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The effects of high-intensity interval training on adipose tissue pathophysiology, reproductive and metabolic health in women with polycystic ovary syndrome

Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
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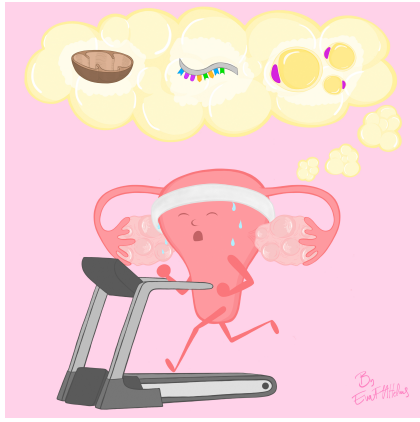

Thesis for the Degree of Philosophiae Doctor

Trondheim, June 2021

Norwegian University of Science and Technology
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Sammendrag

Polycystisk ovariesyndrom (PCOS) er den vanligste endokrine forstyrrelsen hos kvinner i fertil alder med en prevalens på opptil 13% globalt. PCOS diagnostiseres på bakgrunn av uregelmessig/manglende menstruasjon, hyperandrogenisme og/eller polycystiske ovarier, og syndromet er assosiert med en rekke reproduktive, kardiometabolske, og psykologiske risikofaktorer. Til tross for dette er den bakenforliggende etiologien og den optimale behandlingen for PCOS fortsatt usikker. Selv om livsstilsendringer (som inkluderer fysisk aktivitet) anbefales som førstelinjebehandling for å forbedre symptomer og risikofaktorer hos kvinner med PCOS, finnes det ennå ingen spesifikk treningsanbefaling for denne gruppen. Hovedmålet med studien «IMproving Reproductive function in women with Polycystic OVary syndrome with high-intensity Interval Training (IMPROV-IT)» (Artikkel I og II) var å undersøke om 16 ukers delvis veiledet høy-intensiv intervalltrening (HIT), enten med lavt eller høyt treningsvolum, etterfulgt av 36 ukers hjemmebasert HIT kunne øke menstruasjonsfrekvensen hos kvinner med PCOS. Sekundære utfallsmål var om treningen påvirket kondisjon, antallet graviditeter, helkropps-insulinfølsomhet og livskvalitet.

IMPROV-IT var en randomisert kontrollert klinisk studie med to senter som inkluderte 64 kvinner med PCOS. Deltakerne ble tilfeldig fordelt (1:1:1) til enten: lav-volum HIT (LV-HIT, 3 dager/uke; 10 x 1 min drag med maksimal intensitet); høy-volum HIT (HV-HIT, 3 dager/uke; 4 x 4 min drag med 90-95% av maksimal hjertefrekvens); eller en kontrollgruppe. Deltakerne gjennomgikk omfattende testing ved inklusjon, etter 16 uker og 12 måneder etter inklusjon. Hovedfunnene i IMPROV-IT var at det ikke var noen forskjell mellom gruppene i menstruasjonsfrekvens ved 12 måneder, men at der var en signifikant økning i menstruasjonsfrekvens fra inklusjon til 12 måneder i alle tre grupper (inklusiv i kontrollgruppen). Videre ble flere kvinner gravide i LV-HIT-gruppen sammenlignet med kontrollgruppen. Få kvinner i treningsgruppene fulgte treningsprotokollene gjennom hele studien og dette kan være en av årsakene til at vi ikke observerte en forskjell imellom gruppene i menstruasjonsfrekvens og at vi kun fant begrenset forbedringer i sekundære utfallsmål som kroppssammensetning, insulinsensitivitet og kondisjon. En annen årsak kan være at kontrollgruppen viste forbedring i flere av utfallsmålene etter både 16 uker og 12 måneder. Livskvaliteten var

forbedret i to ut av fem PCOS-relaterte domener (problemer med infertilitet og kroppsbehåring) i HV-HIT-gruppen sammenlignet med kontrollgruppen etter 12 måneder.

Det kompliserte samspillet mellom flere biologiske systemer kan delvis forklare den begrensede kunnskapen om PCOS-etiologien. Flere har foreslått at dysfunksjonelt fettvev kan spille en viktig rolle i de metabolske problemene hos kvinner med PCOS. Formålene med artikkel III og IV var å sammenligne uttrykket av forskjellige microRNA i fettvev og i blod, fettcellestørrelse, helkropps-fettoksidasjon under submaksimalt arbeid og mitokondrierespirasjon i fettvev hos kvinner med og uten PCOS. Vi undersøkte også respons på 16 ukers HIT på disse utfallsmålene i begge grupper. Hovedfunnene fra artikkel III var at uttrykket av microRNA-27b målt i blodet var høyere hos kvinner med PCOS og at uttrykket av sirkulerende microRNA-27b var redusert etter 16 ukers LV-HIT. Vi fant ingen forskjeller i de åtte utvalgte microRNAene vi målte i fettvev hos kvinner med eller uten PCOS, eller etter 16 ukers HIT. Hovedfunnene i artikkel IV var at fettoksidasjon under submaksimalt arbeid kun var forbedret hos kvinner uten, men ikke med PCOS, til tross for lik økning i kondisjon. Dette tyder på en metabolsk infleksibilitet hos kvinner med PCOS. Vi fant også at kvinner med PCOS hadde lavere mitokondrierespirasjon i subkutant abdominalt fettvev sammenlignet med kvinner uten PCOS. Dette underbygger hypotesen om at dysfunksjonelt fettvev muligvis spiller en viktig rolle i patofysiologien til PCOS.

Sofie Buurgaard Lionett
Institutt for sirkulasjon og bildediagnostikk, Det Medisinske Fakultet, Norges teknisk-naturvitenskapelige universitet (NTNU).

Hovedveileder: Trine Moholdt, PhD, Forsker
Biveileder: John A. Hawley, PhD, Professor

Finansiering: Samarbeidsorganet Helse Midt-Norge.

Summary

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-aged women, affecting up to 13% of women worldwide and has wide-ranging reproductive, metabolic and psychological health implications. Still, the etiology and optimal therapies for PCOS remain unclear. Even if lifestyle modification, including exercise training, is the first-line therapy for improving reproductive, metabolic and psychological features in PCOS, there is currently no specific exercise prescription for the management of PCOS. Therefore, the primary aim of the IMproving Reproductive function in women with Polycystic OVary syndrome with high-intensity Interval Training (IMPROV-IT) trial (Paper I and II) was to investigate whether 16 weeks of either low-volume or high-volume semi-supervised high-intensity interval training (HIT), followed by subsequent 36 weeks of home-based HIT could increase menstrual frequency in women with PCOS during a 1-year period. Secondary aims were to examine the effects of HIT on cardiorespiratory fitness, rates of pregnancies, whole-body insulin sensitivity, and quality of life.

IMPROV-IT was a two-center randomized controlled trial (RCT) that included 64 women with PCOS. The participants were randomly allocated (1:1:1) to either: low-volume HIT (LV-HIT, 3 days/week of 10 x 1 min work bouts at maximal, sustainable intensity); high-volume HIT (HV-HIT, 3 days/week of 4 x 4 min work bouts at 90-95% of maximal heart rate); or non-exercise control (Non-Ex). Outcome measures were assessed at baseline, after 16 weeks and 12 months after baseline. The main findings in IMPROV-IT were that there were no between-group differences in menstrual frequency at 12 months but a significant increase in menstrual frequency from baseline to 12 months in all three groups including in the Non-Ex (control) group. Furthermore, pregnancy rates increased significantly after LV-HIT. Adherence to the exercise protocols was poor which may explain the lack of difference between groups in menstrual frequency as well as limited findings in secondary outcome measures (including insulin sensitivity and cardiorespiratory fitness). Quality of life improved in two out of five PCOS-related domains (infertility problems and body hair) in the HV-HIT group compared to the control group at 12 months follow-up.

The complex relationship between multiple biological systems may partly explain the limited understanding of the etiology of PCOS. Adipose tissue dysfunction has been suggested to play a central role in the metabolic abnormalities observed in PCOS. The aims of Paper III and IV were to compare the expression of selected microRNAs in adipose tissue (and the circulation), fat cell size, rates of whole-body fat oxidation during submaximal exercise and rates of mitochondrial respiration in adipose tissue taken from women with and without PCOS. We also investigated the response to 16 weeks of HIT in these outcome measures in both groups of women. The main findings of Paper III were that the expression of circulating microRNA-27b was increased in PCOS, and that the expression of this microRNA was reduced after 16 weeks of LV-HIT. No differences were observed in the eight selected microRNAs in adipose tissue taken from women with and without PCOS, or following 16 weeks of LV- or HV-HIT. The main finding of Paper IV was that rates of whole-body fat oxidation during submaximal exercise only improved in women without, but not with PCOS, despite similar increases in cardiorespiratory fitness, indicating metabolic inflexibility in women with PCOS. Furthermore, we found that at baseline, women with PCOS had lower mitochondrial respiration through complex I + II in subcutaneous abdominal adipose tissue compared to women without PCOS, which supports the notion that abnormal adipose tissue may play a central role in PCOS pathophysiology.

Acknowledgements

I started the journey as a PhD student in March 2017 at the Department of Circulation and Medical Imaging, Faculty of Medicine, Norwegian University of Science and Technology (NTNU) in Trondheim, Norway. In January 2018, I went to the Mary MacKillop Institute for Health Research at the Australian Catholic University in Melbourne, Australia. Due to the covid-19 pandemic, I moved back to Denmark, my home country, in April 2020 where I completed my thesis. The research was funded by the Liaison Committee for education, research and innovation in Central Norway.

Firstly, thank you for opening my thesis. My thesis is a product of four years of hard work that are reduced to these pages. I know that many of you will not make it past the acknowledgements (you will be bored), but I am still grateful and proud. I have so many special and dear people I need to thank for supporting me during these last four years. I ended up completing my PhD in three different countries and labs, and my acknowledgements will follow the chronological order of the countries; Norway, Australia and Denmark. I am so grateful to all the people I met during the years.

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Copenhagen, March 2021
Sofie Buurgaard Lionett

Table of Contents

List of Papers

Paper I:

Ida Almenning Kiel, Sofie Lionett, Evelyn Bridget Parr, Helen Jones, Maria Aurora Hernandez Røset, Øyvind Salvesen, Eszter Vanky & Trine Moholdt.

Improving reproductive function in women with polycystic ovary syndrome with high-intensity interval training (IMPROV-IT): study protocol for a two-centre, three-armed randomised controlled trial.

BMJ Open 2020;10:e034733.

Paper II:

Ida Almenning Kiel*, Sofie Lionett*, Evelyn Bridget Parr, Helen Jones, Maria Aurora Hernandez Røset, Øyvind Salvesen, John Alan Hawley, Eszter Vanky & Trine Moholdt.

Improving reproductive function in women with polycystic ovary syndrome with high-intensity interval training (IMPROV-IT): a two-centre, three-armed randomised controlled trial.

In review.

Paper III:

Sofie Lionett, Ida Almenning Kiel, Donny Michael Camera, Eszter Vanky, Evelyn Bridget Parr, Stian Lydersen, John Alan Hawley & Trine Moholdt.

Circulating and Adipose Tissue miRNAs in Women With Polycystic Ovary Syndrome and Responses to High-Intensity Interval Training.

Front Physiol. 2020 Jul 30;11:904

Paper IV:

Sofie Lionett, Ida Almenning Kiel, Ragnhild Røsbjørgen, Stian Lydersen, Steen Larsen & Trine Moholdt.

Absent exercise-induced improvements in fat oxidation in women with polycystic ovary syndrome after high-intensity interval training.

Accepted in Front Physiol (26.02.2021).

* shared first authorship.

Abbreviations

ACU: the Australian Catholic University
ADP: Adenosine diphosphate
AE-PCOS: The Androgen Excess and PCOS Society
AMH: Anti-Müllerian hormone
ATP: Adenosine triphosphate
AUC: Area under the curve
BMI: Body mass index
DXA: Dual X-ray Absorptiometry
FAI: Free androgen index
FSH: Follicle stimulating hormone
GLUT-4: Glucose transporter type 4
GnRH: Gonadotropin releasing hormone
HDL: High density lipoproteins
HIT/HIIT: High-intensity interval training
HOMA-IR: Homeostatic Model Assessment for Insulin Resistance
HR_{max}: Maximal heart rate
HV-HIT: High-volume high-intensity interval training
HA: Hyperandrogenism
iAUC: Incremental area under the curve
IMPROV-IT: IMproving Reproductive function in women with Polycystic OVary syndrome with high-intensity Interval Training
LDL: Low density lipoproteins
LH: Luteinizing hormone
LV-HIT: Low-volume high-intensity interval training
MICT: Moderate-intensity continuous exercise training
NIH: The National Institutes of Health
OA: Oligo-/anovulation
OGTT: Oral glucose tolerance test
PCO: Polycystic ovaries
PCOS: Polycystic ovary syndrome
RCT: Randomized controlled trial
RER: Respiratory exchange ratio
RT-PCR: Real-time polymerase chain reaction
SHBG: Sex hormone binding globulin
NTNU: the Norwegian University of Science and Technology
T2DM: Type 2 diabetes mellitus
U: Ubiquinone
VO_2max: Maximal oxygen uptake
VO_2peak: Peak oxygen uptake
WHO: World Health Organization

1.0 Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-aged women, affecting up to 13% of women worldwide (1). PCOS has substantial and wide-ranging health implications including reproductive (hyperandrogenism, hirsutism, irregular menstrual cycle and infertility), metabolic (obesity, increased abdominal adiposity, dyslipidemia, hypertension, insulin resistance, impaired glucose tolerance and type 2 diabetes mellitus (T2DM)), and psychological features (increased anxiety and depression, and impaired quality of life) (2, 3). Despite the high prevalence and adverse health implications of PCOS, the etiology and optimal treatment for PCOS are still unclear. The scope of this thesis is to explore exercise training, and specifically high-intensity interval training (HIT), as a primary treatment to improve selected aspects of metabolic and reproductive health in women with PCOS, and whether aberrant adipose tissue plays a role in the pathophysiology of PCOS. The first section of the Introduction will describe general aspects related to exercise prescription. This will be followed by a detailed review of exercise training in women with PCOS. The second section of the Introduction will describe PCOS and includes diagnostic criteria, prevalence, phenotypes, possible etiology and pathophysiology as well as current recommended treatment of PCOS.

Throughout this thesis, the terms *physical activity* and *exercise* will be used. These terms might appear synonymous, but there are important differences, and therefore a need to define the nomenclature. *Physical activity* is defined as any bodily movement produced by skeletal muscles that requires energy expenditure and includes informal, unstructured activities such as walking to the train, going shopping, walking a dog, and climbing stairs (4). In contrast, *exercise* is planned, structured and repetitive physical activity with the aim to maintain or improve components of physical fitness (e.g. being active with a purposeful intention including going to the gym/for a run and playing a sport) (4).

1.1 Exercise training

Human physical activity patterns and behavior have evolved greatly since the time of our ancestors >10,000 years ago. Before the agricultural, industrial and digital ages, humans

expended large amounts of energy on a daily basis through activities required to sustain life, including hunting and gathering, and escaping predators (5). Such a lifestyle markedly contrasts current activity patterns, in which sedentary behavior, supported by mechanized forms of transport and labor-saving devices at home and at work, are a common part of our everyday life. Physical activity for the vast majority of people in the modern era is an optional activity, separated from daily tasks of living, and what we define as exercise (5).

The World Health Organization (WHO) reports that physical inactivity is the fourth leading global risk for mortality in the world and increases the risk of developing cancer, T2DM and cardiovascular diseases (6). However, engaging in regular exercise training and being physically active is a potent tool to reduce the risks for developing metabolic and cardiovascular-related disorders (7). Therefore, increasing participation in regular exercise and physical activity is vital from a public health perspective. However, the volume and intensity of exercise training required to reduce the risk of metabolic and related diseases states is unknown. Determining the dose and intensity of exercise training required to improve cardiorespiratory and cardiometabolic health outcomes is important in order to enable researchers and clinicians to recommend an exercise strategy for prevention and therapy in healthy and diseased populations.

1.1.1 Exercise therapy

There are various types of aerobic-based exercise that can be differentiated by their duration and intensity. Two common types of aerobic exercise protocols are traditional moderate-intensity continuous exercise training (MICT) and HIT. These two exercise modalities will be described briefly, followed by a comparison of some of the physiological adaptations to these different training modes in healthy and diseased populations, including in women with PCOS.

Traditional moderate-intensity, continuous exercise training

MICT is performed as continuous exercise undertaken at a moderate intensity (**Figure 1A**, 40-60% of maximal oxygen uptake (VO_2max) or between 55 and 70% of maximal heart rate (HR_{max})) and typically lasting between 30-60 min (8).

High-intensity Interval training

HIT broadly refers to exercise that is characterized by relatively short bursts of vigorous activity, interspersed by periods of rest or low-intensity exercise for recovery (9). A common, yet simplified, classification scheme subdivides this type of training into 1) HIT (**Figure 1B**, comprising near maximal efforts performed at the power output/speed that elicits 85-95% of HR_{max} for 1-4 min, with 1-3 min rest or active recovery), and 2) sprint interval training (SIT **(Figure 1C**), which comprises supramaximal efforts performed at power outputs/speeds > VO_2peak, for 30-60 sec, with ~4 min rest or active recovery) (10, 11). The focus in this thesis will be on HIT.

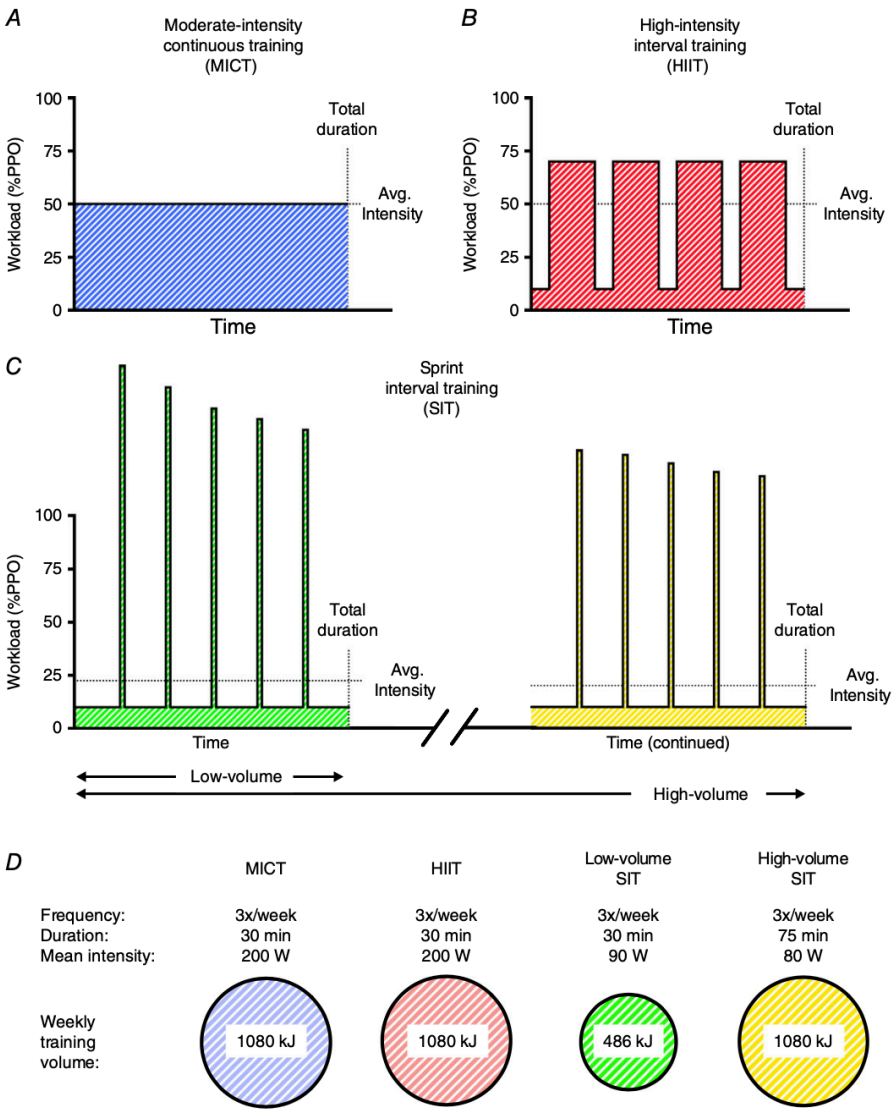

*Figure 1. A graphical depiction of the main types of aerobic exercise. Reproduced from MacInnis and Gibala (2017) (10) with permission from J Physiol. (**A-C**) representative examples of moderate-intensity continuous training (MICT), high-intensity interval training (HIIT), and low and high volumes of sprint interval training (SIT). The intensity is depicted as a percentage of the peak power output (PPO) obtained during a standard ramp VO_2peak test. **(D)** the training volume associated with each protocol based on the durations and training frequencies provided. The MICT and HIIT protocols shown in **A** and **B** are work-matched when performed for the same*

duration and at the same frequency. The low-volume SIT protocol requires less total work to complete relative to HIIT and MICT, whereas performing three sessions of the high-volume SIT protocol matches the training volume in the MICT and HIIT protocols.

Several HIT protocols exist and they can broadly be divided into low-volume HIT (LV-HIT) and high-volume HIT (HV-HIT). LV-HIT normally has at least a 1:1 ratio in work bout and recovery time whereas HV-HIT has a shorter work:recovery ratio.

The majority of HIT interventions use work bouts lasting between 1 and 4 min (12). Two common HIT protocols are the LV-HIT 10 x 1 min protocol (**Figure 2A**) (13) and the HV-HIT 4 x 4 min protocol (**Figure 2B**) (14). The 10 x 1 min LV-HIT protocol consists of 10 x 1 min work bouts at the maximal intensity participants can sustain with 1 min of passive or low-intensity recovery between work bouts. The 4 x 4 min HV-HIT protocol consists of four work bouts of 4 min, with a goal to reach 90-95% maximal heart rate (HR_{max}) during the first 2 min of each bout. These work bouts are typically separated by up to 3 min of active recovery at ~70 of HR_{max}.

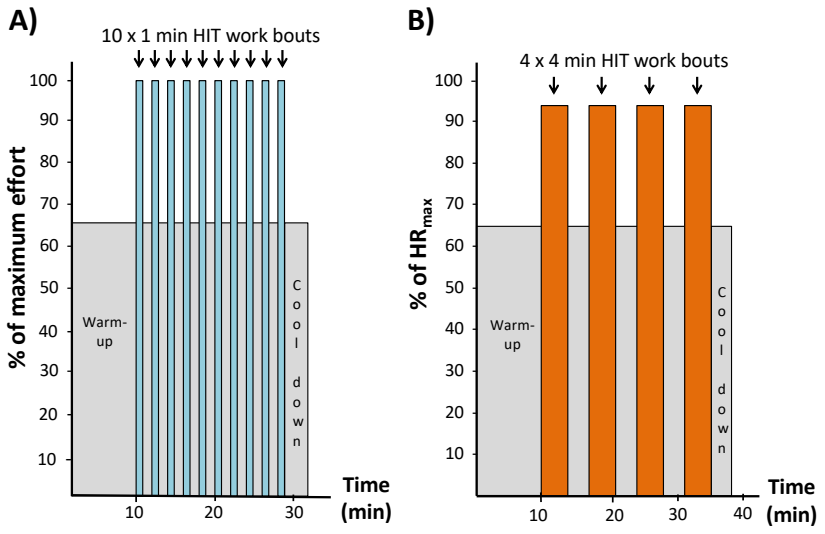

*Figure 2. Two common HIT protocols: **(A)** Low-volume HIT consisting of 10 x 1 min work bouts at maximal, sustainable intensity separated by 1 min of passive recovery or low-intensity exercise, **(B)** High-volume HIT consisting of 4 x 4 min work bouts reaching 90-95% HR_{max} during the first 2 minutes of each bout, separated by 3 min of active recovery at approximately 70% of HR_{max}.*

MICT versus HIT

A large body of evidence demonstrates that in both healthy individuals and diseased populations, HIT can provide similar or greater benefits to a number of cardiorespiratory measures and health markers compared to MICT (12, 14-18). Helgerud and colleagues reported greater improvement in VO_2max after eight weeks of three weekly sessions of HIT (4 x 4 min at 90-95% of HR_{max}) compared to MICT (continuous running at 70% HR_{max} for 45 min) in healthy male university students (14). The training protocols employed in that study were matched for total work and frequency, suggesting that training intensity is an important consideration with regard to improving cardiorespiratory fitness. The superior improvements in VO_2max following HIT are important since poor cardiorespiratory fitness is strongly associated with increased risk of developing the metabolic syndrome and T2DM (19). Furthermore, Farrell and colleagues showed that poor cardiorespiratory fitness is an independent predictor of all-cause mortality in women (20).

