5 (5.5)	36.4 (5.1)	36.3 (5.8)	38.9 (4.4)
Leisure-time physical activity				
Self-reported, MET-h/d	4.5 (2.3–7.5)	4.5 (3.4–6.8)	4.4 (2.3–7.5)	2.9 (1.5–4.5)
Accelerometry, MVPA min/d[a]	49.0 (26.0), n = 40	71.5 (28.8), n = 5	49.2 (23.6)	32.4 (15.8)
Glucose tolerance				
Fasting glucose, mmol/l	5.3 (5.1–5.5)	5.5 (5.2–5.7)	5.2 (5.0–5.5)	5.4 (5.2–5.5)
2-h glucose, mmol/l	5.6 (4.7–6.9), n = 41	6.1 (5.3–7.0)	5.1 (4.7–6.3), n = 21	7.4 (6.0–7.8)
Fasting insulin, IU/l	4.0 (2.4–5.7)	3.1 (1.5–4.4)	4.3 (2.2–7.0)	3.8 (3.2–8.0)
2-h insulin, IU/l	45.4 (38.5–57.9), n = 41	41.0 (36.6–52.2)	44.6 (35.0–51.6), n = 21	57.9 (49.0–72.5)
HOMA-IR	0.85 (0.49–1.36)	0.81 (0.37–1.06)	0.75 (0.46–1.44)	0.91 (0.62–1.94)
Glucose AUC, mmol/l/h	12.9 (11.3–14.5)	13.8 (12.0–15.0)	12.3 (10.8–13.5)	16.0 (13.8–16.4)
Insulin AUC, IU/l/h	75.7 (66.8–112.2)	75.8 (64.2–101.2)	72.0 (61.4–114.9)	106.7 (76.1–131.2)
Matsuda index	7.3 (5.4–9.4), n = 41	8.5 (6.9–10.7)	7.2 (5.4–11.1), n = 21	6.3 (3.7–8.8)
Cardiorespiratory fitness				
$\dot{V}O_{2PEAK}$, l/min	2.1 (0.3), n = 39	2.2 (0.4), n = 6	2.2 (0.3)	2.0 (0.3), n = 7
$\dot{V}O_{2PEAK}$, ml/kg/min	31.7 (5.1), n = 39	31.3 (4.0), n = 6	32.5 (5.3)	27.7 (3.5), n = 7
$\dot{V}O_{2PEAK}$, ml/kg LM/min	51.9 (6.9), n = 39	51.2 (5.4), n = 6	53.0 (7.7)	48.3 (6.4), n = 7
W_{MAX}, W	183 (26), n = 41	188 (23), n = 6	185 (24)	167 (29)

Data as means (standard deviation) or medians (interquartile range).
AUC, area under the curve; BMI, body mass index; E2, 17β-estradiol; FSH, follicle-stimulating hormone; HOMA-IR, homeostatic model assessment of insulin resistance; LM, lean mass; MET, metabolic equivalent of task; SHBG, sex hormone-binding globulin; $\dot{V}O_{2PEAK}$, peak oxygen uptake, W_{MAX}, maximal workload.
[a] moderate-to-vigorous intensity leisure-time physical activity minutes per day.

3. Results

The participants were 52- to 58-year-old women, free of chronic diseases and metabolism affecting medication verified by a medical examination performed at the first visit (Table 1). On the basis of OGTT, two HT users had elevated fasting glucose levels (>6.0 mmol/l), and five women (POST, $n = 2$; HT, $n = 3$) had impaired glucose tolerance (2-h glucose measurement value, 7.8–11.0 mmol/l).

Age ($P = 0.56$), percent body fat ($P = 0.46$), and self-reported leisure-time physical activity ($P = 0.74$) did not significantly differ across the menopausal groups (Supplementary Table 1). As expected, the PRE/PERI and HT groups had higher E2 levels and lower FSH levels than the POST group (Fig. 1). Testosterone ($P = 0.25$) and sex hormone-binding globulin levels ($P = 0.39$) did not differ between the groups; however, the PRE/PERI group had higher progesterone levels compared with the POST group ($P = 0.002$) and the HT group ($P = 0.026$) (Supplementary Table 1).