HIT confers greater health benefits compared to MICT in several clinical populations (12, 15, 16). Tjønna and colleagues demonstrated superior improvements in cardiovascular fitness, fasting glucose concentrations and insulin sensitivity (based on Homeostatic Model Assessment for Insulin Resistance (HOMA-IR)) in patients with the metabolic syndrome after 16 weeks of three weekly HIT sessions (4 x 4 min at 90% of HR_{max}) compared to work-matched MICT (47 min at 70% of HR_{max}) (17). Improvements in glucose control and insulin sensitivity have also been reported following HIT in patients with T2DM (18, 21). Little and colleagues demonstrated that LV-HIT (6 sessions of 10 x 1 min at ~90% of HR_{max}) reduced average 24 h blood glucose concentration, area under the 24 h blood glucose curve and the sum of three-hour postprandial area under the glucose curve for breakfast, lunch and dinner in patients with T2DM. The latter study (21) was an uncontrolled trial and did not directly compare the effects of HIT with MICT. However, a randomized controlled trial (RCT) undertaken by Karstoft and colleagues demonstrated superior effects of four months of interval walking compared to energy-expenditure matched continuous walking on VO_2max, body composition and glycemic control (decreased fasting glucose levels and mean continuous glucose monitoring concentration) in patients with T2DM (18).

To include HIT as a type of training can result in greater health benefits gained in less time, which makes HIT (and especially LV-HIT with a total exercise time of ~30 min including warm-up and cool-down) a time-efficient and attractive option to include for individuals who are time-poor. From a public health perspective, these findings are important as lack of time is an often-cited barrier to regular exercise participations (22). Training volumes usually differ when the physiological effects of LV-HIT versus MICT have been compared (23), whereas training volumes are usually matched in studies that have compared the physiological effects of HV-HIT versus MICT (14, 17, 18). Although HV-HIT (such as 4 x 4 min intervals with a total exercise time of 38 min including a 10 min warm-up and 3 min cool-down) is a more time-efficient approach compared to volume matched MICT (total exercise time of ~45-50 min), HV-HIT is still time-demanding. Apart from being time-efficient, evidence suggests that HIT is also perceived to be more enjoyable than MICT despite higher perceived exertion during HIT (24). Therefore, HIT may prove as a feasible strategy to improve long-term exercise adherence and participation.

1.1.2 Exercise therapy in PCOS

Lifestyle interventions, including exercise training, are recommended as first-line therapy for women with PCOS (3). However, there are few well-designed RCTs that have investigated the effect of exercise on reproductive and metabolic outcomes in women with PCOS. Accordingly, clinicians' face many challenges and lack a solid evidence-base when attempting to prescribe specific modes of exercise to women with PCOS. **Table 1** summarizes the results from RCTs including different types of aerobic exercise interventions in women with PCOS. RCTs that included the use of pharmacotherapy were excluded.

Table 1. Summary of RCTs with aerobic exercise intervention(s) in women with PCOS

Study	Definition of PCOS	Exercise group characteristics at baseline	Exercise protocol	Diet intervention	Cardio-respiratory outcomes	Body composition outcomes	Cardiometabolic outcomes	Hormonal and reproductive outcomes
Almenning et al. (2015) **(25)**	Rotterdam	*N* = 8 Age = 27.2 ± 5.5 yrs BMI = 26.1 ± 6.5 kg/m^2 VO_{2peak} = 35.6 ± 5.6 mL/min/kg	Type: Aerobic (HIT) Dose: 19 (10 x 1 min with 1 min recovery) and 25 min/session (4 x 4 min with 3 min recovery) Frequency: 3/wk Intensity: 4 x 4 min at 90-95% HR_{max} (2/wk) and 10 x 1 min with maximal intensity (1/wk) Duration: 10 wks	No diet intervention	↑ VO_{2peak}	↓ FM ↓ BF%	↓ HOMA-IR ↓ Fasting insulin ↑ HDL	↓ DHEAS
Brown et al. (2009) **(26)**	NIH	*N* = 8 Age = 36.5 ± 5.0 yrs BMI = 37.9 ± 9.4 kg/m^2 VO_{2peak} = 22.5 ± 6.0 mL/min/kg	Type: Aerobic (continuous) Dose: ~228 min/wk Frequency: NR Intensity: 40-60% VO_{2peak} Duration: 20-24 wks	No diet intervention	↑ VO_{2peak}	→	→	Not measured
Bruner et al. (2006) **(27)**	Rotterdam	*N* = 7 Age = 32.3 ± 1.0 yrs BMI = 36.2 ± 2.0 kg/m^2 VO_{2peak} = 22.6 ± 2.1 mL/min/kg	Type: Combined aerobic (continuous) + Resistance training Dose: 30 min/session (MICT) and ~60 min/session (RT) Frequency: 3/wk Intensity: 70-85% HR_{max} Duration: 12 wks	Nutritional counseling 1hr/wk (in the exercise & control group)	↑ VO_{2peak}	↓ WC	↓ Fasting insulin	→

Costa et al. (2018) **(28)**	Rotterdam	*N* = 14 Age = 27.6 ± 4.5 yrs BMI = 32.0 ± 4.2 kg/m^2 VO_{2peak} = 27.9 ± 3.3 mL/min/kg	Type: Aerobic (continuous) Dose: 40 min/session Frequency: 3/wk Intensity: 60-85% HR_{max} (progressing with ~5% HR_{max} every 4 wks) Duration: 16 wks	No diet intervention	↑ VO_{2peak}	↓ BMI ↓ WC	↓ SBP ↓ DBP ↓ TC	Not measured
Jedel et al. (2011) **(29)** and Stener-Victorin et al. (2012) **(30)**	Rotterdam	*N* = 30 Age = 30.2 ± 4.7 yrs BMI = 27.7 ± 6.4 kg/m^2 VO_{2peak} = 33.9 ± 8.5 mL/min/kg	Type: Aerobic (continuous) Dose: 30 min/session Frequency: 3/wk Intensity: faster than normal walking pace Duration: 16 wks	No diet intervention	↑ VO_{2peak}	→	↓ DBP	↑ Menstrual frequency ↓ Testosterone ↓ DHEAS
Nagelberg et al. (2016) **(31)**	NIH	*N* = 10 Age = NR BMI = ≥25-29.9 kg/m^2 (n=3) ≥30-39.9 kg/m^2 (n=7) VO_{2peak} = NR	Type: Aerobic (continuous) Dose: NR Frequency: 7/wk Intensity: NR Duration: 4 wks	Participants contacted weekly to discuss nutritional goals	Not measured	Not measured	Not measured	→
Orio et al. (2016) **(32)**	Rotterdam	*N* = 39 Age = 25.9 ± 2.7 yrs BMI = 26.7 ± 2.8 kg/m^2 VO_{2peak} = 19.0 ± 2.1 mL/min/kg	Type: Aerobic (continuous) Dose: 45 min/session Frequency: 3/wk Intensity: 60-70% VO_{2peak} Duration: 24 wks	800 kcal/day deficit	↑ VO_{2peak}	↓ BMI ↓ WHR	↓ TC ↓ LDL ↓ Fasting insulin ↓ $AUC_{insulin}$ ↓ HOMA-IR ↑ HDL	↑ Menstrual frequency → Sex hormones

Ribeiro et al. (2020) **(33)**	Rotterdam	*Continuous group / Intermittent group* *N* = 28 / 29 Age = 29.1 ± 5.3 / 29.0 ± 4.3 yrs BMI = 28.4 ± 5.6 / 28.7 ± 4.8 kg/m^2 VO_{2peak} = NR	Type: Aerobic (continuous or intermittent) Frequency: 3/wk Duration: 16 wks *Continuous group* Dose: 30-50 min (progressing with 5 mins every 3 wks) Intensity: 65-80% HR_{max} (progressing with ~5% HR_{max} every 4 wks) *Intermittent group* Dose: 30-50 min (2 min work bouts with 3 min recovery, progressing from 6 to 10 series from wk 1 to 16) Intensity: 70-90% HR_{max} (progressing with ~5% HR_{max} every 4 wks)	No diet intervention	Not measured	*Intermittent group:* ↓ WHR *Continuous group:* ↓ HC *Both groups:* ↓ WC	*Intermittent group:* → *Continuous group:* ↓ TC ↓ LDL	*Intermittent group:* ↓ FAI *Both groups:* ↓ Testosterone
Turan et al. (2015) **(34)**	Rotterdam	*N* = 14 Age = 24.5 ± 2.8 yrs BMI = 21.8 ± 1.0 kg/m^2 VO_{2peak} = 587.9 ± 21.7 mL/min/kg *(*The values reported for VO_{2peak} are strange).	Type: Combined aerobic (continuous) + Resistance training Dose: 50-60 min/session (with 5-20 min being MICT). Frequency: 3/wk Intensity: 65-70% HR_{max} Duration: 8 wks	General dietary and behavioral advice at baseline (no calorie restriction program).	↑ VO_{2peak}	↓ WC ↓ HC	↓ DBP ↓ Fasting glucose ↓ Fasting insulin ↓ HOMA-IR ↓ TC ↓ LDL ↑ HDL	↓ Menstrual cycle length → Sex hormones

Vigorito et al. (2007) **(35)**	Rotterdam	*N* = 45 Age = 21.7 ± 2.3 yrs BMI = 29.3 ± 2.9 kg/m^2 VO_{2peak} = 17.6 ± 2.5 mL/min/kg	Type: Aerobic (continuous) Dose: 30 min/session Frequency: 3/wk Intensity: 60-70% VO_{2peak} Duration: 12 wks	General dietary and behavioral advice at baseline (no calorie restriction program).	↑ VO_{2peak}	↓ BMI ↓ WC ↓ WHR	↓ Fasting insulin ↓ $AUC_{insulin}$ ↑ $AUC_{glucose}$/ $AUC_{insulin}$ ratio	↑ Menstrual cyclicity (in 60% of participants) → Sex hormones

AMH, Anti Müllerian hormone; AUC, Area under curve; BF%, Body fat percentage; BMI, Body mass index; BP, Blood pressure; BW, Body weight; DHEAS, Dehydroepiandrosterone sulfate; DBP, Diastolic blood pressure; FAI, Free androgen index; FFM, Fat-free mass; FM, Fat mass; FSH, Follicle stimulating hormone; HC, Hip circumference; HDL, High density lipoprotein cholesterol; HIT, High-intensity interval training; HOMA-IR, Homeostatic model assessment of insulin resistance; HR_{max}, Maximal heart rate; LDL, Low density lipoprotein cholesterol; LH, Luteinizing hormone; NIH, National Institutes of Health; NR, Not reported; PCOS, Polycystic ovary syndrome; SBP, Systolic blood pressure; SHBG, Sex hormone binding globulin; TC, Total cholesterol; TG, Triglycerides; VO_{2peak}, Peak oxygen uptake; WC, Waist circumference, WHR, Waist-to-hip ratio.

↑ illustrates an increase, ↓ a decrease and → no change. Exercise dose does not include warm-up and cool-down but does include recovery time in between work bouts in HIT sessions. N reflects the number of participants included in the analysis. Changes are based on within- and between-group differences.

As summarized in **Table 1**, there are few well-designed RCTs investigating the effect of exercise training on reproductive and metabolic outcomes in women with PCOS. The published studies to date have several limitations including, but not limited to: small sample sizes, and therefore insufficient statistical power to detect clinically relevant changes in outcome measures; short intervention periods; insufficient details regarding the exercise protocols; not measuring and reporting VO_2peak (and thereby unable to report changes in VO_2peak after the exercise intervention); inconsistent reproductive outcome definitions (pregnancy, ovulation rate, ovulatory cycle, menstrual frequency and menstrual cycle length (36)); varied and numerous methods of reporting outcome measures (i.e., insulin sensitivity); interventions combining diet and exercise; a lack of a control group; and the inclusion of participants taking metformin, oral contraceptives and other medications. Taken collectively, these factors make it difficult to interpret the isolated effect of exercise on reproductive and cardiometabolic health in PCOS.

The studies by Nybacka et al. (37, 38) and Thomson et al. (39-41) have been characterized as RCTs but are not included in **Table 1** since they were not RCTs but rather randomized, non-controlled trials. The study by Nybacka and colleagues (37, 38) compared the effects of energy restriction only (≥600 kcal/day deficit), exercise only (45-60 min/session at moderate to vigorous exertion level, two-three days/week for 16 weeks) and the combined effect of energy restriction and exercise on body composition, reproductive (menstrual pattern and ovulation rates) and cardiometabolic outcomes. This study is defined as an RCT by the authors where the diet alone group is referred to as the control group, but this is questionable since they received an energy restricted diet intervention. Similarly, it can be argued that the study by Thomson and colleagues (39-41) is a randomized, uncontrolled trial as their "control" group is also a dietary intervention group. In this study, participants were randomized to three groups; diet only, diet and aerobic exercise, and diet and combined aerobic + resistance exercise. The diet intervention was an energy-restricted, high protein diet (5000-6000kJ/day with 30% of the total energy coming from protein) with an aim of 8-12 kg weight loss over the 20 weeks study period. The purpose of the study was to assess the additive effect of aerobic and combined aerobic + resistance exercise training combined with a hypocaloric, high-protein diet on body composition, cardiometabolic, hormonal and reproductive outcomes.

Even if they were able to report results based on their aim, this was not an RCT as they did not include a no-intervention/usual care control group.

HIT versus MICT in PCOS

In addition to lack of well-designed RCTs on the (isolated) effects of exercise training in women with PCOS, there is a lack of RCTs comparing the effects of different aerobic exercise modes in women with PCOS. Only two studies have published results on the effects of HIT versus MICT on metabolic parameters (and hormonal parameters for Ribeiro et al. (33)) in women with PCOS (33, 42), and only one of these was an RCT (33). In the three-armed RCT by Ribeiro and colleagues, 110 women with PCOS were allocated in a 1:1:1 manner to one of three groups: 1) intermittent aerobic training, 2) continuous aerobic training, or 3) control group (no training). The 16-week training protocols for the two exercise groups were matched for exercise volume throughout the study (**Table 1)**. Briefly, both exercise groups undertook three weekly sessions of 30-50 min, in which the intermittent training group performed 2 min work bouts at intensities progressing from 70% of HR_{max} in week 1 to 90% of HR_{max} in week 13, interspersed with 3 min of low-intensity recovery. The continuous group exercised continuously at intensities progressing from 65% of HR_{max} in week 1 to 80% of HR_{max} in week 13. The aim was to investigate whether different intensities of aerobic exercise would provide diverse health benefits in women with PCOS. Testosterone levels and waist circumference decreased in both exercise groups, while the free androgen index (FAI) and waist-to-hip ratio decreased in the intermittent exercise training group only. Total cholesterol, LDL-cholesterol and hip circumference decreased only in the continuous exercise group. All the changes were within-group changes, with no statistically significant differences between training groups. This RCT has its limitations as they used the HR_{max} formula (220-age) to calculate the exercise intensity, rather than performing a maximal exercise test to estimate HR_{max}. Furthermore, Ribeiro et al. (33) did not measure cardiorespiratory fitness (i.e., VO_2peak) before and after their interventions.

Aktas and colleagues reported that 12 weeks of HIT decreased fasting insulin levels, triglycerides, total cholesterol and low density lipoprotein (LDL)-cholesterol, as well as increased high-density lipoproteins (HDL)-cholesterol and serum adiponectin (42). Furthermore, body mass index (BMI) decreased in the HIT group. No improvements

were observed following 12 weeks of MICT, however, there were no significant between-group differences. There are several limitations to this study. Insufficient details were provided regarding the exercise intensities of the two interventions. The authors described that both groups exercised for 30 min three days a week for 12 weeks and that the HIT group ran for 2 minutes, then walked for 2 minutes, while the MICT group ran for 30 minutes at a moderate tempo and at a constant speed. Furthermore, this was a combined exercise and diet intervention, in which both exercise groups followed a diet program that induced a 2-4 kg weight loss per month. There was no control group in this study, which makes it difficult to determine whether the observed effects were a result of the diet- and/or exercise intervention.

One RCT determined the effects of HIT as the sole intervention on metabolic and reproductive outcomes in women with PCOS (25). In a pilot study examining the effects of HIT, ten weeks of two weekly sessions of 4 x 4 min at 90-95% of HR_{max} and one weekly session of 10 x 1 min at maximal intensity improved VO_2max, HOMA-IR, fasting insulin concentrations, HDL-cholesterol, and body fat percentage, compared to a non-exercising control group. Of note, these improvements occured in the absence of any change in body mass (25).

The results of a recent meta-analysis conclude that vigorous intensity exercise is superior to moderate intensity exercise for improvements in cardiometabolic outcomes including VO_2peak, BMI and waist circumference in women with PCOS (43). Similarly, data from a cross-sectional study in women with PCOS showed that vigorous exercisers had lower HOMA-IR, higher HDL and sex hormone binding globulin (SHBG), and reduced prevalence of the metabolic syndrome, compared with those who exercised at moderate intensity or were inactive (44). In summary, the majority of evidence to date suggests that HIT is superior to MICT for inducing improvements in cardiorespiratory fitness, body composition and hormonal outcomes in women with PCOS. However, further research is required to determine whether exercise protocols utilized as part of clinical trials (8-12 weeks of supervised exercise) is realistic in a real-world scenario, and whether unsupervised training should be considered in order to promote long-term sustainability of exercise behaviors. Furthermore, research on the impact of various HIT protocols is necessary in order to determine the most successful interventions for retaining engagement and improving health.

Low-volume HIT versus high-volume HIT

Superior changes in a range of physiological and health-related markers have been reported after HIT compared to MICT in both healthy and clinical populations. However, far less is known regarding which type of HIT protocol is most effective in free-living, unsupervised conditions in women with PCOS. Baekkerud and colleagues compared the effects of three popular exercise modalities on VO_2max in men and women with overweight/obesity (23). Participants were randomly allocated to six weeks of either 4 x 4 min HIT at 85-95% of HR_{max} (HV-HIT), 10 x 1 min at VO_2max load (LV-HIT), or 45 min MICT at 70% of HR_{max}. All groups completed three supervised, sessions per week (18 sessions in total). Only the 4 x 4 min HIT group increased their VO_2max, whereas plasma volume and hemoglobin mass increased only in the 10 x 1 min HIT group. Skeletal muscle citrate synthase activity, a surrogate marker of mitochondrial content, increased in all three groups. These findings illustrated divergent adaptations following LV- and HV-HIT, and support previous findings suggesting that greater volumes of high-intensity work result in greater improvements in VO_2max (45, 46). In addition to a high volume of high-intensity exercise (≥15 min/session), a recent meta-analysis on the effects of different HIT protocols on improvements in VO_2max showed that HIT with longer work bouts (≥ 2 min) and longer training periods (4-12 weeks) improved VO_2max the greatest in healthy individuals and those with overweight/obesity (46). No previous studies have compared the effects of LV-HIT and HV-HIT on reproductive and cardiometabolic outcomes in women with PCOS.

1.2 Polycystic ovary syndrome (PCOS)

1.2.1 Definitions of PCOS

Almost ninety years ago, Stein and Leventhal described the disorder we now know as PCOS (47). They described a reproductive disorder in which they observed an association between polycystic ovaries and amenorrhea. Over time, the definition of PCOS has evolved, and hyperandrogenism has since been acknowledged as an important component of the condition. Today, PCOS is considered a 'syndrome' with no single diagnostic test, but rather a condition based on a collection of clinical signs and features (2). Over the past three decades, three sets of criteria have been developed to standardize the diagnosis of PCOS: 1) the National Institutes of Health (NIH) criteria, 2) the Rotterdam criteria, and 3) the Androgen Excess and PCOS Society (AE-PCOS) criteria (**Table 2**). Currently, diagnosis of PCOS is most often based on the Rotterdam criteria, and this is the criteria used in the work presented in this thesis. The NIH and the AE-PCOS criteria are still used in the literature, therefore, all three sets of criteria are outlined below.

Table 2. The features of the three diagnostic criteria sets of PCOS.

Features	NIH criteria (1990)	Rotterdam criteria (2003)	AE-PCOS criteria (2006)
Hyperandrogenism (HA; clinical and/or biochemical-HA)	✓	±	✓
Oligo-/anovulation (OA)	✓	±	±
Polycystic ovaries (PCO)	–	±	±
Diagnosis characteristic	HA and OA are required features for diagnosis	Any 2 of the 3 features for diagnosis	HA is required, accompanied by OA and/or PCO

✓ reflects a required diagnostic feature of PCOS for the given diagnostic criteria; ± reflects that this feature may or may not be present; – reflects that this feature may be present, but it is not identified or a suggested feature in the diagnostic criteria set.
NIH, National Institutes of Health; AE-PCOS, Androgen Excess and PCOS Society; HA, hyperandrogenism; OA, oligo-/anovulation; PCO, polycystic ovaries.

The NIH criteria were the first described diagnostic criteria set and were developed at the National Institutes of Health consensus conference on PCOS in 1990 (48). According to these criteria, a PCOS diagnosis requires clinical and/or biochemical signs of hyperandrogenism and oligo-/anovulation. Following the NIH criteria, it was recognized that the syndrome also encompasses signs and symptoms of ovarian dysfunction (polycystic ovaries). This lead to a new set of criteria developed at the 2003 consensus workshop in Rotterdam, also known as the Rotterdam criteria (49). The Rotterdam criteria require at least two out of the following three features: 1) clinical and/or biochemical signs of hyperandrogenism, 2) oligo- or anovulation and 3) polycystic ovary morphology. In 2006, after reviewing the available data, the Androgen Excess and PCOS (AE-PCOS) Society proposed a new definition for clinically diagnosing PCOS (2). According to the AE-PCOS criteria, clinical and/or biochemical signs of hyperandrogenism were proposed as the indispensable feature of PCOS and should be accompanied by oligo-/anovulation and/or polycystic ovaries. Thereby, the AE-PCOS criteria were a compromise between the NIH and Rotterdam criteria and excluded the non-hyperandrogenic phenotype proposed by the Rotterdam criteria (phenotype D in **Table 3**). All three sets of criteria are based on the assumption that other etiologies (congenital adrenal hyperplasia, androgen-secreting tumors and Cushing's syndrome) are excluded before the diagnosis of PCOS.

Although the Rotterdam criteria are most commonly used to diagnose PCOS today, the NIH and AE-PCOS criteria are still used in the scientific and clinical literature. Using three different sets of diagnostic criteria for diagnosing PCOS complicates PCOS research by decreasing the comparability of studies and their research findings, and in addition causes confusion in clinical practice.

1.2.2 Diagnostic features of PCOS

Although the NIH, Rotterdam and AE-PCOS criteria are different, they all require combinations of these three features; clinical and/or biochemical hyperandrogenism, menstrual irregularities, and polycystic ovaries. These diagnostic features will be described in the following paragraphs.

Hyperandrogenism

Hyperandrogenism is a feature of PCOS that can manifest clinically or biochemically. Clinical manifestations of hyperandrogenism are hirsutism, acne and androgenic alopecia, with hirsutism being the most predominant feature of clinical hyperandrogenism (50). Hirsutism is assessed by using the modified Ferriman-Gallway scoring system where seven areas of the body (the upper lip, chin, chest, chest, upper and lower back, and upper and lower abdomen) are scored from 0 to 4, where 0 is no excess hair growth and 4 is severe hair growth (51).

Biochemical hyperandrogenism can be assessed when clinical signs of hyperandrogenism are unclear or absent. To assess biochemical hyperandrogenism, women need to have withdrawn from the use of hormonal contraceptives for a minimum of three months before the measurements are performed (52). Biochemical hyperandrogenism is determined by elevated serum levels of androgens including total testosterone, calculated free testosterone or by FAI (52). FAI is calculated as 100 x (total testosterone/SHBG).

Oligo-/anovulation

Ovulatory dysfunction usually manifests as irregular menstrual cycles (oligo/amenorrhea) resulting from chronic oligo- or anovulation (53). Oligomenorrhea is defined as an intermenstrual interval >35 days and <8 menstrual cycles during a year. Amenorrhea is defined as absent menstruations in the past 90 days. Approximately 75% of women with PCOS have irregular menstrual cycles (54).

Polycystic ovaries

According to the 2003 consensus workshop in Rotterdam, polycystic ovaries are defined as 12 or more 2-9 mm follicles and/or increased ovarian volume (>10 mL) in at least one ovary measured by ultrasound scans (49). In 2018, the criteria for polycystic ovaries were revised in line with advances in technology allowing for increased sensitivity. The revised criteria recommend that the diagnostic threshold for polycystic ovaries should be based on the presence of ≥20 follicles (2-9 mm) per ovary and/or an ovarian volume ≥10 mL when using ultrasound transducers with a frequency bandwidth of ≥8MHz (52).

1.2.3 Prevalence of PCOS

PCOS is the most common endocrine disorder in reproductive-aged women. A meta-analysis by Bozdag et al. demonstrated that the prevalence of PCOS varies depending on diagnostic criteria; 6% according to the NIH criteria and 10% using the Rotterdam criteria or the AE-PCOS Society criteria (1). An Australian PCOS prevalence study carried out in a community sample of 728 women (mean age of 30.2 years) reported a prevalence of PCOS of ~9% using the NIH criteria, ~12% according to the Rotterdam criteria and ~10% using the AE-PCOS criteria (55). Of note, approximately 70% of the women with PCOS were unaware of having PCOS (i.e. not previously diagnosed). This indicates that many women with PCOS remain undiagnosed, possibly resulting in an even higher prevalence than reported by Bozdag et al. (1). As a consequence, the opportunity to manage symptoms of PCOS and initiate treatments, like lifestyle changes with the potential to reduce long-term health complications (3), is missed in a large number of undiagnosed women with PCOS.

1.2.4 Phenotypes of PCOS

PCOS can present as one of four phenotypes (**Table 3**) (2) illustrating the clinical heterogeneity of the disorder. Of note, some of the diagnostic sets for PCOS do not include phenotype C and/or D.

Table 3. Phenotypes of PCOS and diagnostics sets.

	Phenotypes			
	A	**B**	**C**	**D**
Hyperandrogenism (clinical and/or biochemical)	+	+	+	
Oligo-/anovulation	+	+		+
Polycystic ovaries	+		+	+
	Diagnostic sets			
NIH (1990)	✓	✓		
Rotterdam (2003)	✓	✓	✓	✓
AE-PCOS (2006)	✓	✓	✓	

NIH, National Institutes of Health; AE-PCOS, Androgen Excess and PCOS Society.

The large number of undiagnosed women with PCOS makes it difficult to estimate the actual distribution of the four phenotypes. The Australian PCOS prevalence study (55) also reported on phenotype distribution. That study reported that out of the 87 women with PCOS according to the Rotterdam criteria, ~72% had phenotype A or B (**Table 3**), while ~13% had phenotype C and ~15% were identified with phenotype D (55). It was not possible to give exact numbers of women with phenotype A or B, as some of the women presenting hyperandrogenism and oligo-/anovulation did not undergo an ultrasound examination. Of note, relatively few women were included in that study.

The PCOS phenotypes appear to demonstrate different metabolic disturbances, with phenotype A and B reported to have greater prevalence of the metabolic syndrome (29.6% and 34.5%) compared to phenotype C and D (10% and 8.3%) (56). These findings are supported by others who have linked PCOS phenotypes A and B to the presence of the metabolic syndrome and insulin resistance (57).

1.2.5 Etiology and Pathophysiology

Despite decades of research, the etiology of PCOS remains largely unclear due to the highly complex and multifactorial nature of the syndrome (58). PCOS is believed to be caused by a combination of genetic and environmental factors leading to hormonal disturbances of androgens and insulin (**Figure 3**). Obesity exacerbates these hormonal disturbances (3, 59).

Interactions between hyperandrogenism and hyperinsulinemia lead to the clinical features of PCOS; hirsutism and acne, polycystic ovaries, and oligo-/anovulation (menstrual disturbances). Hyperandrogenism is detected in 65-85% of women with PCOS, and is a well-established contributor to the PCOS etiology (60). Both the ovaries and the adrenal glands contribute to increased circulating androgens in women with PCOS (61). In the ovary, androgen production is regulated by luteinizing hormone (LH) in the theca cells, while follicle stimulating hormone (FSH) regulates the conversion of androgens to estrogens in the granulosa cells (62). The frequency of gonadotropin releasing hormone (GnRH) pulses undergo cyclical changes and stimulate the synthesis and secretion of LH and FSH. Women with PCOS have persistently rapid GnRH pulse frequency, favoring the secretion of LH over FSH, resulting in an elevated LH:FSH ratio

(62, 63). The elevated LH level stimulates theca cells to synthesize androgens leading to hyperandrogenism observed in women with PCOS, while the low levels of FSH inhibit follicular maturation (62).

Insulin resistance and the resulting hyperinsulinemia are major contributors to the PCOS etiology, with insulin resistance reported to be present in approximately 75% of lean and 95% of overweight women with PCOS (64). In addition to contributing to metabolic features, hyperinsulinemia also plays an indirect role in hyperandrogenism by reducing SHBG production in the liver, which results in increased ovarian theca cell androgen production (65, 66). As a consequence of insulin resistance, PCOS is associated with long-term health risks, including impaired glucose tolerance, the metabolic syndrome and T2DM (67, 68). Furthermore, PCOS is associated with an increased incidence of cardiovascular events, even after adjusting for BMI (69). Combined, the health consequences accompanying PCOS lead to psychological issues including impaired quality of life and increased prevalence of anxiety and depression (3).

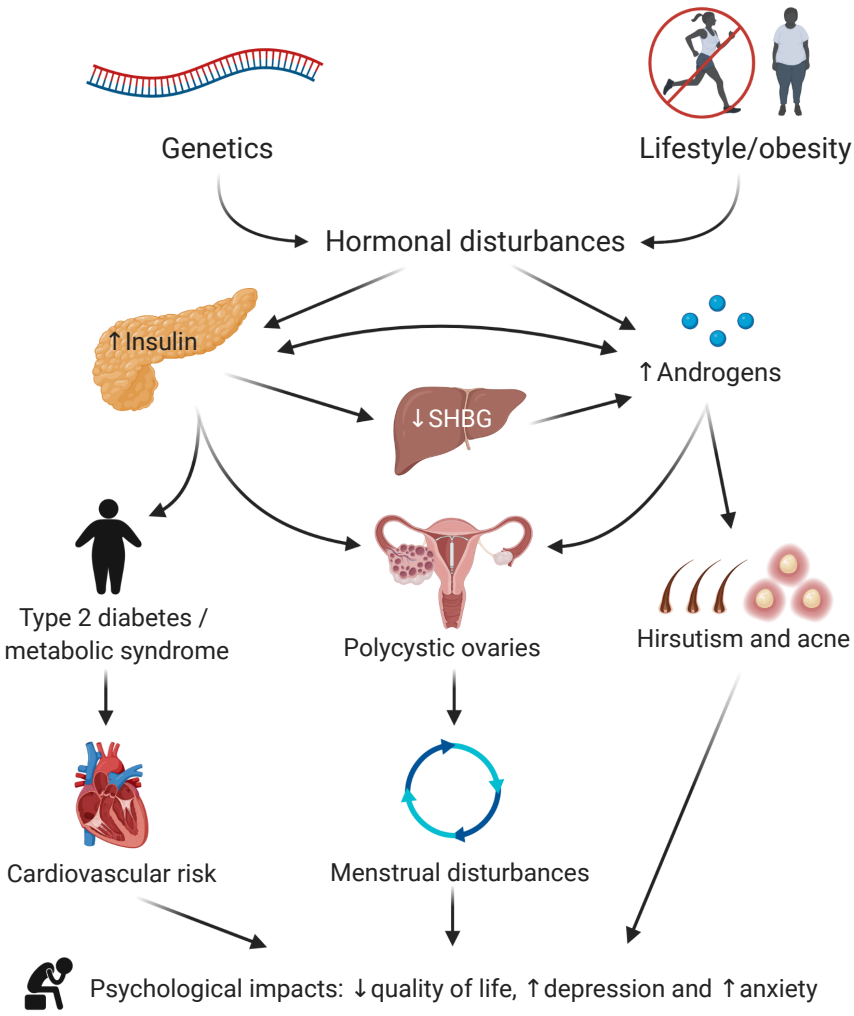

Figure 3. The etiology and clinical features of PCOS. PCOS is caused by a combination of genetic and lifestyle factors that lead to an underlying imbalance between androgens and insulin which is further exacerbated by obesity. The increased androgens lead to the clinical features of PCOS including hirsutism and acne. The hyperinsulinemia leads to the metabolic features associated with PCOS such as increased risk of the metabolic syndrome, type 2 diabetes and cardiovascular risk whilst both the hyperinsulinemia and elevated androgens result in reproductive features including anovulation, menstrual irregularities and subfertility. Combined, these features often result in psychosocial issues including reduced quality of life and increased symptoms of anxiety and depression. SHBG, Sex hormone binding globulin. This figure was created with Biorender.com.

1.2.6 PCOS, obesity and insulin resistance

The number of people with overweight and obesity (i.e. BMI ≥ 25 kg/m^2) has increased dramatically on a global scale over the past decades. In 2016, the World Health Organization estimated that 39% of the adult population had overweight or obesity (70). In parallel with the rise in overweight and obesity observed in the general population, the prevalence of overweight and obesity is also increasing in women with PCOS. The percentage of women with PCOS and obesity (i.e. BMI ≥ 30 kg/m^2) increased from 51% to 74% in just 10-15 years (71). Obesity *per se* does not cause PCOS, but exacerbates some of the metabolic and reproductive dysfunctions linked to PCOS including hyperandrogenism, hirsutism and infertility (72). Obesity also worsens insulin resistance in women with PCOS (64, 73). A meta-analysis showed that women with PCOS have a 27% reduction in insulin sensitivity as measured by the gold standard euglycemic-hyperinsulinemic clamp compared to women without PCOS, independent of BMI and age (73). Furthermore, this meta-analysis found that a higher BMI exacerbated the reduction in insulin sensitivity in women with PCOS. Although the prevalence of insulin resistance is exacerbated by obesity, insulin resistance is still more prevalent in lean women with PCOS compared to BMI-matched women without PCOS (64, 74, 75). The underlying mechanisms for the observed insulin resistance in women with PCOS remain elusive.

Insulin resistance and the compensatory hyperinsulinemia is believed to play a central role in the etiology of PCOS, and to contribute to the metabolic and reproductive features of the condition (66). Furthermore, adipose tissue dysfunction has been suggested to play a central role in the metabolic abnormalities observed in PCOS (63, 76). The potential role of adipose tissue in PCOS pathophysiology is described in paragraph 1.2.8.

1.2.7 Metabolic inflexibility in PCOS

Metabolic inflexibility, characterized by distorted nutrient sensing and blunted substrate switching in response to physiological stimuli including the transition from fasting to fed states/insulin stimulation or exercise, is linked to insulin resistance (77). A recent systematic review reported metabolic inflexibility in the rested, insulin-stimulated state

in women with PCOS compared with healthy women (78). It is well-documented that individuals with obesity and T2DM are metabolically inflexible (77). Broskey and colleagues found that women with PCOS and obesity (28.8 ± 4.7 years) were as metabolically inflexible as middle-aged women with obesity and T2DM (58.2 ± 9.9 years) (79). Metabolic inflexibility has also been reported in adolescent girls with PCOS (80) and in lean women with PCOS (75), which suggests an association between metabolic inflexibility and PCOS independent of age and BMI.

Previous studies have reported increased rates of whole-body fat oxidation during submaximal exercise at 60% of VO_2peak in healthy recreationally active women after seven sessions of HIT (10 x 4 min bouts at 90% of VO_2peak separated by 2 min of rest) (81), and in untrained recreationally active men and women after six weeks of HIT (using the same HIT protocol but for three days/week for six weeks) (82). This increased fat oxidation reflects improved metabolic flexibility. Whether HIT results in increased fat oxidation during submaximal exercise in women with PCOS, thereby reflecting the same metabolic flexibility as in healthy women, has not been investigated in previous studies (**Figure 4**).

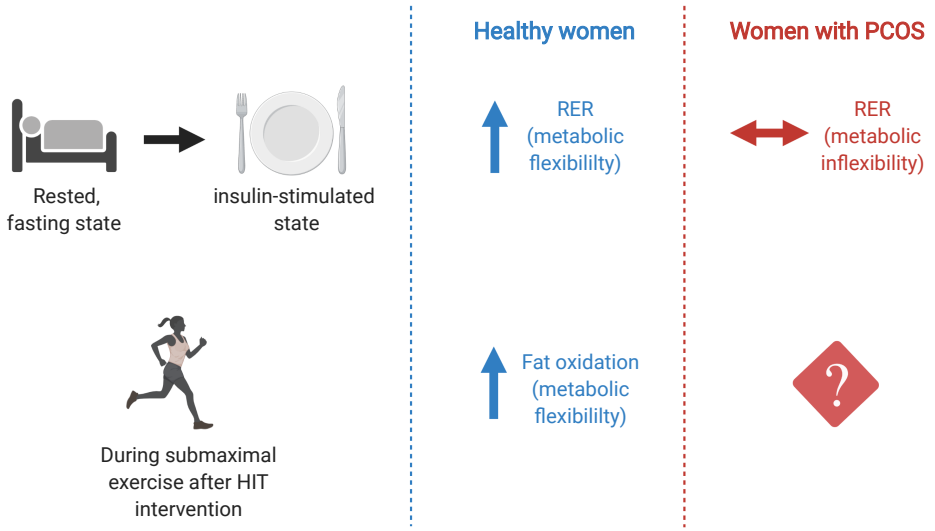

Figure 4. Changes in fat oxidation in healthy women and women with PCOS from the fasting to insulin-stimulated state, and during submaximal exercise (60% of VO_2peak) after HIT intervention. The respiratory exchange ratio (RER) increases in healthy women in the transition from fasted to insulin-stimulated state, whereas RER is unchanged in women with PCOS which reflects metabolic inflexibility in women with PCOS. Previous studies have reported improved whole-body fat oxidation during submaximal exercise in healthy women after HIT intervention, but this is yet to be investigated in women with PCOS. RER, respiratory exchange ratio; HIT, high-intensity interval training. This figure was created with Biorender.com.

1.2.8 The potential role of adipose tissue in PCOS pathophysiology

The understanding of the role of adipose tissues in metabolic homeostasis has evolved considerably over the past two decades. Adipose tissue was once considered an inert energy storage that provided isolation from the cold, but recent discoveries have led to the concept that white adipose tissue is a dynamic endocrine organ that produces several endocrine, paracrine and neuroendocrine signals through the production and release of adipokines (83). Adiponectin, which is the most abundant of these adipokines, increases insulin sensitivity through a combination of decreased hepatic gluconeogenesis and increased fatty acid oxidation (83). Reduced adiponectin levels have been reported in women with PCOS compared with age- and BMI-matched control women (75, 84). Such

reduced levels of circulating adiponectin is one of the reasons why adipose tissue dysfunction has been suggested to play a central role in the pathophysiology of insulin resistance and metabolic disturbances observed in PCOS (63, 76).

Carmina and colleagues evaluated fat quantity and distribution in 110 women with PCOS and 112 age- and body weight-matched, healthy women (85). They found that PCOS was associated with greater abdominal fat distribution, with greater percent trunk fat and amounts of visceral fat. Abdominal fat accumulation (abdominal obesity), and in particular greater visceral fat quantity, is associated with cardiometabolic risk (insulin resistance, T2DM and cardiovascular disease), whereas gluteal fat accumulation is paradoxically protective when it comes to metabolic risk (86). Excess abdominal fat has been suggested as one explanation for the metabolic abnormalities in women with PCOS, due to the association between abdominal fat and metabolic risk. However, insulin resistance also exists in lean women with PCOS (64). Thus, it is unlikely that fat distribution is the sole reason for the metabolic abnormalities reported in PCOS.

Not only is reduced whole-body insulin sensitivity present in women with PCOS, but they also present with lower abdominal adipocyte insulin sensitivity (87). Such abdominal adipocyte insulin resistance may be linked to decreased expression of the insulin-regulated glucose transporter type 4 (GLUT-4) in adipocytes in women with PCOS (88). Insulin-stimulated glucose uptake in adipose tissue is largely facilitated by GLUT-4 (89). Other features of aberrant adipose tissue may be present in women with PCOS. The potential role of adipose tissue morphology (fat cell size), mitochondrial respiration and microRNAs (miRNAs) in PCOS pathophysiology (**Figure 5**) will be discussed, followed by the reported effects of aerobic exercise on these outcomes.

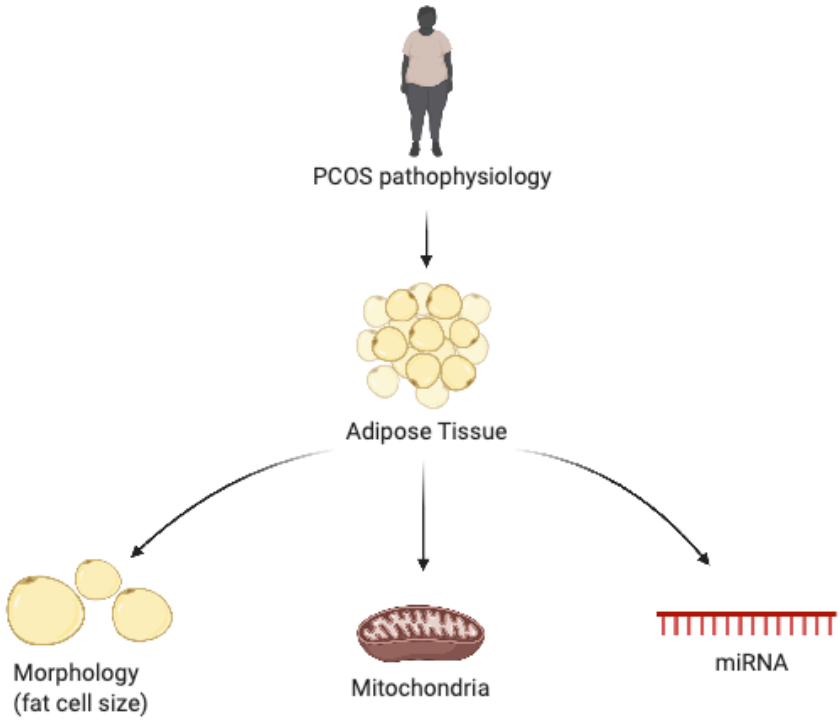

Figure 5.The pathophysiology of PCOS is complex due to the multifactorial nature of the syndrome. Adipose tissue dysfunction has been suggested to play a central role in the pathophysiology of insulin resistance and metabolic disturbances observed in PCOS, yet further research is required to determine the link between PCOS pathophysiology and adipose tissue. The role of fat cell size, mitochondrial respiration, and miRNAs in PCOS pathophysiology will be explored in this thesis along with the effects of 16 weeks of high-intensity interval training on these outcomes. This figure was created with Biorender.com.

Adipose tissue morphology

The mechanisms underlying the insulin resistance in women with PCOS are potentially related to aberrant adipose tissue morphology. Obese individuals with hypertrophic abdominal adipocytes are reported to be more insulin resistant than individuals with smaller adipocytes but with the same body fat percentage (90). Thus, adipocyte size may affect insulin resistance. A case-control study including women with PCOS and age- and BMI-matched women without PCOS reported enlarged abdominal adipocytes in women

with PCOS (84). Hypertrophic abdominal adipocytes were strongly associated with insulin resistance in women with PCOS (84), suggesting that aberrant adipose tissue morphology may play a central role in the pathogenesis of the observed insulin resistance in PCOS.

In a secondary analysis of an RCT, the effects of exercise on adipose tissue morphology were investigated in women with PCOS (30). No changes in abdominal adipose tissue volume were observed after 16 weeks of aerobic, continuous training (3 days/week for 30 min/session). However, the exercise intensity in that study is poorly described and was a self-selected pace instructed to be "*faster than normal walking at a pace that could be sustained for at least 30 minutes*". The exercise protocol did not induce any changes in cardiometabolic outcomes, including insulin sensitivity measured by the hyperinsulinaemic-euglycemic clamp. Hence, the intensity, frequency and duration of the exercise protocol was potentially insufficient to induce changes in adipose tissue morphology. It is important to note that most studies have only investigated abdominal adipose tissue morphology. However, adipose tissue from different depots express distinctive molecular, cellular and metabolic properties, and respond in different ways to exercise and nutritional challenges (91-93). No prior investigations exist on gluteal adipose tissue cell size and its response to exercise training in women with PCOS. To investigate multiple adipose tissue depots is of great importance to achieve a more comprehensive understanding of the aberrant adipose tissue function and morphology observed in women with PCOS.

Mitochondrial respiration in adipose tissue

Mitochondria are involved in multiple vital cellular activities, where energy production is the most critical. Mitochondria are the major site of adenosine triphosphate (ATP) production through oxidative phosphorylation, and are often referred to as the powerhouse of the cell. Mitochondria are present in most eukaryotic cells and are double-membraned organelles that contain an outer and inner membrane. The inner membrane is folded into cristae, and surrounds the inner space of the mitochondria called the matrix (94, 95).

The oxidative phosphorylation is the metabolic process in which ATP is generated as a result of the transfer of electrons from NADH and $FADH_2$ to O_2 in the electron

transfer chain (**Figure 6**) (96). NADH and $FADH_2$ are generated by the oxidation of macronutrients in food through the glycolysis, fatty acid oxidation, and the citric acid cycle. NADH and $FADH_2$ are then transported to the inner membrane of the mitochondria where the oxidative phosphorylation takes place. The electron transfer chain is series of enzymes (Complex I to IV) that are embedded into the inner mitochondrial membrane. Briefly, NADH donates electrons to complex I which powers the active transport of protons from the matrix to the intermembrane space. The pumping of protons across the membrane, driven by energy of electron transfer, produces an electrochemical gradient referred to as the proton-motive force. From complex I the electrons are transported via ubiquinone (Q) to complex III. $FADH_2$ donates electrons to complex II that are similarly transported via Q to complex III. Complex III transfers the electrons from Q to cytochrome c with the transport of protons from the matrix to the intermembrane space, and cytochrome c then transfers the electrons to complex IV. Complex IV carries the electrons to O_2 and reduces it to H_2O while using protons. Furthermore, complex IV also pumps one proton from the matrix to the intermembrane space for every electron that passes, which adds to the proton-motive force produced by complexes I and III. The protons then flow down the electrochemical gradient from the intermembrane space to the matrix via. ATP synthase which creates kinetic energy to phosphorylate adenosine diphosphate (ADP) to ATP (95).

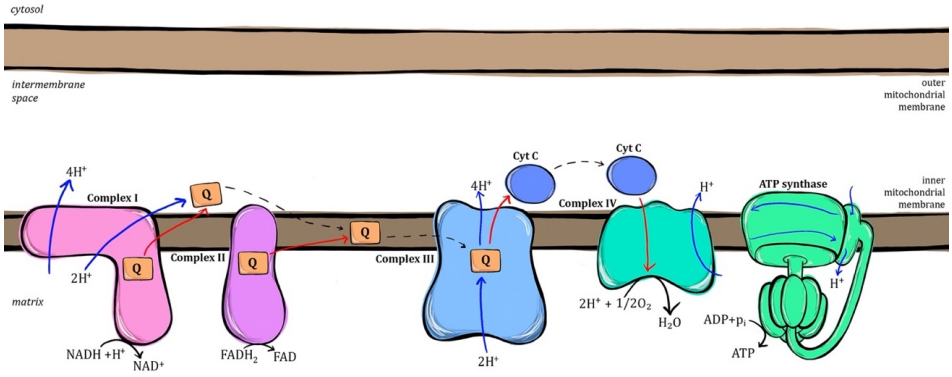

Figure 6. The electron transfer chain and oxidative phosphorylation. The oxidative phosphorylation begins when NADH and $FADH_2$, derived from glycolysis, fatty acid oxidation and citric acid cycle, are oxidized by feeding electrons into complex I and II of the electron transport chain. The electrons are then transferred via ubiquinone (Q) to complex III, and subsequently via cytochrome c (Cyt C) to complex IV where the electrons reduce O_2 to H_2O (red arrows). The flow of electrons from NADH and $FADH_2$ to O_2 through the protein complexes lead to the pumping of protons from the mitochondrial matrix to the intermembrane space by complex I, III and IV (blue arrows). The resulting uneven distribution of protons creates a pH gradient and an electrochemical gradient referred to as the proton-motive force. The proton-motive force drives the protons back from the intermembrane space to the matrix via ATP synthase and leads to phosphorylation of ADP to ATP. This figure was drawn by Eva Frederikke Høy Helms.