3.1. Resting and peak fat oxidation determinants and mediators

The indirect calorimetry, dietary, and metabolite data are shown in Table 2. Fat mass was positively, and prior energy

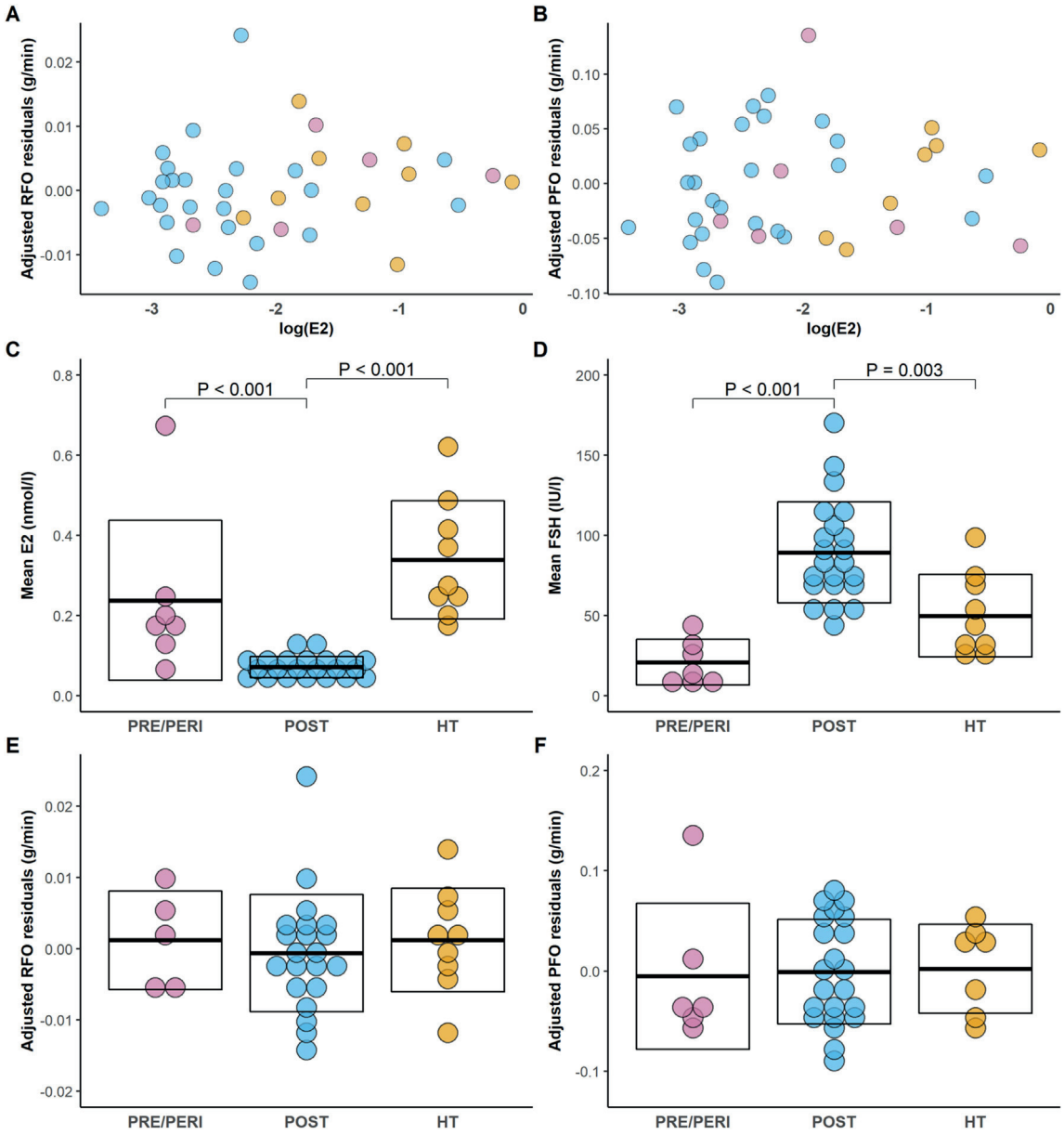

Figure 1 The associations of log-transformed E2 levels with adjusted resting fat oxidation residuals (RFO; Fig. 1A) and peak fat oxidation residuals (PFO; Fig. 1B), and comparison of female sex hormone levels (Fig. 1C and D) and adjusted RFO and PFO residuals (Fig. 1E and F) between the menopausal groups. The adjusted residuals represent the difference between the measured fat oxidation and the fat oxidation predicted by the linear regression when adjusted for fat oxidation main determinants (RFO: lean mass, fat mass, and energy intake; PFO: lean mass, cardiorespiratory fitness, and self-reported leisure-time physical activity).

intake was negatively associated with RFO (Table 3). The explanatory model including lean mass ($\beta = 0.26$; $P = 0.075$), fat mass ($\beta = 0.47$; $P = 0.002$) and energy intake ($\beta = -0.43$; $P = 0.005$) explained 40% of the RFO variance (adjusted $R^2 = 0.35$, $P < 0.001$). NEFA and 3-hydroxybutyrate levels were positively associated with RFO (Table 3).

RFO and PFO, measured on separate days, were not significantly associated with each other ($\beta = 0.10$, $P = 0.53$). VO_{2PEAK}, W_{MAX}, FATMAX, and self-reported and accelerometer-measured leisure-time physical activity were positively associated with PFO (Table 3). In the explanatory model, lean mass ($\beta = 0.16$; $P = 0.33$), W_{MAX} ($\beta = 0.46$; $P = 0.032$) and self-reported leisure-time physical activity ($\beta = 0.21$; $P = 0.23$) explained 43% of the PFO variance (adjusted $R^2 = 0.39$; $P < 0.001$). Pre-exercise NEFA and pre- and post-exercise glycerol levels were positively associated with PFO when post-exercise analyses were adjusted for metabolite pre-exercise concentration (Table 3).