Insulin resistance has been linked to reduced mitochondrial respiration in skeletal muscle (97). Only one prior study has measured mitochondrial function in women with and without PCOS using high-resolution respirometry (98). Rabol and colleagues (2011) measured skeletal muscle mitochondrial function and reported that skeletal muscle mitochondrial respiratory capacity was not reduced in women with PCOS. However, as outlined above, the insulin resistance observed in PCOS may be linked to adipose tissue dysfunction rather than skeletal muscle.

Skeletal muscle and adipose tissue are crucial tissues in energy metabolism and play a major role in metabolic flexibility in humans (77). However, little research has been reported on metabolic flexibility of white adipose tissue. Metabolic flexibility is

driven by cellular processes that may be linked to mitochondrial function (77). Research on mitochondrial respiration in adipose tissue are sparse, and the first protocol to quantify mitochondrial respiration in adipose tissue using high-resolution respirometry was developed only a decade ago in 2010 by Kraunsoe and colleagues (99).

In addition to their central role in energy metabolism, mitochondria in adipose tissue play an important role in adipocyte differentiation, lipogenesis and adipokine secretion (96). Furthermore, obesity and insulin resistance has been linked to mitochondrial dysfunction in adipose tissue (96).

Conflicting findings have been reported on exercise-induced changes in adipose tissue mitochondrial respiration. Larsen and colleagues investigated the effects of 6 weeks of LV-HIT on mitochondrial respiration in subcutaneous abdominal adipose tissue and skeletal muscle in overweight but otherwise healthy men and women (100). The participants exercised 15 min three times weekly, and each session included five 1 min maximal effort work-bouts. Skeletal muscle mitochondrial respiratory capacity increased, but with no change in mitochondrial respiration in abdominal adipose tissue. In contrast, Dohlmann and colleagues (2018) reported decreased mitochondrial respiration in subcutaneous abdominal adipose tissue in healthy men and women after 6 weeks of LV-HIT (18 HIT sessions with 7 x 1 min exercise bouts at ~100% of VO_2peak) (101). Mendham and colleagues (2020) reported increased mitochondrial respiration in subcutaneous abdominal adipose tissue in obese, black South-African women after 12 weeks of combined aerobic and resistance exercise training (four days/week of 40-60 min with aerobic exercise at 75-80% HR_{peak} and resistance training that included upper- and lower-body exercises at 60-70% HR_{peak}) (102). There are no prior publications on mitochondrial respiration and the effects of HIT in this metabolically active endocrine organ in women with PCOS.

MicroRNAs

Micro-RNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional regulators through two divergent mechanisms depending on the complementarity between the miRNA and its target mRNA (**Figure 7**). Perfect base-pairing between the

miRNA and mRNA results in mRNA degradation, whereas imperfect binding with mRNA transcripts inhibits protein and gene translation (103).

miRNAs are remarkably stable and can be detected in a wide range of body fluids including plasma and follicular fluid (104). Moreover, some miRNAs also exert specificity in tissue expression within the body and can either be released or taken up by these tissues from the bloodstream (105). As miRNAs can function within the tissue of origin, adjacent and/or distant cells/tissues and each miRNA is able to attenuate the expression of multiple target mRNAs (103), they play crucial roles in many biological processes and have been implicated in several metabolic disorders including obesity, diabetes and cardiovascular diseases (106).

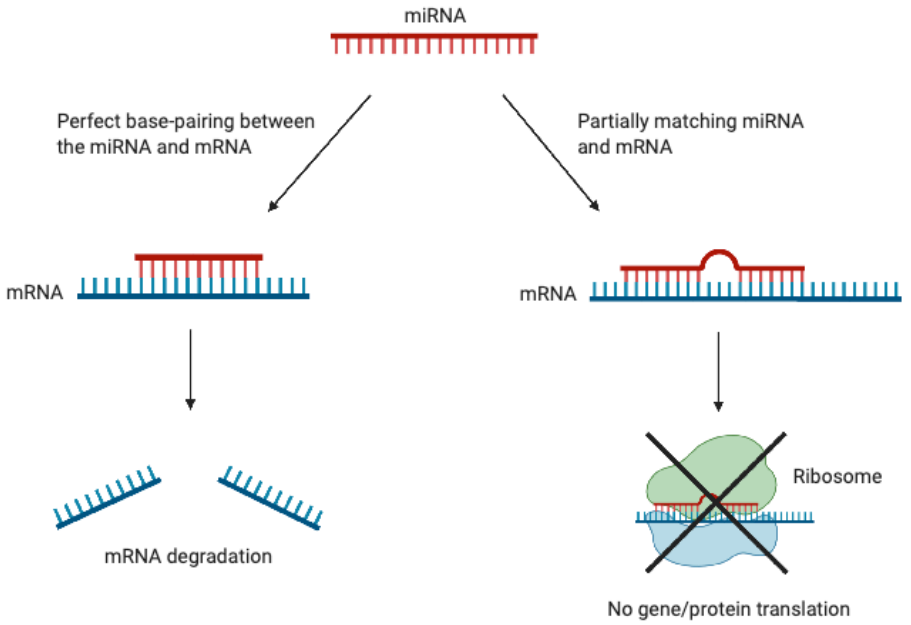

Figure 7. The post-transcriptional regulation of miRNAs. miRNAs are short non-coding RNAs that act as post-transcriptional regulators and repress mRNA translation either via perfect base-pairing between miRNA and mRNA target which leads to mRNA degradation, or base-pairing with partial complementarity to the target mRNA which inhibits gene and protein translation. miRNAs can act within the tissue of origin, or be released to the circulation and taken up by adjacent and/or distant tissues where they exert their effects on cell processes. This figure was created with Biorender.com.

Several studies have reported altered expression of miRNAs in distinct tissues in women with PCOS including in the circulation (107-116), adipose tissue (114, 117-119), follicular fluid and granulosa cells (114, 120). The abnormal expression of miRNAs observed in women with PCOS compared to women without PCOS suggest a potential role for miRNAs in the pathophysiology of PCOS. Specifically, abnormally expressed miRNAs with putative roles in regulating glucose metabolism in adipocytes have been reported in subcutaneous abdominal adipose tissue in women with PCOS and in insulin resistant women regardless of PCOS status (117, 119, 121). As such, it is likely that miRNAs are implicated in the adipose tissue abnormalities and adipocyte insulin resistance in women with PCOS.

An overlooked and understudied area of adipose tissue miRNA research is the anatomical location of adipose tissue sampling. As outlined previously, adipose tissue from different depots exhibit different molecular, cellular and metabolic properties, therefore, research on miRNAs in adipose tissue should include tissue from more depots than subcutaneous abdominal adipose tissue. There are no publications on miRNA expression in gluteal adipose tissue in women with PCOS or any that have compared the miRNA expression between abdominal and gluteal adipose tissue in this population.

Exercise training can modulate the expression of circulating miRNAs in healthy individuals and in persons with overweight and obesity (122-125). However, no prior studies have investigated the effect of exercise training on the expression of circulating or adipose tissue miRNAs in women with PCOS. This is of interest, as exercise training could potentially regulate and normalize the miRNAs that are abnormally expressed in women with PCOS. The effect of aerobic exercise training on abdominal and gluteal adipose tissue miRNA expression has also only been sparsely investigated in other populations (93). Tsiloulis and colleagues reported no effect of 6 weeks of aerobic exercise training (four supervised session/week; three moderate intensity sessions and one HV-HIT session) on the expression of 526 miRNAs in abdominal or gluteal adipose tissue in overweight males. However, in that study they had a very low sample size (only five complete data sets were included) which likely reduced the statistical power to detect small differences and increased the risk of type 2 errors.

1.2.9 Treatment of PCOS

Despite the high prevalence of PCOS in reproductive-aged women, the optimal therapies for PCOS remain unclear. The treatment of PCOS includes addressing reproductive, metabolic, and psychological features and needs to be individualized and tailored to the clinical presentation of each individual (52). Insulin resistance is a major contributor to the PCOS etiology, driving hyperandrogenism as well as clinical features, and is present in the majority of women with PCOS (64). Therapeutic strategies targeting insulin resistance improve clinical features (126). The first line treatment for women with PCOS is lifestyle modifications including a healthy diet and regular physical activity (52). However, the studies used to develop guidelines for women with PCOS were limited to a small number of RCTs, resulting in a general consensus recommendation of exercise and healthy eating principles rather than a specific exercise prescription and/or dietary strategy for the management of PCOS (52, 127). When lifestyle modifications do not achieve the desired goals, pharmacological treatments for women with PCOS exist, and the most widely used pharmaceuticals are oral contraceptives and metformin (52).

Lifestyle modifications

In overweight women with PCOS, lifestyle intervention including diet, exercise and behavioral strategies are recommended to reduce weight, central obesity and insulin resistance (52). Currently, recommendations and approaches for lifestyle modifications are ambiguous. With regard to diet interventions, current recommendations are to implement general healthy eating habits across the life span. For those with excess body weight, a diet intervention with an energy deficit of 30% of total daily energy intake or 500-750 kcal/day (taking individual energy requirements, physical activity levels and body weight into considerations) is recommended to achieve weight loss (52).

The current recommendations for exercise state that women with PCOS should be physically active for at least 30 min/day which should include a minimum of 150 min/week of moderate intensity or 75 min/week of vigorous intensity (or an equivalent combination of both) and include activities for muscle strengthening twice per week in order to prevent weight gain and maintain health (52). To achieve weight loss in those women with overweight and obesity, prevent weight regain and induce greater health

benefits, a minimum of 250 min/week of moderate intensity or 150 min/week of vigorous intensity is recommended in combination with muscle strengthening twice weekly (52). It is also recommended that behavioral strategies (goal-setting, problem solving, confidence training, slower eating) be reinforced to prevent relapse during lifestyle interventions to improve weight management, healthy lifestyle and wellbeing in women with PCOS (52). Strategies to enhance engagement and adherence to a healthy lifestyle and thereby improve long-term health outcomes for women with PCOS need consideration as these are currently limited.

1.2.10 PCOS, exercise and reproductive function

PCOS is the primary cause of anovulatory infertility and menstrual disorders in reproductive-aged women (3). A higher prevalence of infertility in women with PCOS (72%) compared to women without PCOS (16%) was reported in a large Australian community-based cohort study (128). Infertility was defined as trying unsuccessfully to conceive for ≥ 12 months. Exercise training has been shown to positively affect metabolic and cardiovascular outcomes in women with PCOS (36, 43, 44), and may provide a non-pharmacological strategy to enhance fertility in these women. Indeed, some studies report improved pregnancy and ovulation rates following an exercise intervention (29, 32, 34, 37, 129). Palomba and colleagues (129) compared the efficacy of a structured exercise training program versus a hypoenergetic, hyperproteic diet intervention on reproductive function in anovulatory, infertile women with PCOS and obesity. The exercise group (n=20) completed three weekly sessions of 30 minutes ergometer cycling at 60-70% of VO_{2max} for 24 weeks. The diet group (n=20) had a daily energy deficit of 800 kcal and consumed a high protein diet (35% protein, 45% carbohydrate and 20% fat) for 24 weeks. After 24 weeks, menstrual frequency and ovulation rate were higher in the exercise group compared to the diet group. Furthermore, there was a tendency towards more pregnancies in the exercise group (35%) versus in the diet group (10%; P= 0.058), although, the study was underpowered to detect a difference in pregnancy rate (their primary outcome) (129). That study was a non-randomized trial in which the participants could self-select which group they wanted to attend (self-selection bias), and without a control group, therefore the findings should be interpreted with caution (129).

Nybacka and colleagues (37) reported that diet alone (≥600 kcal/day energy deficit), exercise alone (45-60 min/session at moderate to vigorous exertion level, 2-3 days/week for 16 weeks) or the combination of diet and exercise intervention, were equally effective in improving menstrual pattern (shifting from oligo-/amenorrhea to a regular menstrual cycle) in women with PCOS. However, women in the exercise only group also reduced their daily calorie intake by ~270 kcal, which resulted in a 3% weight loss, thereby making it difficult to conclude that exercise alone can improve the menstrual pattern in women with PCOS. Furthermore, this study was a randomized, non-controlled trial as noted previously.

Improved menstrual cycle length (48 days to 27 days) following eight weeks of combined aerobic continuous exercise training and resistance training was reported in normal weight women with PCOS (BMI<25 kg/m^2) (34). However, findings from this RCT are limited by several factors in the research design; 1) the exercise intervention only lasted eight weeks (arguably not long enough to determine the exercise-induced effects on menstrual cycle length), 2) the exercise protocol was not described in detail e.g. the aerobic exercise dose depended on the exercise tolerance of the patient and the prescribed intensity was vague (determined by ratings of perceived exertion of 10-15 on the Borg 6-20 Scale), 3) participants were limited to normal-weight women with PCOS, so the findings may not apply to women with PCOS and overweight/obesity.

Well-designed, long-term RCTs investigating the effects of exercise alone on reproductive function in women with PCOS are lacking. Furthermore, data on exercise type, intensity and duration of exercise for improved reproductive function and fertility in women with PCOS are lacking, making recommendations for exercise in this population unclear.

2.0 Gaps in the literature

There are few well-designed, long-term RCTs on the effect of exercise training on reproductive and metabolic outcomes in women with PCOS. The published studies to date are limited by inconsistent reproductive-outcome definitions, small sample sizes, short intervention periods, interventions combining diet and exercise, the lack of an actual control group, and the inclusion of participants taking metformin, oral contraceptives and other medications. Combined, these factors make it difficult to interpret the isolated effect of exercise on reproductive and metabolic health in PCOS. Furthermore, no study to date has compared the effects of different HIT protocols on reproductive and cardiometabolic outcomes in women with PCOS.

We have little knowledge as to whether exercise protocols implemented in clinical trials under controlled laboratory conditions, typically comprising 8-12 weeks of supervised exercise training, are realistic and could be successfully performed in a 'real-world' setting (efficacy versus effectiveness and ecological validity). Long-term adherence to exercise training is necessary to obtain its benefits and we have no knowledge of which exercise training protocol induces the best long-term adherence when the women are no longer supervised.

Despite decades of research, the etiology of PCOS remains largely unclear. Currently, PCOS is believed to be caused by a combination of genetic and environmental factors leading to hyperandrogenism and hyperinsulinemia. The mechanisms underlying the insulin resistance are unclear, but are potentially linked to aberrant adipose tissue function and morphology. There is little prior knowledge about gluteal adipose tissue cell size, mitochondrial respiration or miRNA profiling in women with PCOS. Furthermore, the effect of HIT on abdominal and gluteal adipose tissue morphology, mitochondrial respiration and miRNA expression in women with PCOS is still lacking in the literature.

3.0 Aim of the thesis

The overall aim of the experiments conducted for this thesis was to explore the effects of two different HIT protocols on reproductive, cardiometabolic and quality of life outcomes in women with PCOS and explore whether aberrant adipose tissue plays a role in the pathophysiology of PCOS.

3.1 Aims of the studies:

3.1.1 Paper I:

The aim of this paper was to provide details of the study protocol and methods for the IMproving Reproductive function in women with Polycystic OVary syndrome with high-intensity Interval Training (IMPROV-IT) trial.

3.1.2 Paper II:

The primary aim of the first study was to investigate whether 16 weeks of semi-supervised HIT, followed by a subsequent 36 weeks of home-based HIT could increase menstrual frequency in women with PCOS during a 1-year period. Secondary aims were to examine the effects of HIT on cardiorespiratory fitness, rates of pregnancies, whole-body insulin sensitivity, and quality of life.

3.1.3 Paper III:

The primary aim of the second study described in Paper III was to compare the expression of selected miRNAs in the circulation, gluteal and abdominal adipose tissue taken from women with PCOS to an age- and BMI-matched control group of women without PCOS. Secondary aims were to investigate the effects of two chronic HIT protocols on the "expression signature" of these miRNAs in the circulation and in gluteal and abdominal adipose tissue in women with PCOS.

3.1.4 Paper IV:

The primary aims of the third study were to compare rates of whole-body fat oxidation during submaximal exercise, fat cell size and rates of mitochondrial respiration in adipose

tissue taken from women with and without PCOS. In addition, the response to 16 weeks of HIT were assessed.

4.0 Methods and methodological considerations

This thesis is based on two studies; 1) a two-center RCT including women with PCOS (the IMPROV-trial) and 2) a randomized, non-controlled trial performed in women without PCOS (the HITFAT trial). In the following chapter, the study design and methods used in the two trials are described together with some methodological considerations.

4.1 Study design

The IMPROV-IT trial was a two-center RCT with three parallel groups. **Figure 8** shows an overview of the study protocol. Women with PCOS (according to the Rotterdam criteria) were included in the study and stratified for BMI $<$ or $\geq$ 27 kg/m^2 and study center, and allocated in a 1:1:1 manner to: 1) Low-volume HIT (LV-HIT), 2) High-volume HIT (HV-HIT) or 3) a non-exercising control group (Non-Ex). The study was conducted at the Department of Circulation and Medical Imaging at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway from June 2015 to March 2020, and at the Mary MacKillop Institute for Health Research at the Australian Catholic University (ACU) in Melbourne, VIC, Australia from March 2018 to April 2019. The participants were assessed at baseline, after 16 weeks and 12 months after baseline.

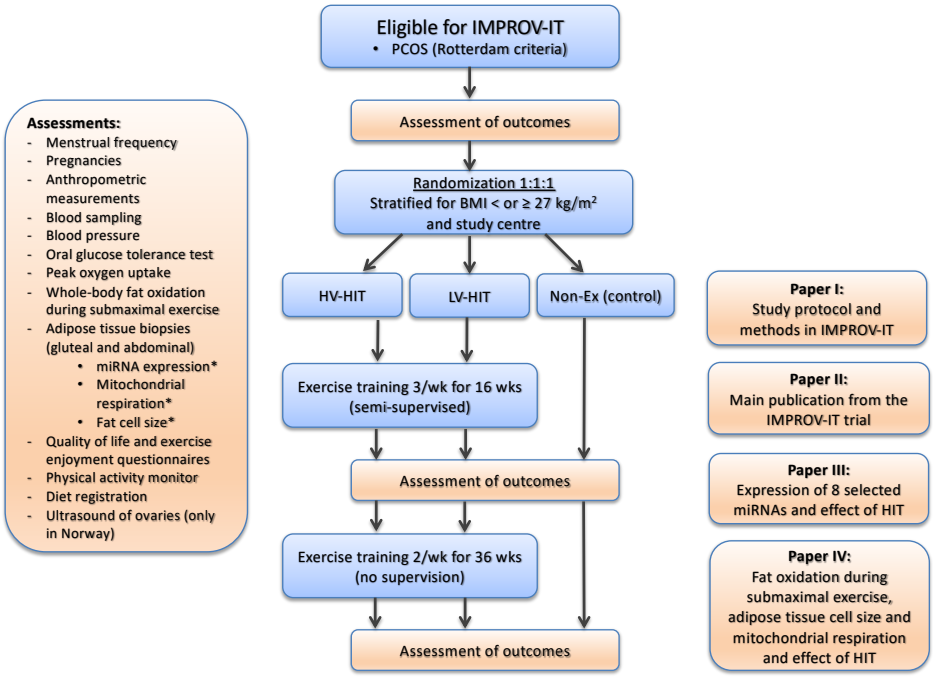

*Figure 8. An overview of the IMPROV-IT trial, assessments of outcomes and papers included in the thesis. Women with PCOS according to the Rotterdam criteria were included in the trial. Assessments were conducted at baseline (before randomization), after 16 weeks of intervention and after 12 months from baseline. The assessments marked with * were only measured at baseline and after 16 weeks of HIT. Exercise training was semi-supervised the first 16 weeks of the intervention, and the remaining 36 weeks were non-supervised. Paper I included the study protocol and methods for the IMPROV-IT trial, and data from the IMPROV-IT trial were included in Paper II, III and IV in this thesis.*

The HITFAT trial was a randomized, non-controlled trial with two parallel groups. **Figure 9** shows an overview of the trial in which women without PCOS were individually age- and BMI-matched with women in the IMPROV-IT trial. Women with less than five years of age difference and a BMI difference of maximum 2 kg/m^2 compared to a participant in the IMPROV-IT trial were included. After inclusion and baseline testing, women without PCOS were stratified for BMI < or ≥ 27 kg/m^2 and allocated 1:1 to: 1) Low-volume HIT (LV-HIT) or 2) High-volume HIT (HV-HIT). The study was conducted at the Department of Circulation and Medical Imaging at NTNU in Trondheim, Norway

from October 2016 to July 2019. Assessments were conducted at baseline and after 16 weeks.

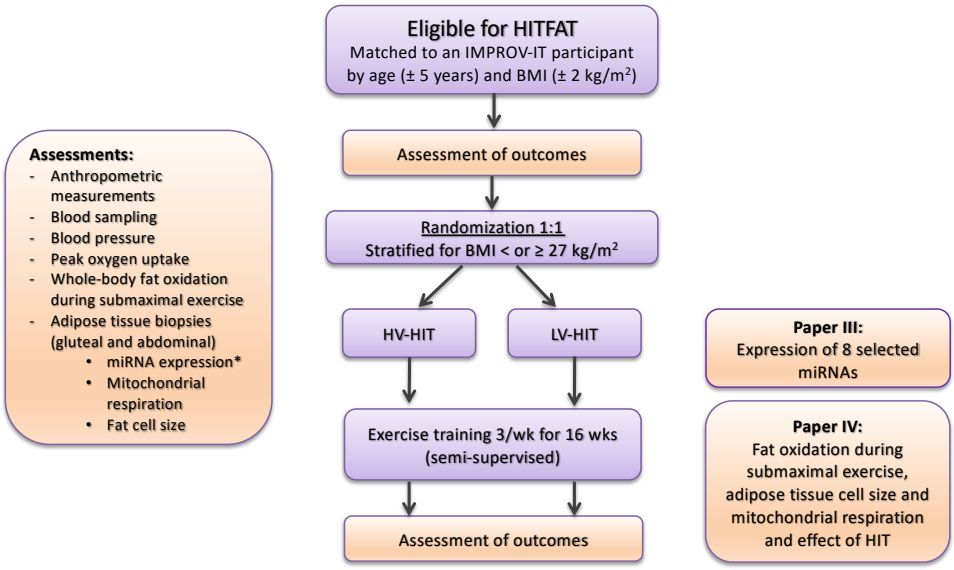

*Figure 9. An overview of the HITFAT trial and the included assessments. Women without PCOS were individually age- and BMI-matched with women with PCOS included in the IMPROV-IT trial. Assessments were conducted at baseline (before randomization) and after 16 weeks of HIT intervention. Assessments marked with * were only measured at baseline. Data from the HITFAT trial were included in paper III and IV in this thesis.*

A combination of randomized trials and case-control studies were used to serve as complimentary forms of research for the scope of this thesis. RCTs are considered to be the gold standard design to assess cause-effect relationship and outcomes of a treatment, and provide precise measures of efficacy of an intervention under ideal conditions, with a low risk of bias (130). The aim of randomization is to ensure that any risk factors of participant's that could affect the investigated outcomes are balanced between the intervention-arms (groups) to make sure that any observed differences in outcomes between groups are effects of the intervention (130). Observational studies, such as case-control studies, are in particular appropriate for studying rare outcomes and diseases, as case-control studies start with participants known to have the outcome/disease (130, 131).

Another advantage of case-control studies are that they are essential in advancing the understanding the pathophysiology of a disease such as PCOS. To this end, women without PCOS were included to perform a case-control comparison between women with and without PCOS, to identify whether miRNAs, fat oxidation rates during submaximal exercise, mitochondrial respiration and/or fat cell sizes differed in women with and without PCOS. The overall aim of these comparisons was to assess whether these outcomes may play a role in PCOS pathophysiology. The randomized design of the IMPROV-IT trial and the HITFAT trial allowed us to also investigate the effect of LV- and HV-HIT on reproductive and metabolic outcomes in women with and without PCOS.

The IMPROV-IT trial was a two-center RCT including women with PCOS in Trondheim, Norway and Melbourne, Australia. Some of the advantages of running multi- versus singlecenter studies are faster rates of participant recruitment and the improved generalizability (external validity) (132). Thereby, the multicenter nature of the IMPROV-IT study including women with PCOS from different parts of the world provides unique scope to the investigation of PCOS. There are also disadvantages of running multicenter rather than singlecenter studies, and one of them is that it can be difficult to align all methods used for assessments. In the IMPROV-IT trial, body composition was estimated using two different methods; bioelectrical impedance analysis (InBody720, Biospace CO, Korea) was used in Norway, and Dual-energy X-ray absorptiometry (DXA; GE Lunar iDXA Pro, Encore software version 16, General Electric, Boston, MA, USA) in Australia. For further discussion on the use of these two methods to estimate body composition, please see the Assessments paragraph.