3.2. Sex hormone profile associations with resting and peak fat oxidation

Neither E2 (Fig. 1) nor the other sex hormone levels (Supplementary Fig. 3) were significantly associated with RFO or PFO. Moreover, they did not contribute to RFO or PFO explanation when included in the explanatory models with the main fat oxidation determinants (Table 4).

We could not show that women with different menopausal statuses differed in absolute (F [2,32] = 1.46, $P = 0.25$, $\eta^2p = 0.08$) or lean mass adjusted (F [2,31] = 1.68, $P = 0.25$, $\eta^2p = 0.10$) RFO. The lack of group differences appeared even more clear when we included the main RFO determinants as covariates (F [2,29] = 0.25, $P = 0.78$, $\eta^2p = 0.02$) (Fig. 1). Neither did the menopausal groups differ in PFO (F [2,33] = 0.32, $P = 0.73$, $\eta^2p = 0.02$), whether adjusted for lean mass (F [2,32] = 0.18, $P = 0.84$, $\eta^2p = 0.01$), or the main PFO determinants (F [2,29] = 0.01, $P = 0.99$, $\eta^2p = 0.00$) (Fig. 1). The explorative analyses pararelled the results from primary analyses and did not reveal significant RFO or PFO differences between the HIGH E2 and LOW E2 groups (Supplementary Table 2).

3.3. Associations of resting and peak fat oxidation with blood glucose regulation

Neither RFO nor PFO was significantly associated with Matsuda on HOMA-IR indexes (Table 5, Supplementary Fig. 4). However, RFO was positively associated with glucose and insulin AUCs. The associations between RFO and glucose AUC ($\beta = 0.50$; $P = 0.007$) and insulin AUC ($\beta = 0.39$; $P = 0.042$) remained significant when adjusted

Table 2 Fat oxidation, preceding diet, and lipolysis-related metabolites in the whole study sample and in the pre- or perimenopausal (PRE/PERI), postmenopausal (POST), and postmenopausal hormone therapy user (HT) groups. Four POST women were excluded from group comparisons for having high estradiol levels.