4.2 Study population

In Norway, participants for the IMPROV-IT and HITFAT trials were recruited through public announcement at the university and hospital's homepages, at local shops and public places, and on social media. In Australia, participants were recruited for the IMPROV-IT trial via social media.

To be eligible for inclusion, the women had to be between 18 and 45 years old. In the IMPROV-IT trial, women were diagnosed with PCOS according to the Rotterdam criteria (49), with at least two of the following three features present: polycystic ovary

morphology (≥12 follicles (2-9 mm) per ovary and/or an ovarian volume of ≥10 mL), hyperandrogenism (either clinical signs such as acne or hirsutism, or biomedical) and/or oligo/amenorrhea. In the HITFAT trial, the PCOS diagnosis was ruled out in all the women as they were normally menstruating, with no evidence of hyperandrogenism or polycystic ovaries. Women in both trials were excluded if they were undertaking regular endurance training with an intensity that induce heavy breathing ≥ 2 sessions/week, had any cardiovascular diseases or endocrine disorders, were pregnant, had been breastfeeding within the last 24 weeks, or if they were using hormonal contraceptives, insulin sensitizers or drugs known to affect gonadotropin or ovulation (with a washout period of 3 months prior to inclusion).

Women in all BMI-categories were included to have a representative population of women and to increase participant enrollment. In both trials, women were randomized for BMI < or ≥ 27 kg/m^2 to ensure more homogenous intervention groups and to avoid that any observed differences in outcomes between groups were due to baseline differences in BMI. Similarly, women without PCOS were BMI-matched with women in the IMPROV-IT trial (±2 kg/m^2) to rule out the effect of BMI on potential differences in the assessed outcomes.

4.3 Interventions

In the IMPROV-IT trial, the two HIT protocols consisted of three weekly exercise sessions during the first 16 weeks, and for the remaining 36 weeks of the intervention period the participants were instructed to perform at least two weekly HIT sessions according to the HIT protocol they were allocated to. The exercise sessions were semi-supervised during the first 16 weeks of the intervention, where participants attended at least one weekly supervised training session at the study centers, with the opportunity to perform the remaining two exercise sessions under supervision or unsupervised. No supervision was provided during the remaining 36 weeks (from 16 weeks to 12 months). The two HIT protocols in the HITFAT trial consisted of three exercise sessions per week during the 16 weeks intervention period, and similar to the IMPROV-IT trial, participants attended supervised exercise at least once weekly at the study center, while the two

remaining exercise sessions were performed either supervised or unsupervised based on the participant's preference.

For both the IMPROV-IT and the HITFAT trials, the participants walked or ran on treadmills during the supervised exercise sessions (**Figure 10**), while they could choose to exercise on treadmills or outdoors during the unsupervised exercise sessions. The HR_{max} recorded during the VO_2peak test was used to estimate exercise intensity (133), and the workload was adjusted during the intervention period to account for improvements in cardiorespiratory fitness. The LV-HIT protocol started with a 10 min warm-up at low-to-moderate intensity (60-70% of VO_2peak), followed by 10 x 1 min work-bouts at the maximal intensity the participants were able to sustain. The work-bouts were separated by 1 min of passive recovery or low-intensity walking, and the exercise sessions were completed after a 3-min cool-down, which resulted in a total exercise time of 32 min. The HV-HIT protocol consisted of a 10 min warm-up at low-to-moderate intensity (60-70% of VO_2peak), followed by 4 x 4 min work-bouts at 90-95% of HR_{max}. The work-bouts were interspersed by 3 min recovery at low-to-moderate intensity (60-70% of HR_{max}), and the exercise sessions were completed after a 3-min cool-down, which resulted in a total exercise time of 38 min.

Figure 10. The supervised training sessions were performed as treadmill walking or running.

All participants wore heart rate monitors (Polar M400) during all training sessions, and registered these using an online exercise diary (www.polar.flow.com). The researchers had access to records of the exercise sessions through this online tool and were able to supervise adherence to the protocols.

The semi-supervised nature of the IMPROV-IT and HITFAT trials meant that some participants performed all exercise sessions supervised, while others only attended supervised exercise sessions once weekly. Less than ten women in the IMPROV-IT study and less than five women in the HITFAT trial chose to perform all exercise sessions at the study centers. A semi-supervised exercise program was chosen with the intention to increase practicality for the participant and make it easier to implement the exercise in their everyday life. Another reason for choosing the semi-supervised approach was to investigate the real-world efficacy of the HIT protocols, which have previously been studied mainly under strict supervision in a laboratory setting. Since our main research question in IMPROV-IT required a long follow-up period, we chose programs that we believed would promote long-term sustainability of exercise behaviors. Walking or running in the HIT protocols were chosen because these types of exercise require minimum equipment and are easy to perform unsupervised. Additionally, it is easier to reach the prescribed exercise intensities by walking/running on an incline compared to cycling, as walking/running requires activation of a greater muscle mass. The only requirement during the exercise sessions was to follow the prescribed exercise intensities, which the participants were able to by using the heart rate monitors they received.

The women with PCOS allocated to the control group (Non-Ex) in the IMPROV-IT trial were asked to continue their daily physical activities and informed about the current recommendations for physical activity for achieving health benefits; be physically active for at least 150 min/week of moderate intensity or 75 min/week of vigorous intensity (or an equivalent combination of both). By informing the women in the Non-Ex group about the current recommendations for physical activity, some of the women may have been inspired to engage in a healthier lifestyle during the study period, and thereby reduce the chance of finding possible effects of the HIT interventions on the measured reproductive and metabolic outcomes. The information was given for ethical reasons. We assessed physical activity using physical activity monitors (Sensewear Armband, APC

Cardiovascular, UK) at all timepoints to control for physical activity. All participants were encouraged to maintain their habitual diet throughout the study period.

4.4 Assessments

Outcomes for the IMPROV-IT trial were assessed at baseline and after the 16-week intervention at both study centers. The same assessments were executed in Norway after 12 months, while only data on menstrual frequency and questionnaires were collected in Australia at 12 months. Outcomes for the HITFAT trial were assessed at baseline and after the 16-week intervention. The majority of assessments were identical in the IMPROV-IT and HITFAT trials apart from the reproductive outcomes, including menstrual frequency diaries, ovarian morphology and pregnancies, and the quality of life and enjoyment questionnaires that were only collected in the IMPROV-IT trial.

4.4.1 Reproductive outcomes

As noted above, the reproductive outcomes were only assessed in the IMPROV-IT trial and these are reported in Paper II, with a description of the methodology used also included in Paper I.

Menstrual frequency

The primary outcome in the IMPROV-IT trial was menstrual frequency, used as a proxy of ovulation. Participants completed a menstruation diary (either on paper or using an electronic application) to register the first day of their menstrual cycle, and the length (number of days) during the 12-month intervention period, and sent information to the study personnel after each menstrual cycle. The study personnel sent out reminders to fill out the diary on a regular basis to ensure accuracy and increase reliability with the self-reported menstrual frequency data. In addition to the self-reported menstrual frequency data, the participants also completed questionnaires about their menstrual pattern at each assessment timepoint (at baseline, and after 16 weeks and 12 months). We compared groups using the number of menstrual cycles per year, and the number of observed menstrual cycles divided by the number of expected menstrual cycle per year (with n=13

for the number of expected menstrual cycles per year, given a regular cycle length of 28 days).

We used menstrual frequency as a proxy of ovulation, which is a limitation as it is possible to ovulate without a later bleeding and to have a regular cycle despite ovulatory dysfunction (52). Due to practical reasons, we were not able to assess ovulation as that requires daily temperature measurements, frequent urine samples to assess LH or blood samples to assess progesterone concentrations as markers of ovulation. Menstrual bleedings have been used by others to assess possible improvements in reproduction in women with PCOS after an exercise intervention (29).

Ovarian morphology

Ovarian morphology was only assessed at the Norwegian study center, and all imaging and measurements were executed by an experienced gynecologist. The ultrasound assessments of the ovaries were performed using a multifrequency transvaginal transducer, and included measurements of ovarian volume with and without dominant follicles, and the number of follicles in each ovary.

The diagnostic criteria for polycystic ovaries were ≥12 follicles (2-9 mm) per ovary and/or an ovarian volume of ≥10 mL as the study commenced in 2015, before the criteria were revised to the presence of ≥20 follicles (2-9 mm) per ovary and/or an ovarian volume ≥10 mL when using ultrasound transducers with a frequency bandwidth of ≥8 MHz (52) (see 1.2.2 Diagnostic features of PCOS).

Fertility and pregnancies

Participants completed a questionnaire providing background information regarding fertility. The questionnaire included questions about number of children (natural or assisted fertilization), whether the participant was actively seeking pregnancy, if they had tried to become pregnant (if yes, for how long), and if they had previously experienced miscarriages or had abortions. We registered the number of pregnancies during the 12-month study period.

4.4.2 Physiological outcomes

Anthropometric measures and blood pressure

After an overnight fast (≥12 hours), body composition was estimated using InBody720 for the HITFAT trial, and either using Inbody720 or DXA in the IMPROV-IT trial. Waist and hip circumference were measured to the nearest 0.5 cm in duplicate using measuring tape, and waist-to-hip ratio was calculated by dividing waist circumference by hip circumference. BMI was calculated as weight in kg divided by height in meters squared; BMI = kg/m^2.

After participants had rested for 15 min, systolic and diastolic blood pressure was measured in triplicate on the left arm while participants were in a seated position. The three measurements were recorded with two min break in between each measurement and the mean of these three measurements was used in the analysis.

We had to use two different methods to estimate body composition in the IMPROV-IT trial as we were not able to align the assessment at the two study centers; we only had an InBody720 available in Norway and a DXA in Australia. We acknowledge that it was not ideal to use two different methods, and it is one of the difficulties that can occur in a multicenter study. However, both methods have been reported reliable to estimate body composition in individuals with healthy weight and obesity (134, 135), although, it has been reported that the Inbody 720 overestimates fat-free mass, and underestimates body fat percentage and fat mass compared to DXA (134). The same method was used to estimate body composition of each individual participant at all timepoints, which should minimize the risk of it affecting our results. While the same method for assessing body composition would have been optimal, we posit that the multi-center nature of our study from different parts of the world provides unique scope to the investigation of PCOS.

Cardiorespiratory fitness

Participants performed an incremental test to exhaustion on a treadmill to assess VO_2peak and HR_{max}. We used an individualized protocol with the goal of a total test time of 8-12 min for each participant. After 10 min warm-up at low-to-moderate intensity (individually adjusted), the treadmill speed or inclination was increased by 0.5-1.0 km/h

or 1-2% every 1-2 min until volitional exhaustion. VO_2peak was assessed via indirect calorimetry (Oxycon Pro, Jaeger, Germany in Norway and TrueOne 2400, ParvoMedics, USA in Australia), and the maximum heart rate attained during this test was used to calculate the intensity for the HIT sessions.

Berglund and colleagues (133) found that a standard cardiorespiratory fitness test (with a protocol similar to ours) can serve as a good estimate of HR_{max} compared to an actual HR_{max} test (15 min warm-up at 60-70% HR_{max} followed by 4 x 4 min bouts at 90-95% HR_{max} interspersed with 2 min at 60-70% HR_{max}. After the last 4-min work-bout, treadmill speed or inclination was increased by 0.5 km/h or 1-2% every 30 seconds until volitional exhaustion) in non-athlete individuals. Based on their findings, the HR_{max} recorded during the cardiorespiratory fitness test sufficed to calculate the HIT session intensities and thereby we could include one less assessment in the IMPROV-IT and HITFAT trials.

Rates of whole-body fat oxidation during submaximal exercise

After an overnight fast (≥12 hours), participants performed a submaximal exercise test on a treadmill. The protocol included 20 min warm-up at low-to-moderate intensity without any sampling of gas, followed by 20 minutes of walking/running that elicited a 'steady state' condition at approximately 60% of individual VO_2peak with sampling of expired gas. The participants recorded their dietary intake the day prior to baseline testing and were requested to repeat that diet on the day prior to the subsequent measurement at 16 weeks and 12 months follow-up to reduce any diet-induced effect of rates of substrate oxidation (136). Rates of whole-body fat oxidation (g/min) were calculated from five min of steady oxygen uptake during the last 10 min of the test, and using the following equation; $1.695 \times VO_2 - 1.701 \times VCO_2$ (where VO_2 is oxygen uptake and VCO_2 is expired carbon dioxide) (137).

Rates of fat oxidation were measured at ~60% of VO_2peak based on previous research. Achten and colleagues reported that the exercise intensity that elicits maximal rates of fat oxidation is ~64% of VO_2max in moderately trained men, and that the range of intensities where fax oxidation rates are within 10% of the maximal rate, were detected between 55 and 72% of VO_2max (138). Apart from exercise intensity, rates of fat oxidation during exercise can be affected by several factors such as gender, training status

and exercise training (137); maximal fat oxidation appears to be elicited at lower exercise intensities in untrained individuals compared to trained individuals (137), maximal fat oxidation occurs at higher exercise intensities for females compared to men (139), and exercise training has the ability to modify fat oxidation rates. Previous studies have investigated the effects of HIT protocols (10 x 4 min bouts at 90% of VO_2peak separated by 2 min of rest for seven sessions and three days/week for six weeks, respectively) on rates of whole-body fat oxidation during submaximal exercise at 60% of VO_2peak in healthy recreationally active women (81), and in untrained recreationally active men and women (82). Both studies showed that HIT improved whole-body fat oxidation during submaximal exercise at 60% of VO_2peak.

Blood biochemistry and insulin sensitivity

Resting blood samples were obtained after an overnight fast (≥12 hours), followed by a 2-hour oral glucose tolerance test (OGTT) in which the participants consumed 75 g of glucose diluted in 250 mL water. Blood samples were collected every 30 mins during the OGTT at 0 (fasting prior to OGTT), 30, 60, 90 and 120 min for the determination of glucose and insulin concentrations. Plasma glucose concentrations were analyzed using a Roche Moduclar P (Roche, Switzerland) and serum insulin concentration was measured in duplicate using an enzyme-linked immunosorbent assay (ELISA, IBL-International, Germany). HOMA-IR was used to estimate insulin sensitivity and calculated as; fasting serum insulin (μIU/mL) x fasting plasma glucose (mmol/L) divided by 22.5 (140). To evaluate glycemic control, we calculated the total area under the curve (AUC) using a baseline of 0 mmol/L, and incremental area under the curve (iAUC) using fasting concentration as baseline value. AUC and iAUC were calculated for glucose and insulin concentrations using the trapezoidal method (141).

Fasting blood samples were also analyzed for lipid concentrations including total cholesterol, HDL, LDL and triglycerides. In the IMPROV-IT trial, fasting blood samples were also used to analyze hormone concentrations including androstenedione, 17OH-progesterone (at baseline only), testosterone, albumin, Anti-Müllerian hormone (AMH) and SHBG. FAI was calculated as 100 x (total testosterone/SHBG). The equipment used for the blood analyzes are described in Paper II.

Insulin sensitivity can be assessed using a range of methods, and the gold standard method is the euglycemic-hyperinsulinaemic clamp (142). This method directly measures whole body glucose disposal at a given level of insulinaemia under steady-state conditions (143). However, the clamp method is not typically used in clinical practice or in larger research studies as it is impractical, time- and labor-consuming and more expensive than methods such as the OGTT and indexes that are based on fasting insulin and glucose levels such as HOMA-IR (143). The clamp method also demands qualified medical personnel, and for these reasons we used the OGTT and HOMA-IR to evaluate insulin sensitivity and glycemic response.

Physical activity monitoring, diet recording and physical activity enjoyment scale

Participants wore a physical activity monitor (Sensewear Armband, APC Cardiovascular, UK) for five days, and recorded their dietary intake for four days at each timepoint. We wanted to investigate the isolated effect of the two HIT protocols, and these assessments were undertaken to control for possible changes in the participants' physical activity and diet behaviour.

The Physical Activity Enjoyment Scale (PACES) was introduced in August 2016 in the IMPROV-IT study, and therefore completed by a subset of women (the last 40 participants who were allocated to one of the HIT groups). PACES was completed immediately after one of the weekly supervised exercise sessions and used to assess enjoyment with the two HIT protocols. The PACES contains 18 questions, and each question is rated on a 7-point scale (144). Enjoyment scores range from the minimal total score of 18 to the maximal total score of 126, where higher scores represent greater levels of enjoyment. The PACES is helpful tool to use to compare enjoyment in different exercise protocols and/or changes in enjoyment over time.

Adipose tissue biopsies

Abdominal and gluteal subcutaneous adipose tissue biopsies were obtaining using a 14-gauge needle under local anesthesia (1% Xylocaine) excising ~300-500 mg of tissue from each depot (**Figure 11A**). The adipose tissue was washed on gauze with saline, and capillaries and connective tissues were removed, and the tissue was allocated into

portions for different analyses (**Figure 11B**). Approximately 80 mg was allocated for immediate analysis of mitochondrial respiration using high-resolution respirometry (Oxygraph-2K, Oroboros, Innsbruck, Austria). ~200-300 mg tissue was snap-frozen in liquid nitrogen and stored at -80 °C for later analyses, including miRNA profiling, and the remaining tissue was immediately fixed in phosphate-buffered formalin for subsequent fat cell size analysis. Mitochondrial respiration, miRNA profiling and fat cell size analyses were performed on a subset of women in the IMPROV-IT and HITFAT trials, as we were not always able to obtain adipose tissue biopsies from the two depots and/or unable to excise enough adipose tissue for all analyses.

A) ***B)***

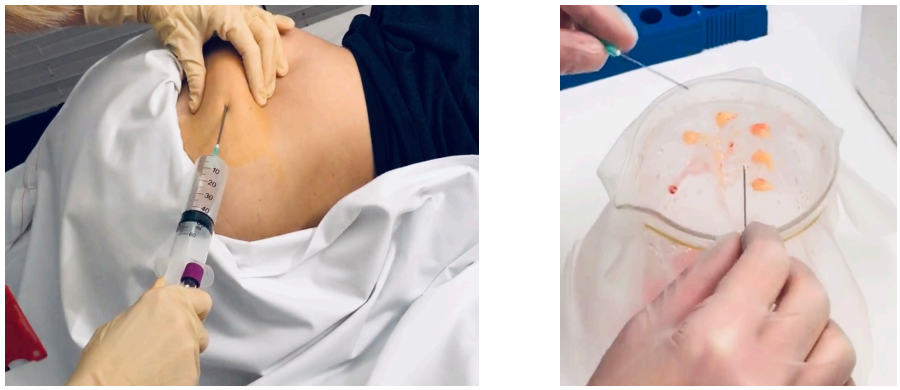

*Figure 11. Adipose tissue biopsies. **A)** depicts a subcutaneous adipose tissue biopsy taken from the gluteal area using a 14-gauge needle, hereafter **B)** the adipose tissue was washed on gauze with saline, and connective tissues and capillaries were removed from the sample before later analyses were performed.*

Adipose tissue was included in the IMPROV-IT and HITFAT trials, as adipose tissue may play a role in the pathophysiology of PCOS. Research on adipose tissue has evolved considerably over the past two decades, yet, most research is performed on abdominal adipose tissue, and no previous studies have investigated gluteal adipose tissue characteristics in PCOS. Adipose tissue in the gluteal region appears to have a more protective role when it comes to metabolic diseases such as insulin resistance and T2DM, whereas abdominally stored adipose tissues appears to be “dangerous” (86). Therefore,

we included gluteal and abdominal adipose tissue to obtain a greater understanding of adipose tissue's possible role in PCOS pathophysiology.

Mitochondrial respiration

Mitochondrial respiration was measured using high-resolution respirometry (Oxygraph-2K, Oroboros, Innsbruck, Austria) and a protocol modified from Kraunsoe et al. (99). **Figure 12** depicts a representative trace of the protocol from the Oxygraph-2k, and a complete description of the protocol is presented in Paper IV. In brief, several substrates and inhibitors were added to measure different states of the electron transfer chain.

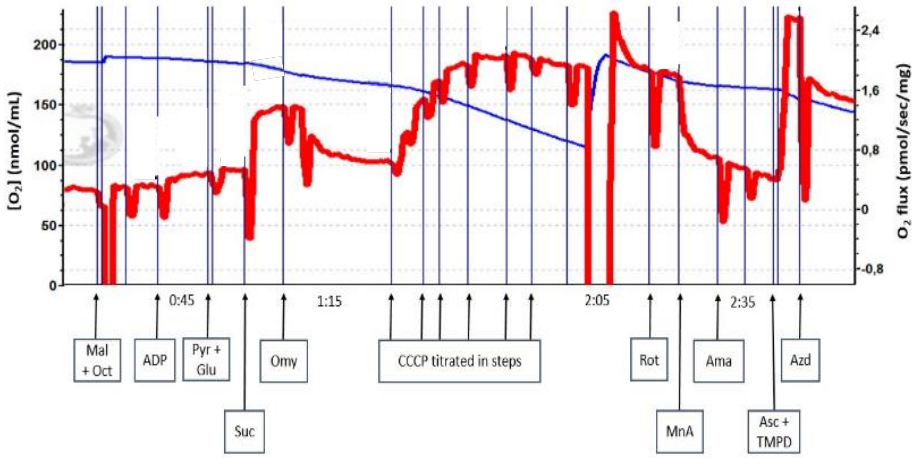

Figure 12. Representative trace of the protocol used to measure different states of the electron transfer chain using high-resolution respirometry. Left Y-axis (blue line): oxygen concentration in the chamber. Right Y-axis (red line): oxygen flux per mg tissue. X-axis: time in hours and minutes. Mal, Malate; Oct, Octanoyl carnitine; ADP, adenosine diphosphate; Pyr, Pyruvate; Glu, Glutamate; Suc, Succinate; Omy, Oligomycin; CCCP, Carbonyl cyanide m-chloro phenylhydrazone; Rot, Rotenone; MnA, Malonic Acid; Ama, Antimycin A; Asc, Ascorbate; TMPD, Tetramethyl-p-phenylenediamine dihydrochloride; Azd, Azide.

We wanted to test if adipose tissue mitochondrial respiratory was reduced in women with PCOS, and used high-resolution respirometry (Oxygraph-2K respirometer; Oroboros, Innsbruck, Austria) on permeabilized adipocytes in women with and without PCOS to characterize rates of mitochondrial respiration, as high-resolution respirometry allows for

detailed measurements of the complexes involved in the electron transport chain by sequential addition of substrates and inhibitors (98, 99). Other studies have also used this method to investigate the effect of an exercise protocol on mitochondrial respiration in abdominal subcutaneous adipose tissue from male and females (100-102).

Fat cell size

Immediately after excision, adipose tissue was fixed in phosphate-buffered formalin, embedded in paraffin and cut into sections of 4 μm, before the sections were mounted on glass slides and dried at 37^{0}C overnight in an incubator. The sections were stained with CD68 (Mouse monoclonal anti-CD68 (Dako, M0814)) in a Dako Autostainer (EnVision™ + Systems HRP (DAB) Mouse (Dako, K4007)) and digital images of the sections were captured with an EVOS FL Auto 2 Imaging System (ThermoFisher Scientific, USA) at x10 objective under guidance of an experienced user. The area of ≥200 adipocytes were analyzed using Fiji (145), and investigators were blinded during the analysis.

miRNA

Eight selected miRNAs were measured from the circulation, and from subcutaneous gluteal and abdominal adipose tissue of a subset of women with and without PCOS using real-time polymerase chain reaction (RT-PCR). A complete description of the steps performed to measure miRNA expression is presented in Paper III. In brief, the following steps are performed in order to measure the expression of miRNAs and in the listed order; extraction, reverse transcription and quantification. We performed miRNA quantification on a Qiagen customized 96-well miScript miRNA PCR Array (339330, Qiagen, Australia) with miScript SYBR using a BioRad CFX96 (BioRad, Australia) following the manufacturer's instructions. The PCR array also contained an inter-plate calibrator, along with four internal control genes (housekeeping genes); RNU1A1, SNORD44, and UniSp3 and UniSp6 RNA Spike-in controls. Adipose tissue miRNA expression was normalized to the geometric mean of RNU1A1, SNORD44, and UniSp3 and UniSp6, while circulating miRNA expression was normalized to the geometric mean of RNU1A1, UniSp3 and UniSp6 (SNORD44 was not used as there was a low circulating expression).

After normalization, we used the $2^{\Delta\Delta CT}$ method of relative quantification to calculate the relative abundance of miRNAs in plasma and adipose tissue (146).

The eight miRNAs measured in the circulation were; hsa-miRNA-21-5p, hsa-miRNA-27b-5p, hsa-miRNA-93-5p, hsa-miRNA-146a-5p, hsa-miRNA-155-5p, hsa-miRNA-222-3p, hsa-miRNA-223-3p and hsa-miRNA-103a-3p. These miRNAs were selectively targeted because previous studies had shown these miRNAs to be altered in the circulation in women with PCOS and/or after exercise training (107, 114, 124, 147). Furthermore, these miRNAs have been reported to play a role in hormone secretion and metabolism, lipid and glucose metabolism, inflammation and adipogenesis (107, 114, 119, 148). As miRNAs can be transported in the circulation and taken up by tissues (105), we chose to measure the same eight miRNAs in abdominal and gluteal subcutaneous adipose tissue, and thereby explore any potential circulation and adipose tissue cross-talk.

The normalization strategy applied for miRNA quantification is vital for accurate gene expression profiling and reliable results, and we normalized to the geometric mean of multiple housekeeping genes rather than using single control normalization. Expression of a housekeeping gene should not vary in the tissues or cells that are investigated, or in response to an experimental intervention, although housekeeping gene expression can vary considerably (149). Therefore, using the geometric mean of several housekeeping genes for normalization has proven to be the best strategy that leads to more accurate gene expression profiling (149).

4.4.3 Quality of life

In the IMPROV-IT trial, quality of life was assessed using the Polycystic Ovary Syndrome Questionnaire (PCOSQ) developed by Cronin and colleagues (150). The PCOSQ was developed to examine PCOS-related dysfunction and is widely used in PCOS research today. It consists of 26 questions that are divided into five domains; Emotions, Body Hair, Weight, Infertility problems and Menstrual problems. Each question is rated on a 7-point scale were higher scores represent higher quality of life. The number of questions in each domain vary between 4-8 questions, thus to ensure that all 26 questions are weighted equally, the developers of the PCOSQ recommended that

results from each domain score should be divided by the number of questions in the that domain (150).

We used the PCOSQ to investigate whether HIT had the potential to improve quality of life in women with PCOS. Women with PCOS face challenges such as infertility, hirsutism, acne, obesity and insulin resistance, and these features likely result in the poor quality of life observed in PCOS (151). Effective treatments that could potentially reduce the burden of these features and improve self-perception and quality of life, would be of great importance for these women's well-being.

4.5 Ethics

The studies were performed according to the Helsinki declaration and approved by The Regional Committee for Medical and Health Research Ethics in Central Norway (REK-midt 2015/468 and 2016/545) and the ACU Human Research Ethics Committee (2017-260H). Additionally, both trials were registered at clinicaltrials.gov (NCT02419482 and NCT02943291). Participants were informed about the experiments and the potential risks verbally, and their written consent was obtained prior to study entry. The two trials were based on voluntary participation, and the participants were informed that they could withdraw from the trial at any time.

The women with PCOS and allocated to the Non-Ex (control) group were asked to continue their habitual daily physical activities and were not discouraged from performing physical activity on their own. The exercise sessions were stopped if the women felt uncomfortable or reported any pain. In case of soreness/minor injuries, the women performed the HIT protocols on a bike ergometer until they were ready to continue exercising on a treadmill.

The investigators were responsible for documentation of any adverse or serious adverse event, and participants were told to contact the investigators if they experienced any unusual symptoms during the study period. During the study period, all medical events were recorded in the Case Report Form and any serious adverse event in a Serious Adverse Events Report Form. All serious adverse events were to be reported to the principal investigator within 24 hours after the site/investigators had gained knowledge of the event.

4.6 Randomization and blinding

After baseline testing, participants in the IMPROV-IT trial were allocated in a 1:1:1 manner to either the control group (Non-Ex) or one of the two intervention groups; LV-HIT or HV-HIT. Participants in the HITFAT trial were allocated 1:1 to either LV-HIT or HV-HIT after baseline testing. Group allocation for the trials were performed by a random number generator, developed and administered at the Faculty of Medicine, Department of Public Health and General Practice, NTNU, Trondheim, Norway. In IMPROV-IT, the randomization sequence had block sizes of 15 and 6, where the first block size was 15 and the remaining block sizes were 6. In the HITFAT trial, the randomization sequence had block sizes of 4. Study personnel received the group allocation results by email after a new participant was registered. The randomization procedure used in the trials reduced the risk of selection bias, and eliminated the influence of unknown confounding factors by balancing risk factors that could affect the investigated outcomes between the groups, thus support the assumption of no systematic differences between the groups at baseline (130).

Randomized double blind placebo control studies are considered to be the gold-standard in intervention based studies such as clinical trials (152). However, due to the nature of our trial intervention (semi-supervised exercise training), participants and study personnel were not blinded to group allocation. It was not possible to blind study personnel as we did not have the capacity and the personnel who supervised the exercise sessions also performed most of the assessments. Some precautions were taken to minimize bias and to ensure high internal validity; all baseline assessments were performed prior to group allocation, analyses of several assessments were done blinded for group allocation (including ovarian morphology, blood biochemistry, diet and physical activity monitoring, mitochondrial respiration, fat cell sizes, miRNA profiling, and quality of life), and all statistical analyses were performed blinded for group allocation.

4.7 Power calculation and sample size

The sample size in the IMPROV-IT trial was calculated based on results reported in one pilot study (153) and one randomized trial (39) that investigated the effect of lifestyle

interventions on ovulation and menstrual frequency. Accordingly, the sample size was computed for a one-way analysis of variance test with three groups for the primary outcome (menstrual frequency). With a statistical power of 0.80, significance level of 0.05, and a standard deviation of two menstrual cycles during a 12-month period, it was computed that we needed 48 women to detect an increase of three menstrual cycles in the HIT groups. We added 15% to the sample size owing to the non-normality of menstrual frequency, and another 15% to allow for expected drop-out, which resulted in that 64 women were required to be included in the IMPROV-IT study.

A power calculation was not performed for the HITFAT trial. Originally, the primary outcome for the HITFAT trial was to compare mitochondrial respiration in adipose tissue of women with and without PCOS, and at the time the study commenced, there were no former studies in women with and without PCOS on this topic. There was one published paper on mitochondrial respiration in abdominal adipose tissue including 10 overweight but otherwise healthy men and women (100), which did not observe a change in adipose tissue mitochondrial respiration after 6 weeks of HIT. The aim was to include 30 women with PCOS, and 30 women without PCOS for the case-control comparison.

We experienced difficulties in recruiting 30 women with PCOS and 30 women without PCOS for the HITFAT trial due to multiple reasons. The speed of inclusion of women without PCOS in the HITFAT trial was low due to strict matching criteria between women with and without PCOS (BMI of ± 2 kg/m^2 and age of ± 5 years). Moreover, we were not always able to obtain adipose tissue biopsies from the two depots and/or unable to excise enough adipose tissue for the analyses (especially the abdominal adipose tissue).

4.8 Statistical analyses

The principal analyses used in the papers included in this thesis are based on 'intention to treat', where all primary and secondary outcomes were analyzed according to which intervention the participants were randomized to, regardless of adherence to the exercise protocol. Thereby, all available data from all timepoints were included in the analyses. Due to the randomization model in the IMPROV-IT and HITFAT trials, we assumed no

systemic differences between groups at baseline. Similar for Paper II, III and IV; all data were tested for normality and log-transformed when necessary to obtain normality, baseline data are presented as mean ± SD, and comparisons within- and between-groups are reported as estimated means with 95% confidence intervals, as number of participants (percentage), and as frequency ratio. All statistical analyses were carried out using SPSS version 25.0 (SPSS Inc., United States) and R version 2.13.1.

In Paper II, we used mixed Poisson regression to compare the number of menstrual cycles during the 12-months study period (primary outcome). Participant ID was set as random factor to account for repeated measurements, and the effect of group allocation and timepoint were set as fixed effects with these levels; Baseline, HV-HIT at 12 months, LV-HIT at 12 months and Non-Ex at 12 months. All the women who reported their menstrual cycles were included in the analysis, and the number of weeks they attended the study (in case of dropouts or pregnancies) was set as the exposure variable to account for difference in observation time. For the within-group analysis of menstrual frequency, we compared the menstrual cycles reported in questionnaires at baseline with the menstrual cycles reported during the 12-months study period. We used the Fisher's exact test to compare the number of pregnancies between groups. Linear mixed models were used to analyze the effect of the interventions for the remaining outcome variables with participant ID as random factor and the effect of timepoint and group allocation as fixed effects with the levels; Baseline, HV-HIT 16 weeks, LV-HIT 16 weeks, Non-Ex 16 weeks, HV-HIT 12 months, LV-HIT 12 months and Non-Ex 12 months. We considered p-values < 0.05 as statistically significant.

In Paper III, we compared group means for women with PCOS vs Non-PCOS women using Student's t-test for independent samples. We used linear mixed models with participant ID as random factor, and with two dummy variables to uniquely identify the two exercise groups (LV-HIT and HV-HIT) and their interactions with time as fixed factors. The outcome variables were adjusted for baseline values as recommended by Twisk and colleagues (154). Using this model, the coefficients for the interaction terms (time x group) give the estimated effects of exercise training in the HV-HIT and LV-HIT groups compared to the Non-Ex groups. We compared the miRNA expression between gluteal and abdominal adipose tissue at baseline using paired t-tests, and the effect of HIT on the two depots using linear mixed models as detailed but using the exercise group's

interaction with AT depots as fixed factors. Due to multiple testing, we considered *p*-values < 0.01 as statistically significant.

In Paper IV, we first compared all the reported outcomes between the LV-HIT and HV-HIT groups (for PCOS and Non-PCOS women, respectively) using linear mixed models with participant ID as random factor, and the effect of time and group allocation as fixed factors with the following levels; PCOS baseline, Non-PCOS baseline, PCOS Non-Ex 16 weeks, PCOS LV-HIT 16 weeks, PCOS HV-HIT 16 weeks, Non-PCOS LV-HIT 16 weeks and Non-PCOS HV-HIT 16 weeks. As there were no between-group differences between LV-HIT and HV-HIT groups, the groups were pooled to increase statistical power, which resulted in three groups post intervention (at 16 weeks); PCOS Non-Ex group, PCOS HIT group and Non-PCOS HIT group. After pooling the groups, we used linear mixed models as detailed but with these levels; PCOS baseline, Non-PCOS Baseline, PCOS Non-Ex 16 weeks, PCOS HIT 16 weeks and Non-PCOS HIT 16 weeks. The outcome variables were adjusted for baseline values as recommended by Twisk and colleagues (154). We compared group means for weekly exercise training sessions for PCOS HIT and Non-PCOS HIT using Student's t-test for independent samples.

5.0 Summary of Results

The results from the IMPROV-IT and HITFAT trials are described in detail in Paper II-IV included in my thesis. The following chapter will be a summary of the main results in Paper II-IV, some additional analyses on data from the IMPROV-IT trial, and completed with the new knowledge regarding PCOS and exercise that my thesis has contributed with.

5.1 Flow diagrams of the trials

5.1.1 The IMPROV-IT trial

We assessed 421 women for eligibility, and of these, 64 women with PCOS were included (~15% of the women assessed for eligibility), while 357 women were excluded due to not meeting the inclusion criteria, declined to participate, or for other reasons (**Figure 13**). The 64 women were randomly allocated to either HV-HIT (n=20), LV-HIT (n=21) or a non-exercising control group (Non-Ex; n=23).

Three women from Non-Ex and three women from LV-HIT did not report their menstrual cycle to the study personnel, and their results were not included in the primary outcome analysis. Fourteen women withdrew from all assessments on secondary outcomes, and women included in Australia (n=20) were not re-tested after 12 months (we only collected data on menstrual frequency and from questionnaires in Australia at 12 months, as previously described). At baseline, the mean age for all the 64 women was 30 ± 5, mean BMI was 30.5 ± 6.5 kg/m^2, and mean VO_2peak was 33.1 ± 7.2 mL/min/kg.

We recorded one adverse event in the IMPROV-IT trial related to the trial intervention; one participant experienced a local inflammation around the insertion site after an abdominal adipose tissue biopsy. This event was reported to the principal investigator within 24 hours and the participant was treated by a medical doctor, with all expenses covered. Study personnel followed up on the participant afterwards and the participant has no after-effects. There were no serious adverse events in the IMPROV-IT trial.

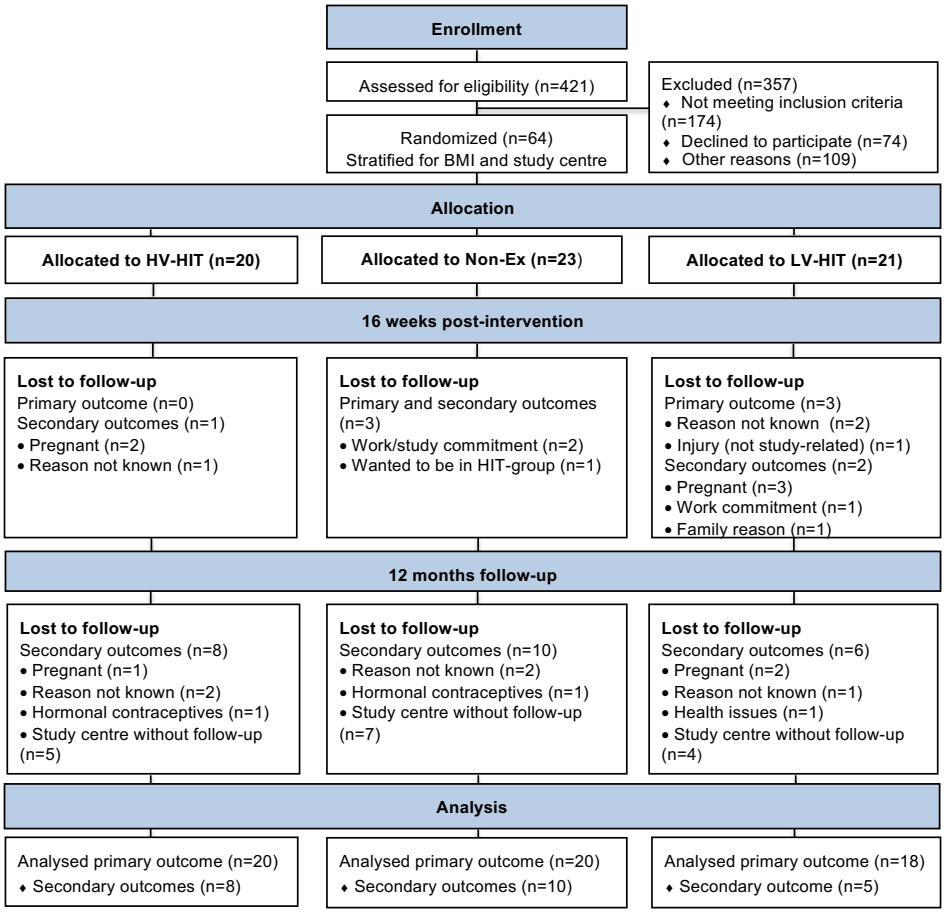

Figure 13. Flow diagram of the IMPROV-IT trial (Consort 2010 Flow Diagram).

5.1.2 The HITFAT trial

In the HITFAT trial, we assessed 64 women for eligibility, and of these, 15 women without PCOS who were age- and BMI-matched with 15 women from the IMPROV-IT trial were included (**Figure 14**). The number of included women corresponds to ~23% of those assessed for eligibility. Forty-nine women were excluded because they did not meet the inclusion criteria (most often because they could not be matched to one of the IMPROV-IT participants) or declined to participate. After randomization, eight women were allocated to 16 weeks of HV-HIT and seven women to LV-HIT. One woman in the LV-HIT group dropped out before study completion due to personal reasons. At baseline,

the mean age for all the 15 women was 31 ± 6 years, mean BMI was 28.4 ± 5.6 kg/m^2, and mean VO_2peak was 36.0 ± 6.8 mL/min/kg. There were no adverse or serious adverse events in the HITFAT trial.

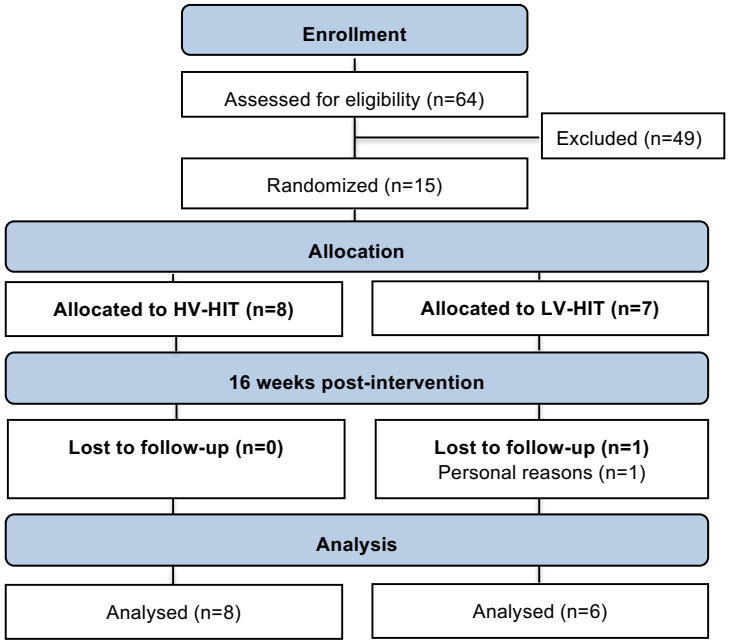

Figure 14. Flow diagram of the HITFAT trial (Consort 2010 Flow Diagram).

5.2 Paper II: Effect of HIT on menstrual frequency during 1-year follow up

5.2.1 Main results

The primary aim of the IMPROV-IT study was to investigate whether 16 weeks of semi-supervised HIT, followed by 36 weeks of home-based HIT, could increase menstrual frequency in women with PCOS during a 1-year period. We found no difference in menstrual frequency at 12 months between the groups; LV-HIT vs Non-Ex (frequency ratio: 1.02, 95% CI 0.73 to 1.42), HV-HIT vs Non-Ex (frequency ratio: 0.93, 95% CI 0.67 to 1.29), or LV-HIT vs HV-HIT (frequency ratio: 1.09, 95% CI 0.77 to 1.56). At baseline, the mean self-reported menstrual frequency was 6.4 menstruations/year (95% CI 5.5 to 7.5) and at 12 months follow-up, the menstrual frequency was 8.7 (95% CI 6.8 to 11.1)

for the Non-Ex group, 8.9 (95% CI 6.8 to 11.6) for the LV-HIT group and 8.1 (95% CI 6.3 to 10.5) for the HV-HIT group. There was a within-group increase in menstrual frequency in all three groups.

Among secondary outcome assessments, more women became pregnant in the LV-HIT group compared to the Non-Ex group (5 versus 0, $p = .02$). Three women in the HV-HIT group fell pregnant, although not statistically significant compared to the Non-Ex group ($p = .23$). Cardiorespiratory fitness only increased in the HV-HIT group after 16 weeks ($p = .015$), with no-between group differences in cardiorespiratory fitness at 16 weeks or 12 months follow-up. We observed a decrease in enjoyment during the first 16 weeks by ~14% in women allocated to HV-HIT ($p < 0.001$) and ~5% in women allocated to LV-HIT (not statistically significant, $p = 0.12$). Furthermore, quality of life improved in two out of five domains that are PCOS-related (infertility problems and body hair) in the HV-HIT group compared to the control group at 12 months follow-up.

5.2.2 Adherence to the exercise protocols

Eleven (52%) women in the LV-HIT group and 16 (80%) women in the HV-HIT group completed the first 16 weeks of the intervention period with an average of 2 sessions per week for both groups (2.2 ± 0.7 sessions/week for LV-HIT and 2.0 ± 0.7 sessions/week for HV-HIT). When excluding the women who became pregnant during the first 16 weeks (n=3 in LV-HIT and n=2 in HV-HIT), 61% of the women in LV-HIT and 89% of the women in HV-HIT completed the 16 weeks intervention period. At 12 months follow-up, nine (43%) women in the LV-HIT group and seven (35%) women in the HV-HIT group reported ≥ 1 exercise session/week. The LV-HIT sessions were completed with a mean intensity of 88% of HR_{max} (the average of the last 30 s of each work bout), and the HV-HIT sessions were completed with a mean intensity of 90% of HR_{max} (the average of the last 2 min of each work bout).

In Paper I, we pre-specified a per protocol analysis for the 12 months follow-up measurements in the IMPROV-IT study with the following criteria; >75% completion of the scheduled exercise sessions during the first 16 weeks followed by at a minimum of one weekly exercise session in the remaining 36 weeks until 12 months follow-up. Only four women in the LV-HIT group, and two women in the HV-HIT group fulfilled these

criteria (~15% of the 41 women allocated to the HIT groups). Due to the low adherence, we were unable to complete a meaningful per protocol analysis.

5.3 Paper III: Circulating and adipose tissue miRNA expression and effect of HIT

We found a 1.8-fold higher basal expression of circulating miRNA-27b in women with PCOS compared to age- and BMI-matched women without PCOS (p = .006). No differences were observed in circulating miRNA-21, - 93, - 103a, -146a, -155, -222 or -223 between women with and without PCOS. We do not report any differences in the eight selected miRNAs in subcutaneous abdominal or gluteal adipose tissue of women with and without PCOS.

Sixteen weeks of LV-HIT induced a 0.5-fold decrease in the expression of circulating miRNA-27b in women with PCOS (p = .007), with no changes observed in the remaining seven miRNAs. None of the eight miRNAs changed in the circulation after 16 weeks of HV-HIT, nor did we observe any changes in the eight selected miRNAs in subcutaneous abdominal or gluteal adipose tissue of women with PCOS after 16 weeks of either LV-HIT or HV-HIT.

5.4 Paper IV: Rates of whole-body fat oxidation during submaximal exercise + adipose tissue mitochondrial respiration and cell size and response to HIT

At baseline, women without PCOS (n=64) had higher rates of whole-body fat oxidation during submaximal exercise at 60% of VO_2peak (p = .008 for g/min, p = .006 for mg/FFM/min) compared to women with PCOS. We did not observe a difference in whole-body fat oxidation rates during submaximal exercise between the women with and without PCOS who were individually matched (n=15 for each group). At baseline, women with PCOS had lower mitochondrial respiration through complex I + II in subcutaneous abdominal adipose tissue compared to women without PCOS (p = .015), while no difference was observed in mitochondrial respiration through complex I + II in subcutaneous gluteal adipose tissue. No differences were found between women with and without PCOS in metabolic outcomes, or subcutaneous abdominal and gluteal adipose tissue cell size at baseline.

Cardiorespiratory fitness increased to a similar degree in women with (~4%, p = 0.006) and without PCOS (~6%, p = 0.003) after 16 weeks of HIT. Interestingly, rates of whole-body fat oxidation during submaximal exercise only improved after 16 weeks of HIT in women without, but not with PCOS (~16% improvement in g/min (p = .026) for women without PCOS, and ~17% in mg/FFM/min (p = .023)). We observed no changes in cell size or mitochondrial respiration in subcutaneous abdominal and gluteal adipose tissue after HIT in either group of women.

Women with PCOS allocated to the HIT groups completed 2.2 ± 0.5 exercise sessions/week while women without PCOS completed 2.6 ± 0.4 weekly sessions (p = .025), which is equivalent to ~6 exercise sessions less in total over the 16 weeks.

5.5 Additional analyses from the IMPROV-IT study

In these additional analyses from the IMPROV-IT study, I first compared reproductive and metabolic outcomes between the women included in Norway and those included in Australia, and secondly compared the same outcomes in these women based on BMI-category (women with BMI < 27 kg/m^2 and women with BMI ≥ 27 kg/ m^2). These analyses were performed as the stratification factors used in the IMPROV-IT study were study center and BMI < or ≥ 27 kg/m^2, and included to ensure equal allocation of subgroups of participants to each intervention arm and thereby homogenous groups.

5.5.1 Norwegian versus Australian women

Table 4 shows baseline characteristics for the women with PCOS included in Norway (n=44) and in Australia (n=20). The Norwegian and Australian women included in the IMPROV-IT trial were fairly homogenous in metabolic and reproductive outcomes. The Norwegian women were on average 2.6 years older (p = .04) than the Australian women, and the Norwegian women had higher total cholesterol (p = .01), LDL-cholesterol (p = .002), and absolute, but not relative, VO_2peak (p = .02) compared to the Australian women. The Norwegian women also had higher FFM (p = .02) compared to Australian, however, FFM was estimated using two different methods in Norway (bioelectrical impedance analysis) and Australia (DXA), which complicates the comparison of this outcome.