	Full sample	PRE/PERI	POST	HT
Resting metabolism	$n = 39$	$n = 5$	$n = 21$	$n = 9$
RER	0.83 (0.03)	0.83 (0.03)	0.84 (0.03)	0.81 (0.02)
RFO, g/min	0.050 (0.010)	0.053 (0.009)	0.048 (0.010)	0.054 (0.009)
RFO, mg/kg LM/min	1.22 (0.23)	1.25 (0.19)	1.18 (0.25)	1.34 (0.18)
Diet two days before testing				
Energy intake, kcal/d	1795 (394)	1884 (324)	1853 (458)	1595 (197)
Energy intake, kcal/kg/d	27 (6)	26 (5)	28 (6)	23 (2)
Food quotient	0.86 (0.03)	0.83 (0.05)	0.86 (0.02)	0.87 (0.01)
Metabolites				
Non-esterified fatty acids, μmol/l	351 (255–493)	216 (213–256)	379 (255–489)	482 (325–727)
Glycerol, μmol/l	123 (92–150), $n = 36$	89 (82–95), $n = 4$	123 (94–153), $n = 19$	129 (125–166)
3-hydroxybutyrate, μmol/l	47 (23–83)	93 (38–168)	35 (19–54)	73 (58–117)
Exercise metabolism	$n = 40$	$n = 7$	$n = 22$	$n = 7$
PFO, g/min	0.22 (0.07)	0.23 (0.10)	0.22 (0.06)	0.21 (0.05)
PFO, mg/kg LM/min	5.3 (1.4)	5.4 (2.1)	5.3 (1.3)	5.0 (1.2)
FAT_{MAX}, $\%VO_{2PEAK}$	34 (9)	37 (15), $n = 6$	33 (8)	33 (5)
Diet two days before testing				
Energy intake, kcal/d	1887 (360)	1984 (388)	1898 (356)	1624 (224)
Energy intake, kcal/kg/d	28 (6)	29 (5)	28 (6)	23 (2)
Food quotient	0.86 (0.03)	0.85 (0.04)	0.86 (0.03)	0.88 (0.03)
Metabolites pre-exercise				
Non-esterified fatty acids, μmol/l	408 (319–566)	336 (277–553)	400 (266–531)	444 (382–724)
Glycerol, μmol/l	99 (84–136), $n = 34$	93 (86–112), $n = 6$	103 (89–142), $n = 19$	103 (84–164), $n = 6$
3-hydroxybuturate, μmol/l	61 (24–102), $n = 39$	46 (28–213)	42 (22–78), $n = 21$	74 (37–138)
Metabolites post-exercise				
Non-esterified fatty acids, μmol/l	503 (428–817), $n = 38$	601 (463–659)	486 (429–861), $n = 21$	688 (427–927), $n = 6$
Glycerol, μmol/l	269 (219–315), $n = 34$	240 (201–265), $n = 6$	278 (221–323), $n = 21$	304 (259–338), $n = 6$
3-hydroxybuturate, μmol/l	102 (83–128), $n = 38$	107 (94–167)	94 (82–117), $n = 21$	125 (83–152), $n = 6$

Data as means (standard deviation) or medians (interquartile range).
LM, lean mass; PFO, peak fat oxidation; RER, respiratory exchange ratio; RFO, resting fat oxidation.

for NEFA levels. Excluding participants with incomplete OGTT data did not influence the association of RFO and glucose AUC ($\beta = 0.51$; $P = 0.007$). The association with insulin AUC did not remain significant ($\beta = 0.31$; $P = 0.12$).

4. Discussion

This study investigated the associations between sex hormone profile and fat oxidation in middle-aged women. We were unable to show that menopausal status or sex hormone levels explain RFO or PFO in the study sample. We also examined the associations of RFO and PFO with blood glucose regulation during glucose challenge. Higher fat oxidation at rest or during exercise did not relate to insulin sensitivity; however, higher RFO preceded poorer glucose tolerance.

4.1. Sex hormone profile and resting fat oxidation

Fasting substrate use depends on the energy balance and diet composition [39,40]. In agreement, the RFO determinants in this study were variables describing tissue mass and energy intake. NEFA and 3-hydroxybutyrate levels were also positively associated with RFO, further linking RFO to lipolysis rate and energy balance. Therefore unsurprisingly, we did not observe associations between sex hormone levels and RFO. Previous studies also questioned the relationship between E2 levels and RFO in premenopausal [41] and postmenopausal women [42]. Resting substrate use was also unaltered by conjugated estrogens when the diet was controlled before measurements [43].

Ultimately, menopause will unlikely affect RFO directly. In this study, RFO was indeed very similar between the menopausal or HIGH and LOW E2 groups, especially after confounder adjustment. The laws of conservation of mass and energy also make it challenging to accept that the menopause-associated increase in fat mass [44,45] results from decreased fat oxidation ability [39].

4.2. Sex hormone profile and peak fat oxidation

The sex hormone levels or menopausal and E2 group statuses were not associated with PFO. Therefore, we could not reproduce the findings of Abildgaard et al. [16] by using incremental testing. Besides the testing approaches, a key difference between the studies is that our study did not have a pure premenopausal group. Our results agree with those reported by Johnson et al. [18], who observed similar fat oxidation rates in HT users and non-users, although, their selected testing intensity (80% of $\dot{V}O_{2MAX}$) likely influenced their results [46].

Factors other than female sex hormone levels seem to determine PFO in women of all ages. The menstrual cycle phase or circulating E2 levels were not associated with PFO in reproductive women [47]. Moreover, controlling for the menstrual cycle phase did not improve the day-to-day reliability of PFO assessment [48]. In this study, PFO was associated with its known determinants: cardiorespiratory

Table 3 Associations between potential fat oxidation determinants, mediators, and resting fat oxidation or peak fat oxidation during exercise using linear regression models adjusted for lean mass.

	Resting fat oxidation				Peak fat oxidation			
	β	95% CI	*P*-value	*n*	*β*	95% CI	*P*-value	*n*
Univariable model								
Lean mass, kg	0.24	−0.08 to 0.56	0.132	39	0.49	0.20–0.79	**0.002**	40
Multivariable models with lean mass as a covariate								
Fat mass, kg	0.44	0.14–0.75	**0.006**	39	−0.03	−0.33 to 0.27	0.855	40
Leisure-time physical activity								
Self-reported, MET-h/d	−0.17	−0.50 to 0.16	0.300	39	0.37	0.06–0.68	**0.020**	40
Accelerometery, min/d	0.11	−0.24 to 0.45	0.530	39	0.35	0.05–0.65	**0.024**	38
Diet two days before the assessment								
Energy intake, kcal/d	−0.40	−0.73 to −0.07	**0.019**	39	0.05	−0.28 to 0.37	0.760	40
Food quotient	−0.09	−0.41 to 0.24	0.600	39	0.07	−0.22 to 0.37	0.613	40
Exercise test outcomes								
$\dot{V}O_{2PEAK}$, l/min	−0.13	−0.53 to 0.28	0.526	36	0.47	0.14–0.79	**0.006**	39
W_{MAX}, W	−0.03	−0.44 to 0.37	0.864	38	0.59	0.23–0.94	**0.002**	39
FAT_{MAX}, $\%\dot{V}O_{2PEAK}$	−0.16	−0.53 to 0.20	0.364	36	0.33	0.03–0.62	**0.033**	39
Lipolysis-related metabolites at rest or pre-exercise								
NEFA, μmol/l	0.46	0.16–0.75	**0.003**	39	0.33	0.05–0.61	**0.024**	40
Glycerol	0.26	−0.07 to 0.59	0.122	36	0.31	0.04–0.57	**0.024**	34
3-hydroxybuturate, μmol/l	0.52	0.24–0.81	**0.001**	39	0.19	−0.10 to 0.47	0.189	39
Lipolysis-related metabolites post-exercise, also adjusted for metabolite pre-exercise concentration								
NEFA, μmol/l					0.19	−0.12 to 0.50	0.222	38
Glycerol					0.42	0.16–0.68	**0.003**	34
3-hydroxybutyrate, μmol/l					0.37	−0.05 to 0.80	0.084	36

P-values < 0.05 are in bold.
β, standardized regression estimate; CI, confidence interval; MET, metabolic equivalent of task; NEFA, non-esterified fatty acids; Std, standardized; $\dot{V}O_{2PEAK}$, peak oxygen uptake; W_{MAX}, maximal workload.

fitness, lean mass, and leisure-time physical activity [4–7]. Genetic pleiotropy contributes to the associations, and higher PFO does not result solely from an active lifestyle [28,49]. Still, 12-week endurance-focused training increased fat oxidation during exercise but not at rest in postmenopausal women [50], showing that fat oxidation capacity can be improved in E2 deficiency.