Table 4. Baseline characteristics of Norwegian versus Australian women with polycystic ovary syndrome included in the IMPROV-IT trial.

	Norwegian women (n = 44)	**Australian women** (n = 20)	***p-values***
	Mean ± SD	Mean ± SD	
Age (years)	30 ± 5	28 ± 4	**.04**
Body weight (kg)	86.1 ± 20.5	83.0 ± 17.8	.55
Body mass index (kg/m^2)	30.7 ± 6.9	30.2 ± 5.8	.74
Fat free mass (kg) *	52.0 ± 6.9	47.9 ± 5.8	**.02**
Body fat percentage (%) *	37.8 ± 9.0	42.2 ± 8.0	.06
Waist circumference (cm)	101 ± 18	100 ± 15	.90
Hip circumference (cm)	113 ± 14	112 ± 13	.78
Waist/Hip Ratio	0.90 ± 0.08	0.89 ± 0.09	.94
VO_2peak (mL/min/kg)	33.9 ± 7.5	31.3 ± 6.1	.16
VO_2peak (L/min)	2.8 ± 0.4	2.5 ± 0.4	**.02**
Total cholesterol (mmol/L)	4.5 ± 0.8	4.0 ± 0.6	**.01**
HDL (mmol/L)	1.4 ± 0.4	1.3 ± 0.3	.42
LDL (mmol/L)	2.9 ± 0.8	2.4 ± 0.5	**.002**
Triglycerides (mmol/L)	1.1 ± 0.6	1.0 ± 0.5	.41
Fasting glucose (mmol/L)	5.0 ± 0.5	4.8 ± 0.6	.28
Fasting insulin (pmol/L)	125 ± 101	98 ± 47	.16
HOMA-IR	4.9 ± 4.3	3.6 ± 2.0	.12
Glucose AUC (mmol/L x min)	872 ± 165	747 ± 243	.05
Glucose iAUC (mmol/L x min)	217 ± 125	134 ± 177	.07
Insulin AUC (pmol/L x min)	71303 ± 50531	66174 ± 34636	.65
Insulin iAUC (pmol/L x min)	46709 ± 34342	47001 ± 28677	.97
Menstrual frequency the last 12 months	6.5 ± 3.9	6.0 ± 4.9	.70
Ferriman-Gallwey score	8.1 ± 5.4	8.1 ± 3.8	.99

Statistics: outcome variables were analyzed by Independent samples t-test. Statistically significant *p*-values ($p < .05$) are in bold.

* Fat free mass and body fat percentage were estimated with two different methods: bioelectrical impedance analysis in Norway and Dual X-ray Absorptiometry in Australia.

SD; Standard deviation; VO_2peak, peak oxygen uptake; HOMA-IR, Homeostatic model assessment of insulin resistance; AUC; Area under the curve; iAUC, incremental area under the curve.

5.5.2 BMI categories

Table 5 shows baseline characteristics for the women with PCOS and BMI < 27 kg/m^2 (n=23), and for the women with PCOS and BMI ≥ 27 kg/ m^2 (n=41). The women with BMI ≥ 27 kg/ m^2 were on average 1.6 years older than the women with BMI < 27 kg/m^2 (p = .02). Women with BMI < 27 kg/m^2 were in general healthier than women BMI ≥ 27 kg/ m^2, and had higher relative VO_2peak, and lower body weight, BMI, body fat percentage, waist and hip circumference, waist/hip ratio, HDL-cholesterol, triglycerides and fasting glucose and insulin concentrations (**Table 5**). Women with BMI ≥ 27 kg/ m^2 were more insulin resistant compared to women with BMI < 27 kg/m^2 based on HOMA-IR (p < .001), Glucose AUC (p = .005) and iAUC (p = .03), and insulin AUC (p < .001) and iAUC (p = .003).

Table 5. Baseline characteristics of women with polycystic ovary syndrome included in the IMPROV-IT trial, according to body mass index < or ≥ 27 kg/m^2

	Women with PCOS and BMI < 27 kg/m^2 (n = 23)	**Women with PCOS and BMI ≥ 27 kg/m^2 (n = 41)**	***p-values***
	Mean ± SD	Mean ± SD	
Age (years)	28 ± 4	30 ± 6	**.02**
Body weight (kg)	65.8 ± 7.3	96.0 ± 15.6	**<.001**
Body mass index (kg/m^2)	23.8 ± 2.2	34.3 ± 4.8	**<.001**
Fat free mass (kg) *	45.7 ± 4.3	53.5 ± 6.3	**<.001**
Body fat percentage (%) *	30.6 ± 7.1	44.0 ± 5.4	**<.001**
Waist circumference (cm)	83 ± 8	111 ± 12	**<.001**
Hip circumference (cm)	100 ± 6	120 ± 11	**<.001**
Waist/Hip Ratio	0.83 ± 0.05	0.93 ± 0.08	**<.001**
VO_2peak (mL/min/kg)	39.8 ± 6.2	29.3 ± 4.4	**<.001**
VO_2peak (L/min)	2.6 ± 0.4	2.8 ± 0.4	.08
Total cholesterol (mmol/L)	4.2 ± 0.7	4.5 ± 0.8	.18
HDL (mmol/L)	1.5 ± 0.4	1.2 ± 0.3	**.003**
LDL (mmol/L)	2.5 ± 0.6	2.9 ± 0.8	.08
Triglycerides (mmol/L)	0.8 ± 0.3	1.2 ± 0.6	**.001**

Fasting glucose (mmol/L)	4.8 ± 0.5	5.1 ± 0.5	**.02**
Fasting insulin (pmol/L)	61 ± 28	148 ± 96	**<.001**
HOMA-IR	2.2 ± 1.1	5.7 ± 4.1	**<.001**
Glucose AUC (mmol/L x min)	744 ± 156	880 ± 205	**.005**
Glucose iAUC (mmol/L x min)	136 ± 127	220 ± 150	**.03**
Insulin AUC (pmol/L x min)	44852 ± 31383	83356 ± 47009	**<.001**
Insulin iAUC (pmol/L x min)	31391 ± 25396	55320 ± 32944	**.003**
Menstrual frequency the last 12 months	5.8 ± 3.6	6.6 ± 4.5	.41
Ferriman-Gallwey score	6.7 ± 4.1	8.8 ± 5.3	.09

Statistics: outcome variables were analyzed by Independent samples t-test. Statistically significant *p*-values are in bold ($p < .05$).
* Fat free mass and body fat percentage were estimated with two different methods: bioelectrical impedance analysis in Norway and Dual X-ray Absorptiometry in Australia. Body composition was estimated using Dual X-ray Absorptiometry in seven out of 23 (~30%) women with BMI < 27 kg/m^2 and in 13 out of 41 (~32%) women with BMI ≥ 27 kg/m^2.
SD; Standard deviation; VO_2peak, peak oxygen uptake; HOMA-IR, Homeostatic model assessment of insulin resistance; AUC; Area under the curve; iAUC, incremental area under the curve.

5.6 New knowledge

This thesis has contributed with the following new knowledge about PCOS and exercise:

- Semi-supervised HIT does not increase menstrual frequency compared to control after 12 months, but may still lead to increased chances of pregnancy.
- The expression of circulating miRNA-27b is increased in PCOS, and 16 weeks of LV-HIT appears to decrease and normalize the miRNA-27b expression.
- Women with PCOS experience absent exercise-induced improvements in rates of whole-body fat oxidation during submaximal exercise after 16 weeks of HIT.
- Mitochondrial respiration through complex I + II in subcutaneous abdominal adipose tissue is reduced in women with PCOS.

6.0 Discussion

Our findings provide novel data regarding the effects of two HIT protocols on reproductive, cardiometabolic and psychologic outcomes in women with PCOS. Furthermore, we investigated the effect of HIT on whole-body fat oxidation during submaximal exercise and on adipose tissue mitochondrial respiration, fat cell size, and miRNA expression in women with and without PCOS. The following paragraphs will be a discussion of the main findings followed by the strengths and limitations of the studies, clinical relevance and future perspectives.

6.1 Menstrual frequency

We found no between-group difference in menstrual frequency at 12 months but observed a significant increase in menstrual frequency from baseline to 12 months follow-up in all three groups including in the Non-Ex (control) group in the IMPROV-IT trial. Although HV-HIT and LV-HIT did not improve menstrual frequency compared to the Non-Ex group, pregnancy rates increased significantly in LV-HIT. It is difficult to explain the lack of improvement in menstrual frequency after LV-HIT compared to the Non-Ex group despite an increase in pregnancy rates in LV-HIT, and I can only speculate why this was the case.

The major reason for the lack of difference between groups in menstrual frequency is probably poor adherence to the HIT protocols. Only 37% of the participants in the HIT groups completed >75% of the exercise sessions during the first 16 weeks, while 39% reported at least one weekly exercise session at 12 months follow-up. Additionally, our findings indicate that the Non-Ex group most likely did introduce some lifestyle modifications during the intervention period. There was an increase in menstrual frequency of ~2 menstruations/year from baseline to 12 months follow-up in the Non-Ex group, along with improvements in other outcomes including reduced body weight and BMI, and improved blood lipid profiles and FAI. Participants in the Non-Ex group were encouraged to continue their diet and exercise habits throughout the study period. Although we did not observe a change in the 4-day diet recording or five days of physical activity monitoring at each timepoint, these measurements only gave a brief "snap-shot" of the whole study period. Based on the favorable improvements in several outcomes it

is likely that there was a change in behavior in the Non-Ex group. Participants who sign up for lifestyle intervention trials most often hope to be allocated to the intervention group. The participants were possibly particularly disappointed when they were allocated to the Non-Ex group in the IMPROV-IT trial as the chances were high for allocation to the intervention groups (67% chance) and relatively low for allocation to the Non-Ex group (33%). The participants may have been mentally prepared and motivated to introduce lifestyle changes, which could lead to difficulties to continue their previously sedentary lifestyle, and especially over the 12 months required in the IMPROV-IT trial. One solution to this challenge, could be to offer the exercise protocol to the participants allocated to the Non-Ex group at the end of the study period. We were, however, unable to provide such a delayed treatment due to the length and time-demand of the IMPROV-IT study.

Some of the women included in the IMPROV-IT trial had a regular menstrual cycle, as we used the Rotterdam criteria for PCOS diagnosis, in which menstrual dysfunction is not necessarily required for diagnosis. I am aware that the inclusion of women with a regular menstrual cycle limited the chance of finding a difference in this outcome measure; In women with an already regular menstrual frequency at baseline, a further increase would be unlikely. However, there was no statistically significant difference in the proportion of women with a regular menstrual cycle in the three groups (39% in the control group, 33% in LV-HIT and 25% in HV-HIT). Neither was there any statistical difference between groups in the number of women in whom the menstrual pattern changed (i.e. regular cycle, oligo-amenorrhea (35-42 days cycle/ 8-9 cycles per year), oligo-amenorrhea (42 days to 6 months cycle / 2-7 cycles per year), and amenorrhea (> 6 months cycle / 0-1 cycles per year)). We used the Rotterdam criteria because we wanted to increase the comparability of our results to other studies and their research findings. Moreover, if we only were to include women with an irregular menstrual cycle, we would not have been able to include as many women, which would have resulted in lower statistical power for the secondary outcome measures in the trial.

Similar to our findings, Thomson and colleagues found no between-group differences in menstrual frequency after a 20 weeks study period of either diet only, diet and aerobic exercise, and diet and combined aerobic + resistance exercise (39). They did not report whether there were any within-group differences in menstrual frequency from

baseline to 20 weeks. The diet intervention included in all three intervention groups, complicates the interpretation of the effect of exercise on menstrual frequency, and it is only possible to say that the exercise interventions did not have an additional effect on menstrual frequency compared to diet alone.

Nybacka and colleagues (37) compared the effects of diet only, exercise only and the combined effect of diet and exercise for 16 weeks on menstrual pattern and ovulation rates (confirmed by an elevation of progesterone levels in serum during the luteal phase) in women with PCOS. They showed that the interventions were equally effective in improving menstrual pattern (shifting from oligo-/amenorrhea to a regular menstrual cycle), but with no difference in ovulation rates in any group. However, in that study women in the exercise only group also reduced their daily calorie intake by ~270 kcal, which resulted in a 3% weight loss, thereby making it difficult to conclude that the exercise intervention alone was the reason for the improved menstrual pattern.

Jedel and colleagues also found an increase in menstrual frequency after 16 weeks of physical activity compared to a non-intervention, control group (29). Similar to our study, women with PCOS were included based on the Rotterdam criteria, however, at baseline the menstrual frequency (measured as observed menstrual cycles/expected menstrual cycles) was substantially lower compared to in our participants: a menstrual frequency of 0.25 menstruations/month in the Jedel et al. study versus 0.49 menstruations/year in our study. The menstrual frequency in the exercise group in their study increased by 0.14 ± 0.33 during 16 weeks, which means that even after the 16 weeks of exercise, the menstrual frequency of the participants in the exercise group was lower than the menstrual frequency at baseline in our study.

6.2 Exercise adherence

We have little knowledge as to whether exercise protocols implemented in clinical trials under controlled laboratory conditions, typically comprising 8-12 weeks of supervised exercise training, are realistic in a 'real-world' setting. Evidence suggests that HIT is time-efficient (14, 17, 23) and also perceived to be more enjoyable than MICT despite higher perceived exertion during HIT (24). Therefore, HIT may prove as a feasible strategy to improve long-term exercise adherence and participation. However, in our

study we report poor long-term exercise adherence to HIT. We intended to include exercise protocols that were time-efficient, enjoyable and easy to implement in the women's everyday life and assumed that the semi-supervised nature of our intervention would increase flexibility and adherence. I speculate that the poor adherence may be related to too monotonous exercise protocols, as we observed a decrease in enjoyment during the first 16 weeks by ~14% in women allocated to HV-HIT ($p < 0.001$) and ~5% in women allocated to LV-HIT (not statistically significant, $p = 0.12$). A systematic review and meta-analysis on the effectiveness of exercise in the management of PCOS compared to control demonstrated improvements in numerous cardiometabolic outcomes after exercise (VO_2max, fasting insulin, HOMA-IR, total cholesterol, LDL cholesterol, triglycerides, waist circumference and body fat percentage) (155). However, subgroup analyses revealed greater improvements when interventions were supervised and of a shorter duration (≤ 12 weeks), and only VO_2max was improved compared to control when the exercise interventions lasted for more than 12 weeks. The limited improvements in cardiometabolic outcomes after the HIT protocols in our study may be explained by the partly supervised nature, the longer duration, decrease in enjoyment over time and thereby poor adherence.

No previous studies have included and compared different HIT protocols in women with PCOS. Nonetheless, researchers in our laboratory investigated the effects of ten weeks of three weekly sessions of either HIT or strength training on cardiometabolic health outcomes in women with PCOS, and observed improvements in several outcomes in both training groups (25). Similar to ours, the exercise sessions were semi-supervised (at least one weekly supervised exercise session) in that study, but the HIT protocols varied during the week, with two HV-HIT sessions and one LV-HIT session. Both this variety in the training program and the shorter intervention period may explain the higher adherence to the HIT protocols (~90%) in that pilot RCT compared to in the IMPROV-IT trial. In most studies investigating the effect of exercise interventions in women with PCOS, researchers have investigated the effects of the same exercise protocol performed three times per week, with no variation (28, 32-35, 39). It is uncommon in real life to undertake the exact same exercise session multiple times per week with no variation. Therefore, I suggest that future research on exercise training in women with PCOS should focus on the combination of various exercise types/sessions to determine the best exercise

interventions for retaining engagement and improving health. Perhaps this should even be the future direction for exercise interventions in general, at least if the objective is to explore which exercise interventions work in the real world (effectiveness) rather than merely the physiological effect (efficacy) of various exercise sessions.

6.3 Cardiorespiratory fitness

We found a within-group increase in VO_2peak in the HV-HIT group after 16 weeks of HIT, while no significant changes were observed in the LV-HIT group despite the same weekly average of exercise sessions in these groups. These findings are supported by previous findings from Baekkerud and colleagues who compared the effects of 6 weeks of either 4 x 4 min HIT at 85-95% of HR_{max}, 10 x 1 min at VO_2max load, or 45 min MICT at 70% of HR_{max} on VO_2max in men and women with overweight/obesity (23). In that study, only the participants in the 4 x 4 min HIT group increased their VO_2max, with a significant between-group difference versus both 10 x 1 min HIT and MICT protocols (23). Similarly, others have shown that greater volumes of high-intensity exercise result in greater improvements in VO_2max (45, 46). A meta-analysis on the effects of different HIT protocols on VO_2max improvements demonstrated that in addition to a high volume of high-intensity work (≥ 15 min/session), HIT with longer work bouts (≥ 2 min) and longer training periods (4-12 weeks) resulted in the greatest improvements in VO_2max in healthy individuals and those with overweight/obesity (46). The partly supervised nature of our study during the first 16 weeks and no supervision in the remaining 36 weeks along with the long study duration, a decrease in enjoyment, and the poor adherence to the HIT protocols can probably explain why we did not observe any difference in VO_2peak in any group after 12 months in the IMPROV-IT trial.

6.4 Fat oxidation and metabolic flexibility

We show that women with PCOS had lower rates of whole-body fat oxidation during submaximal exercise compared to women without PCOS at baseline, however, no difference was observed when we compared baseline fat oxidation rates between women with and without PCOS who were individually age- and BMI-matched (n=15 in each group). After 16 weeks of HIT, rates of whole-body fat oxidation during submaximal

exercise improved in women without PCOS, whereas no improvements were observed in women with PCOS, despite similar increases in VO_2peak.

To my knowledge, no previous study has investigated the effect of a HIT intervention on whole-body fat oxidation during submaximal exercise in women with PCOS. The absent exercise-induced improvements in whole-body fat oxidation during submaximal exercise in women with PCOS, despite improved VO_2peak, suggest metabolic inflexibility. Metabolic inflexibility in PCOS has been reported previously in the fasted, insulin stimulated state (as reviewed by Rimmer and colleagues) (78). The association between PCOS and metabolic inflexibility was indicated through unchanged RER in the transition from fasted to insulin-stimulated state in women with PCOS whereas there was an increase in RER in healthy women (78). Metabolic inflexibility has also been described in adolescent girls with PCOS (80) and in lean women with PCOS (75), suggesting an association between metabolic inflexibility and PCOS, independent of age and BMI. Moreover, metabolic inflexibility is associated with insulin resistance (77), and the degree of metabolic inflexibility was shown to be similar between women with PCOS and obesity (28.8 ± 4.7 years) and middle-aged women with type 2 diabetes and obesity (58.2 ± 9.9 years) (79).

Studies have demonstrated improved rates of whole-body fat oxidation during submaximal exercise at 60% of VO_2peak in recreationally active, healthy women after seven sessions of HIT (10 x 4 work bouts that elicited ~90% of VO_2peak separated with 2 min of rest) (81), and in recreationally active, healthy men and women after six weeks of HIT (three days/week of 10 x 4 work bouts that elicited ~90% of VO_2peak separated with 2 min of rest) (82). Consistent with these findings, we found improved rates of whole-body fat oxidation during submaximal exercise at 60% of VO_2peak in women without PCOS after 16 weeks of HIT. Contrary, we did not observe an exercise-induced improvement in fat oxidation in women with PCOS. This difference may be explained by the fact that women with PCOS exercised less, at least statistically, than the women without PCOS (2.2 ± 0.5 versus 2.6 ± 0.4 weekly HIT sessions, equivalent to ~6 exercise sessions less in total over 16 weeks). However, VO_2peak increased to a similar degree in women with (~ 4%) and without PCOS (~ 6%) indicating that the fewer HIT sessions performed by women with PCOS cannot fully explain the difference in exercise-induced improvements in fat oxidation rates after 16 weeks of HIT. A recent study by Hansen and

colleagues also reported absent training-induced improvements in whole-body insulin sensitivity and glucose tolerance in women with PCOS compared to age-, BMI- and VO_2peak-matched control women without PCOS (156), despite a similar increase in VO_2peak. Their data implied that the absent training-induced improvement in whole-body insulin action in women with PCOS was owed to a diminished ability to upregulate glucose uptake in skeletal muscle. Collectively, the findings by Hansen and colleagues and those from our study indicate an impaired response to exercise training in some metabolic outcomes related to insulin resistance in women with PCOS. Further investigation is needed to determine the underlying mechanisms behind the absent training-induced improvements in rates of whole-body fat oxidation during submaximal exercise in women with PCOS.

6.5 Adipose tissue in PCOS pathophysiology

In the following paragraphs, I will discuss some of the main findings in Paper III and IV, and the potential role of adipose tissue cell size, mitochondrial respiration and microRNAs in PCOS pathophysiology, as well as the response to 16 weeks of HIT in these outcomes.

6.5.1 Mitochondrial respiration

We found that women with PCOS had reduced mitochondrial respiration through complex I + II in subcutaneous abdominal adipose tissue compared to women without PCOS at baseline, with no differences in subcutaneous gluteal adipose tissue. Moreover, mitochondrial respiration through complex I + II in both adipose tissue depots were unaffected by 16 weeks of HIT in both groups of women. Previous studies have reported aberrant adipose tissue in women with PCOS (75, 84, 87, 88, 157). We found that women with PCOS had reduced mitochondrial respiration in subcutaneous abdominal adipose tissue, supporting the notion that abnormal adipose tissue may play a central role in PCOS pathophysiology (63, 76).

The mitochondria are the major site of ATP production via oxidative phosphorylation, and insulin resistance has been linked to diminished mitochondrial function in skeletal muscle (97). One prior study has compared skeletal muscle

mitochondrial function in women with and without PCOS using high-resolution respirometry (98). Rabøl and colleagues observed no difference in skeletal muscle mitochondrial respiration between women with and without PCOS in either those who were lean (BMI < 25 kg/m^2) or in those with overweight or obesity (BMI > 25 kg/m^2) (98). The findings from Rabøl et al, together with ours, suggest that mitochondrial impairment in PCOS may be evident in (abdominal) adipose tissue rather than skeletal muscle. Thereby, the insulin resistance observed in PCOS could be linked to dysfunctions in the adipose tissue and not in skeletal muscle.

There are no previous publications on exercise-induced changes in subcutaneous gluteal and abdominal adipose tissue mitochondrial respiration in women with PCOS. However, a couple of studies have investigated exercise-induced changes in subcutaneous abdominal mitochondrial respiration in healthy individuals, albeit with conflicting findings. Similar to our findings, Larsen and colleagues found no change in subcutaneous abdominal adipose tissue mitochondrial respiration in men and women with overweight but who were otherwise healthy after 6 weeks of LV-HIT (three days/week of 5 x 1 min maximal effort work-bouts), despite an increase in cardiorespiratory fitness (100). In contrast, Dohlmann and colleagues observed reduced mitochondrial respiration in subcutaneous abdominal adipose tissue taken from men and women with obesity after 18 LV-HIT sessions completed in 6 weeks (7 x 1 min work bouts with workloads corresponding to ~100% of VO_2peak) (101). Contrary to these and our findings, Mendham and colleagues reported increased subcutaneous abdominal adipose tissue mitochondrial respiration in black South-African women with obesity after 12 weeks of combined aerobic and resistance exercise training (four weekly sessions lasting 40-60 min with aerobic exercise at 75-80% HR_{peak} and upper- and lower-body resistance exercises at 60-70 HR_{peak}) (102). Similar to our findings, Mendham and colleagues reported no change in subcutaneous gluteal adipose tissue after the exercise intervention (102). I am unsure of the reasons for these discrepant findings, yet they may be explained by divergent exercise modalities and intensities, as well as different study populations.

6.5.2 Fat cell size

We found no differences in subcutaneous gluteal and abdominal adipose tissue cell size between women with and without PCOS, along with no changes in cell size in neither adipose tissue depots after 16 weeks of HIT in either group of women. Contrary to our findings, Manneras-Holm and colleagues reported enlarged abdominal adipocytes in women with PCOS compared to individually age- and BMI-matched women without PCOS (84). The discrepancy between our current work and the findings by Manneras-Holm et al. may be explained by higher power (27 women in both groups) and a lower mean BMI (< 25 kg/m^2) in their study.