Overall, we could not show that the sex hormone profile influences PFO in middle-aged women. Our finding possesses uncertainty, and more work is needed to clarify whether menopause modifies fat oxidation during exercise and, if so, whether the effect has clinical significance. Cross-sectional designs may not be sensitive enough to accomplish this task. Ideally, longitudinal fat oxidation changes over the menopausal transition or after HT initiation should be measured. Duplicate measurements would benefit PFO assessment [7,48].

4.3. Fat oxidation and blood glucose regulation

The role of RFO as a metabolic health marker is controversial [51]. Some [25] but not all [52] studies have reported that RFO is inversely associated with fasting insulin levels. The relationship is believable as insulin levels decline and fat oxidation increases according to fasting duration [53]. However, these findings are likely caused by differences in metabolic status rather than metabolic health. In this study, higher RFO preceded poorer glucose tolerance when OGTT followed the RFO assessment, as in our previous study in 30-year-old men [28]. Because NEFA and 3-hydroxybutyrate levels were positively associated with RFO, we thought the observed association resulted from fat oxidation-induced inhibition of glucose use [54]. However, we could not explain the association by NEFA adjustment.

The rationale for PFO as a metabolic health marker is more robust compared with RFO because it reflects fiber type distribution [55], concentrations of lipid handling proteins [55–57], and mitochondrial volume density [58] in skeletal muscles. PFO has been shown to be associated with fasting insulin sensitivity surrogates [26,52], although the findings are not unanimous [7,27]. PFO was not associated with insulin sensitivity or glucose tolerance in our present or earlier study [28]. However, PFO was associated with lower insulin AUC in the previous study in men, but we could not replicate the finding in the present study in women. Besides participant characteristics, a difference between our studies was OGTT timing. In the study in men, OGTT was performed the day after the PFO assessment, and in this study in women, 1–3 weeks before PFO testing. As PFO [48] and OGTT outcomes [59] exhibit significant day-to-day variability, their association may be time-dependent. Song et al. [60] recently challenged the concept of metabolic flexibility by showing that insulin resistance does not result from mitochondrial substrate preference, and our results follow their finding.

4.4. Limitations

Our study has several limitations. First, we had to discontinue the participant recruitment early and did not reach our recruitment goal. Therefore, our study may have lacked the power to detect significant group differences between the menopausal groups. We pooled PRE/PERI and HT women into a HIGH E2 group for explorative analyses with larger group sizes to counteract this limitation. This strategy has important limitations as cyclical endogenous hormone production and daily exogenous HT likely affect metabolism differently. For example, besides the apparent difference in how the hormones enter circulation, the two also have discordant temporal kinetics. Second, HT use was not standardized and reflected real-life differences in HT

Table 4 Associations between serum sex hormone levels and resting fat oxidation (RFO) or peak fat oxidation (PFO) during exercise in the whole study sample, with the adjusted R^2 values representing the variance proportions the explanatory variables explain together and the P-values in the last column showing the statistical significance of the whole model adjusted for fat oxidation main determinants.

RFO (n = 39)	Lean mass adjusted				Adjusted for main determinants[a]				
	β	**95% CI**	***P*-value**	R^2**adj**	β	**95% CI**	***P*-value**	R^2**adj**	***P*-value**
Model without the sex hormone				0.03				0.35	0.0004
$_{log}$E2	0.22	−0.10 to 0.53	0.170	0.06	0.10	−0.17 to 0.37	0.439	0.34	0.0010
FSH	−0.21	−0.54 to 0.12	0.200	0.05	−0.09	−0.38 to 0.19	0.504	0.34	0.0011
$_{log}$P4, n = 34	0.20	−0.15 to 0.56	0.251	0.01	0.13	−0.17 to 0.43	0.381	0.32	0.0038
Testosterone	0.27	−0.06 to 0.59	0.105	0.08	0.12	−0.17 to 0.40	0.412	0.34	0.0010
SHBG	0.07	−0.26 to 0.40	0.667	0.01	−0.03	−0.34 to 0.27	0.821	0.33	0.0013
PFO (n = 40)	β	**95% CI**	***P*-value**	R^2**adj**	β	**95% CI**	***P*-value**	R^2**adj**	***P*-value**
Model without the sex hormone				0.21				0.39	0.0002
$_{log}$E2	0.01	−0.28 to 0.30	0.949	0.19	0.04	−0.22 to 0.31	0.736	0.37	0.0005
FSH	0.16	−0.13 to 0.44	0.278	0.22	0.14	−0.11 to 0.40	0.266	0.39	0.0003
$_{log}$P4	0.24	−0.03 to 0.51	0.085	0.25	0.19	−0.06 to 0.44	0.130	0.41	0.0002
Testosterone	0.18	−0.11 to 0.46	0.217	0.22	0.20	−0.05 to 0.45	0.116	0.41	0.0002
SHBG	0.01	−0.28 to 0.31	0.927	0.19	0.01	−0.25 to 0.27	0.940	0.37	0.0005

β, standardized regression estimate; CI, confidence interval; E2,17β-estradiol, FSH, follicle-stimulating hormone; P4, progesterone; SHBG, sex hormone-binding globulin.

[a] RFO: lean mass, fat mass, and energy intake; PFO: lean mass, $W_{MAX,}$ and self-reported physical activity.

Table 5 Lean mass adjusted associations between resting fat oxidation (RFO, n = 39) or peak fat oxidation (PFO, n = 40) and oral glucose tolerance test outcomes, with adjusted R^2 values representing the variance proportions that lean mass and the fat oxidation measure explain together and the P-values in the last column showing the statistical significance of the whole model.

	β	95% CI	*P*-value	R^2adj	*P*-value
HOMA-IR					
RFO	0.21	−0.15 to 0.56	0.244	0.04	0.496
PFO, $n = 39$	−0.37	−0.79 to 0.06	0.092	0.10	0.154
Matsuda index					
RFO, $n = 38$	−0.33	−0.68 to 0.02	0.063	0.10	0.165
PFO, $n = 38$	0.26	−0.19 to 0.72	0.249	0.04	0.507
Glucose AUC					
RFO	0.52	0.21–0.82	**0.002**	0.27	**0.004**
PFO	0.02	−0.34 to 0.38	0.913	0.01	0.786
Insulin AUC					
RFO	0.42	0.10–0.75	**0.012**	0.18	**0.027**
PFO	−0.06	−0.44 to 0.32	0.752	0.04	0.451

P-values < 0.05 are in bold.
AUC, total area under the curve; β, standardized regression estimate; CI, confidence interval; HOMA-IR, homeostatic model assessment of insulin resistance.

prescription. Third, even though we standardized the participant preparation between the two laboratory visits, the metabolic states of the participants may have varied to some extent. The interindividual variation in metabolic states may have also influenced the assessment of the associations between sex hormone levels and fat oxidation.

5. Conclusions

In this study in middle-aged women, sex hormone profile did not explain fat oxidation at rest or during exercise. Higher fat oxidation in either condition did not indicate better blood glucose control. RFO was mainly related to energy balance and PFO to cardiorespiratory fitness. Therefore, we encourage middle-aged women interested in improving their fat oxidation capacity to engage in regular leisure-time physical activity.

Funding

This study was supported by the Academy of Finland (grants 309504, 314181, and 335249 to E.K.L).

Declaration of competing interest

The authors have nothing to disclose.

Acknowledgments

We thank the EsmiRs women for their participation. We also thank Eeva-Maija Palonen, Anja Ahlgren, Mervi Matero, Bettina Hutz, Hanne Tähti, and Sini Jokinen for their help with the data collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2022.06.001.

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