Absent training-induced effects on subcutaneous abdominal adipose tissue cell volume and size have been reported previously (30, 158). Stinkens and colleagues observed no change in subcutaneous abdominal adipocyte size after a 12-week aerobic training program in previously inactive men with overweight or obesity (three weekly exercise sessions which comprised two continuous, aerobic sessions for 30 min at 70% maximal watt and one resistance training session that included upper- and lower-body exercise drills) (158). Similarly, absent training-induced changes in subcutaneous abdominal adipocyte volume has been reported after 16 weeks of continuous, aerobic exercise in women with PCOS (30). The absent training-induced effect on adipose tissue morphology in women with PCOS observed both by others (30) and by us may be explained by less responsiveness in adipose tissue in females. As such, Deprés and colleagues found a sex difference in training-adaptations (159); a 20-week aerobic exercise program (40 min per session at 80% HR_{peak} performed 4-5 days/week) reduced fat percentage and adipose cell weight in men, while no changes occurred in fat percentage or adipose cell weight in women, which suggests that female adipose tissue may be less responsive to adaptations.

6.5.3 microRNAs

Interestingly, we found higher basal expression of circulating miRNA-27b in women with PCOS compared to women without PCOS. miRNA-27b has been implicated in several biological processes including inflammation, fatty acid metabolism, substrate metabolism, and adipocyte differentiation (107, 148, 160). Murri and colleagues found

an interaction between obesity and the group of participants in whole blood miRNA-27b expression, in which obesity (BMI > 30 kg/m^2) was associated with a reduced expression of miRNA-27b in women without PCOS, whereas obesity was associated with an increased expression of the same miRNA in women with PCOS (107). Collectively, the findings by Murri et al. (2013), together with ours, suggest divergent expression of circulating miRNA-27b in women with PCOS compared to women without PCOS. The abnormal expression of miRNA-27b observed in women with PCOS compared to women without PCOS suggests a potential role for this miRNA in the pathophysiology of PCOS.

miRNAs can act within the tissue of origin, or be released to the circulation and taken up by adjacent and/or distant tissues where they exert their effects cell processes (105). As such, we investigated the expression of the eight selected miRNAs in subcutaneous gluteal and abdominal adipose tissue of women with and without PCOS, however, we found no differences. Supporting these findings, previous studies have reported the abundance of some subcutaneous abdominal adipose tissue miRNAs including miRNA-223 to be similar in women with and without PCOS (119, 121), but to be different between insulin resistant women (HOMA-IR ≥ 2.5) compared to insulin sensitive women (HOMA-IR < 2.5). Despite the higher basal expression of circulating miRNA-27b, we found no difference in the expression of this miRNA in either subcutaneous gluteal or abdominal adipose tissue. I can only speculate about this "disconnect" in miRNA expression between the circulation and adipose tissue. Subcutaneous gluteal and abdominal adipose tissue may lack the specific membrane transporters to import miRNA-27b from the circulation (161).

Previous studies have shown that exercise training can modulate the expression of circulating miRNAs in healthy individuals and in individuals with overweight and obesity (122-124). Indeed, we also observed a decrease in the expression of circulating miRNA-27b after 16 weeks of LV-HIT in women with PCOS, indicating that LV-HIT has the potential to regulate and normalize the expression of circulating miRNA-27b in women with PCOS.

The effect of exercise training on gluteal and abdominal adipose tissue miRNAs has only been sparsely investigated in other populations (93). Similar to our findings, Tsiloulis and colleagues reported no effect of 6 weeks of aerobic exercise training (four weekly exercise sessions; three moderate intensity sessions and one HIT session) on the

expression of 526 miRNAs in gluteal and abdominal adipose tissue in men with overweight (93). Collectively, miRNAs in adipose tissue seem to be largely unaltered in response to exercise training lasting up to several months.

6.6 Strengths

A major strength of the IMPROV-IT study was that we compared the effects of LV-HIT and HV-HIT to address selected aspects of metabolic and reproductive health in women with PCOS. Further, we had strict inclusion and exclusion criteria, a long intervention period, and included a control group. The fact that we did not include a dietary intervention in IMPROV-IT allowed us to investigate the isolated effect of exercise training on reproductive and cardiometabolic outcomes in women with PCOS. Furthermore, the multicenter nature of the trial, including women with PCOS from different parts of the world, provides unique scope to the investigation of PCOS and improves generalizability (external validity) of the study.

The strengths of Paper III and IV are that we employed pairwise age- and BMI-matching of women with and without PCOS. In addition, we obtained adipose tissue samples from different adipose depots and were able to assess important metabolic parameters including miRNAs, mitochondrial respiration and fat cell size. Collectively, our findings advance the current knowledge about the possible role of adipose tissue in PCOS pathophysiology.

6.7 Limitations

We used menstrual frequency as a proxy of ovulation, which is a limitation as it is possible to ovulate without a later bleeding and to have a regular cycle despite ovulatory dysfunction (52). Due to practical reasons, we were not able to assess ovulation as that requires daily temperature measurements, frequent urine samples to assess LH, or blood samples to assess progesterone concentrations as markers of ovulation. Menstrual bleedings have been used by others to assess possible improvements in reproduction in women with PCOS after an exercise intervention (29).

The poor adherence to the exercise protocols in the intervention groups with PCOS was the major limitation in the IMPROV-IT trial. The IMPROV-IT trial was based

on the intention to treat principle, in which all primary and secondary outcomes are analyzed according to which intervention the participants are allocated to, regardless of adherence to the protocol. Thereby, to find an effect of the intervention depends heavily on adherence. We intended to add a per protocol analysis to investigate the effect of following the exercise protocols, however, only four women in the LV-HIT group, and two women in the HV-HIT group fulfilled the pre-specified criteria for the per protocol analysis for the 12 months follow-up measurements. Thus, we could not perform a meaningful per protocol analysis. Additionally, our findings indicate that the Non-Ex group introduced some lifestyle modifications during the intervention period.

The limited adipose tissue quantity from the biopsies increased the risk of type II errors (not showing an effect of the intervention even if it was present), which is the main limitation in both Paper III and IV. In Paper III, we compared the expression of miRNAs in subcutaneous gluteal and abdominal adipose tissue between women with and without PCOS in eight women in each group, and secondly determined the response to LV-HIT or HV-HIT in women with PCOS in eight women in each of the groups (total of 24 women including the Non-Ex group). However, the number of participants included in each group (n=8) is comparable or even higher compared to the number of participants in similar studies (93, 121).

Due to the limited adipose tissue biopsy quantity, we were unable to compare fat cell size and mitochondrial respiration between the pairwise age- and BMI-matched women with and without PCOS. To increase power, we pooled the LV-HIT and HV-HIT groups into one group for women with PCOS and one group for women without PCOS after our statistical analyses showed no differences on any outcomes between the LV-HIT and HV-HIT groups. Thereby, we were unable to detect differences in the effects of LV-HIT and HV-HIT in fat cell size and mitochondrial respiration, but instead able to investigate the effect of HIT while increasing the statistical power. The number of participants included in our exercise groups were similar to the number of participants included in previous studies that investigated exercise-induced changes in adipose tissue mitochondrial respiration or morphology (100, 101, 158, 159).

6.8 Clinical relevance and future perspectives

Exercise is widely recommended for women with PCOS (52), and it is important to determine the reproductive and metabolic effects of different types of exercise modalities to improve the prescription of exercise as a therapeutic intervention for this population. The studies included in this thesis have led to new insight about the effects of LV-HIT and HV-HIT as treatment to address selected aspects of reproductive, metabolic and psychologic health in women with PCOS, and adds to the current knowledge regarding the health benefits of various exercise training programs in these women.

The observed improvements in menstrual frequency, pregnancy rates and mental health in the IMPROV-IT trial are of major clinical importance for women with PCOS, since PCOS is the primary cause of anovulatory infertility and menstrual disorders in reproductive-aged women, and since the prevalence of impaired quality of life, anxiety and depression is high among women with PCOS (3). Future research should focus on different exercise protocols to determine their effects on reproductive, metabolic and psychologic outcomes to improve the prescription for this population.

The exercise protocols used in the current studies require no equipment apart from a heart rate monitor and can therefore easily be prescribed by clinicians and implemented in women's everyday life. Yet, we experienced poor adherence and a decrease in enjoyment with the exercise protocols during the study. Strategies to enhance engagement and adherence to a healthy lifestyle and thereby improvements in long-term health outcomes for women with PCOS are needed as these are currently limited. Future studies should examine whether a combination of various exercise modalities could increase adherence and retain long-term engagement in exercise training leading to health benefits and/or if supervised exercise sessions and a close follow-up is the best direction. Furthermore, behavioral change techniques involving goal setting, problem solving, and confidence training should be explored along with motivational support to prevent relapse during lifestyle interventions and thereby improve weight management, healthy lifestyle and wellbeing in women with PCOS.

Further studies are also required to elucidate the underlying mechanisms behind the absent exercise-induced improvements in rates of whole-body fat oxidation during

submaximal exercise and the metabolic inflexibility observed in women with PCOS, as well as to elucidate possible links between adipose tissue and PCOS pathophysiology.

7.0 Conclusion

The overall aim of the experiments conducted for this thesis was to explore the effects of two different HIT protocols on reproductive, cardiometabolic and quality of life outcomes in women with PCOS along with exploring whether aberrant adipose tissue plays a role in the pathophysiology of PCOS. Semi-supervised HIT did not increase menstrual frequency compared to control after 12 months but HIT lead to increased pregnancy rates and improved mental health in women with PCOS. LV-HIT and HV-HIT lead to few improvements in cardiometabolic outcomes in women with PCOS, and the lack of improvements were most likely due to poor adherence to the exercise protocols and behavioral changes in the control group during the study period. We found absent exercise-induced improvements in rates of whole-body fat oxidation during submaximal exercise after 16 weeks of HIT in women with PCOS, which indicates metabolic inflexibility during exercise in these women. Our findings showed that the expression of circulating miRNA-27b was increased in PCOS, and that 16 weeks of LV-HIT decreased and normalized the miRNA-27b expression in the circulation. Mitochondrial respiration through complex I + II in subcutaneous abdominal adipose tissue was reduced in women with PCOS compared with women without PCOS, which supports the notion that abnormal adipose tissue may play a central role in PCOS pathophysiology.

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Paper I

BMJ Open

Improving reproductive function in women with polycystic ovary syndrome with high-intensity interval training (IMPROV-IT): study protocol for a two-centre, three-armed randomised controlled trial

Ida Almenning Kiel ,[1,2] Sofie Lionett,[1,2,3] Evelyn Bridget Parr,[3] Helen Jones,[4] Maria Aurora Hernandez Røset,[2,5] Øyvind Salvesen,[6] Eszter Vanky,[2,5] Trine Moholdt[1,2]

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For numbered affiliations see end of article.

Correspondence to
Dr Trine Moholdt;
trine.moholdt@ntnu.no

ABSTRACT

Introduction Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age and the leading cause of anovulatory infertility. Women with PCOS have a 15-fold higher prevalence of infertility, compared with women without PCOS, independent of body mass index (BMI). A healthy lifestyle is recommended to improve overall health and fertility in PCOS but there is limited evidence on the isolated effects of exercise, especially for reproductive outcomes. Previous findings indicate superior metabolic health benefits after vigorous compared with moderate-intensity exercise. Our primary aim is to determine the effect of high-intensity interval training (HIT) on menstrual frequency, as a proxy of reproductive function, in women with PCOS.

Methods and analysis The study is a two-centre, randomised, controlled trial with three parallel groups. Women (n=64) from Trondheim (Norway) and Melbourne (Australia) with PCOS according to the Rotterdam criteria will be randomly allocated (1:1:1) to high-volume HIT, low-volume HIT or a control group with no exercise after stratifying for BMI < or ≥ 27 kg/m^2 and study centre. Measurements for study end points will be undertaken at baseline, after a 16 week exercise intervention and at 12 months following baseline assessments. The primary outcome measure is menstruation frequency, measured as the number of self-reported menstrual bleedings divided by the number of expected menstrual bleedings during a 12-month period. Secondary outcome measurements include markers of cardiovascular, metabolic and reproductive health, as well as quality of life and adherence to and enjoyment of exercise.

Ethics and dissemination The Regional Committee Medical Research Ethics, Norway, and The Australian Catholic University Human Research Ethics Committee, Australia, have approved the trial protocol. This trial will provide new insight regarding the impact of exercise on fertility in PCOS. We expect this trial to contribute to new therapeutic exercise strategies as part of clinical care for women with PCOS.

Trial registration number Clinical trial gov NCT02419482.

Strengths and limitations of this study

- This will be the first randomised controlled trial to determine the isolated effects of two different high-intensity interval training protocols on reproductive and health-related outcomes in women with polycystic ovary syndrome, with a follow-up time of 12 months.
- The exercise intervention is controlled and monitored through partly supervised sessions for 16 weeks.
- We will include women within all body mass index categories.
- Investigators will not be blinded for all assessments due to the difficulty blinding investigators and participants to a behavioural intervention.
- Menstrual frequency is only a proxy for reproductive function, and we will not undertake ovulation monitoring.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder[1 2] affecting 8%–13% of reproductive-aged women.[3] The ovulatory disturbance is a central diagnostic feature of PCOS,[4] and the syndrome is recognised as the leading cause of anovulatory infertility and menstrual disorders.[5] A large community-based cohort study reported a 72% prevalence of infertility among women with PCOS, compared with 16% among women without PCOS.[6]

According to the most recent guidelines for the assessment and management of PCOS, lifestyle intervention is regarded as first-line therapy to manage reproductive and metabolic outcomes.[4] In addition to reproductive dysfunctions, PCOS is associated with a number of adverse cardiovascular, metabolic and psychological outcomes across all categories of body mass index (BMI).[2 4 7–9] Obesity seems to exacerbate the clinical features in PCOS, but also weight-independent insulin resistance is strongly implicated in the aetiology of PCOS, contributing to the reproductive and metabolic complications.[10–13] Approximately 40% of women with PCOS have a normal BMI (<25 kg/m^2) and the prevalence of insulin resistance among lean women with PCOS is estimated to be around 75%.[14] Adipose tissue dysfunction, such as hypertrophic adipocytes and impairments in lipolysis and insulin action, plays a central role in the metabolic abnormalities observed in PCOS.[15] Hypertrophic adipocytes are more susceptible to inflammation and chronic low-grade inflammation in PCOS.[16] There is limited research on the effect of exercise training on adipose tissue function and low-grade inflammation in PCOS.

Exercise training positively affects metabolic, cardiovascular and psychological outcomes in women with PCOS, but the effect on fertility is unclear.[4 12 17–19] Some trials[20–24] and systematic reviews[12 25] report improved ovulation or menstruation frequency after a period of exercise training or after a combined exercise-diet intervention.[26] Two of these studies indicate a weight-independent effect of exercise on fertility, suggesting insulin sensitivity to be a key factor.[21 23] Only one study to date has investigated the effect of exercise on menstruation frequency in women with PCOS having normal or low BMI (<25 kg/m^2).[22] In that study, they found a significant improvement in menstruation frequency (from 48 to 27 days) after 8 weeks (three sessions per week) of aerobic and resistance training. However, 8 weeks follow-up may be too short to determine any effect of exercise training on menstrual frequency. Furthermore, inconsistent reporting of fertility-related outcomes, small sample sizes and short intervention periods, make it difficult to interpret the effect of exercise on fertility.[2 4 12 25]

Despite growing evidence on health benefits of exercise training in PCOS,[4 12 27] there is a lack of well-designed randomised controlled trials of different exercise protocols including long intervention and follow-up periods to determine the isolated effect of exercise on fertility outcomes

. Most studies to date have only included overweight/obese participants and less is known about the potential benefits in women with PCOS who have BMI ≤25 kg/m^2. Although moderate-intensity exercise provides health benefits, only vigorous, and not moderate-intensity, physical activity was associated with reduced odds of insulin resistance and metabolic syndrome in a cross-sectional study of women with PCOS.[18] Indeed, there is an increased focus on high-intensity interval training (HIT) as a means of improving insulin sensitivity[28] and cardiorespiratory fitness[29–31] in clinical populations, including PCOS.[32] HIT involves brief, repeated work bouts of relatively intense exercise separated by periods of rest or low-intensity exercise. Several different HIT protocols exist, and they can broadly be divided into 'high-volume' (HV) and 'low-volume' (LV) HIT. One of the most common HV-HIT protocols is the Norwegian 4×4 min HIT protocol, which induces superior improvements in cardiorespiratory fitness compared to work-matched moderate-intensity training in both healthy individuals[33] and in various patient groups.[29 34] LV-HIT typically consists of ≤10 min of intense exercise within an exercise session lasting ≤30 min in total, such that the total weekly training time commitment is markedly lower than the current public health guidelines.[35] LV-HIT could therefore have the potential to overcome the most common barriers for women in fertile age, such as time commitment.[36] LV-HIT can improve glycaemic control in people with type 2 diabetes.[37] We have previously reported improved insulin sensitivity (measured with homeostatic assessment of insulin resistance, HOMA-IR), endothelial function and body composition after 10 weeks of HIT in women with PCOS, without any changes in body mass.[32] However, in that pilot study, the participants undertook both LV-HIT and HV-HIT and we did not determine the effects of exercise training on any reproductive outcomes. Based on the positive results from our pilot study with a combined HV-HIT and LV-HIT protocol, we will now compare the two different HIT protocols to investigate potential differences in health outcomes.

Here we describe the design, methodology and potential clinical significance of the 'IMproving Reproductive function in women with Polycystic OVary syndrome by high-intensity Interval Training (IMPROV-IT) trial'.

AIMS

The primary aim of the IMPROV-IT trial is to test the hypothesis that 16 weeks of semi-supervised HIT, followed by home-based HIT for 36 weeks, will increase menstruation frequency, as a proxy measure of ovulation, during 1 year of follow-up in women with PCOS, compared with a non-exercising control group.

Secondary aims are to determine if there are improvements in: ovarian morphology, insulin sensitivity, endothelial function, intima-media thickness, body composition, cardiorespiratory fitness, oxidative capacity, circulation markers of reproductive and metabolic health, low-grade systematic inflammation (in blood and adipose tissue), adipose tissue morphology and function, and quality of life, after 16 weeks of semi-supervised HIT, as well as after the following 36 weeks of home-based HIT.

Additionally, we will record the adherence to exercise training and enjoyment of two different HIT protocols

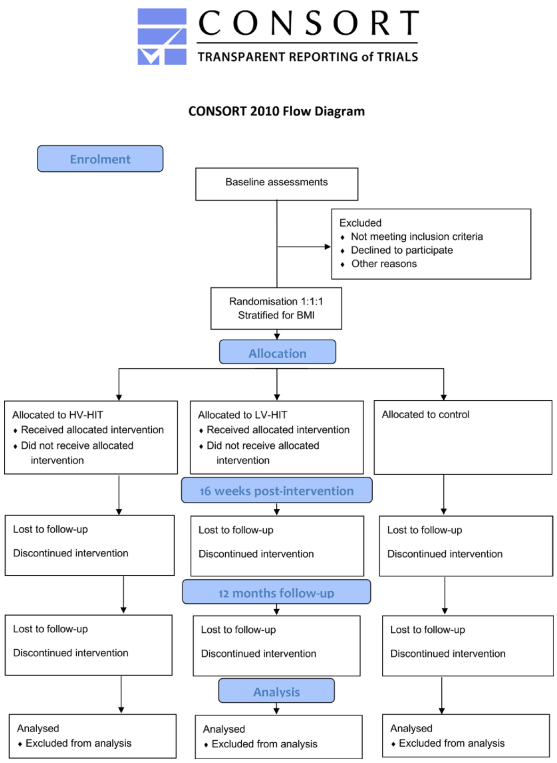

Figure 1 Consort flow diagram. BMI, body mass index; HV-HIT, high-volume high-intensity interval training; LV-HIT, low-volume high-intensity interval training.

and assess if the two protocols induce different effects on the aforementioned measures.

METHODS AND ANALYSIS

Study setting and recruitment

This is a two-centre, randomised controlled trial with three parallel groups; two training intervention groups and one control group. The two study centres are the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway and the Australian Catholic University (ACU) in Melbourne, Australia. Testing and training will take place in the university research laboratories in both Norway and Australia. Participants will be recruited from the public announcement at hospitals and university homepages, at local stores, public places and social media. All participants will sign a written, informed consent. All participants will have a project-specific code that will be used during all analyses of the data. The procedures for data entry, coding and storage have been approved by the Regional Committee Medical Research Ethics in Mid-Norway.

Participants

To be eligible for inclusion in the study, women will have to meet the following criteria:

- Aged 18–45 years old.
- PCOS diagnosis according to the Rotterdam criteria,[38] as confirmed by a gynaecologist, endocrinologist or general practitioner. Thus, a minimum of two of the following criteria have to be present: (1) polycystic ovary morphology (12 or more 2–9 mm follicles or >10 mL in volume, in at least one ovary), (2) hyperandrogenism (either clinical signs as hirsutism or acne, or biochemical), and (3) oligo/amenorrhoea. Oligomenorrhoea is defined as an intermenstrual interval >35 days and/or ≤9 menstrual bleeding in the past year. Amenorrhoea is defined as no vaginal bleeding the last 3 months for women with a previously regular menstrual cycle; no vaginal bleeding the last 12 months for women with an irregular menstrual cycle.[39 40] Hirsutism will be defined as modified Ferriman Gallwey score[41] ≥8.[4]
- At the Norwegian centre, all participants will be screened at baseline for polycystic ovaries, hyperandrogenism and menstruation frequency to confirm the PCOS diagnosis. At the Australian centre, participants will show an ultrasound scan no older than 8 years performed by their general practitioner, gynaecologist or endocrinologist confirming polycystic ovaries. Ferriman Gallway score for hirsutism/hyperandrogenism and information about their menstrual cycle will be obtained before entering the study to confirm PCOS diagnosis.
- Undertaking less than 2 weekly endurance exercise training sessions with an intensity that induce heavy breathing.

Women will be excluded from the study if they meet any of the following criteria:

- Current treatments with hormonal contraceptives including Mirena intrauterine device.
- Insulin sensitisers or drugs known to affect gonadotropin or ovulation (with a washout period of 3 months prior to inclusion).
- On-going pregnancy or breastfeeding within 24 weeks.
- Cardiovascular disease or other endocrine disorders (eg, congenital adrenal hyperplasia, Cushing syndrome or androgen-secreting tumours).

Randomisation and allocation

Participants will be allocated 1:1:1 to HV-HIT, LV-HIT, or control after stratifying for BMI <or ≥ 27 kg/m^2 and study centre (figure 1). A computer random number generator developed and administered at the Faculty of Medicine, Department of Public Health and General Practice, NTNU, Trondheim, Norway, will be used at both study centres. The investigators will be informed about the allocation results by e-mail after the registration of new participants.

Interventions

The first 16 weeks of the exercise training will be semi-supervised, with the remaining 36 weeks of the intervention organised as home-based exercise training without

any supervision. Supervised exercise interventions are effective, but do not resemble a real-life setting, and the long-term adherence often falls once the supervised exercise programme ends. Unsupervised exercise programme are more easily implemented and flexible, but adherence is often low. During the first 16 weeks, the participants will perform at least 1 weekly supervised exercise session at the study centres. They will be given the opportunity to attend up to 3 weekly supervised exercise sessions but can also choose to do one or two of the weekly sessions as home-based training. The participants will be encouraged to continue to complete at least 2 weekly sessions (home-based and unsupervised) for the remaining 36 weeks of the study period. Participants receive a heart rate monitor that they will wear on all sessions and will be required to register all their exercise sessions during the whole study period through an online exercise-training diary (www.polar.flow.com). The researchers will have access to all sessions via the same online tool and will supervise exercise adherence at the home-based sessions; however, no motivational support or instructions will be provided during the follow-up period. We will be advising women in the control group to continue their habitual physical activity and inform them about the current recommendations of a minimum of 150 min of weekly moderate-intensity physical activity.

HIT protocols

The LV-HIT protocol consists of a 10 min warm-up at light to moderate intensity at 60%–70% of HR_{max} followed by ten 1 min work bouts at the maximal intensity the participants can complete for 1 min. In the initial training session, the intensity will be set corresponding to 100% of the workload the participant reached at the baseline VO_2max test. Participants will be instructed to try reaching 90% of maximal heart rate (HR_{max}) during the third or fourth work bout, based on previous findings from Little *et al*.[37] The work bouts are separated by 1 min of passive or low-intensity recovery, trying to reach 60%–70% of HR_{max}. The training session is terminated after a 3 min cool-down at 60%–70% of HR_{max}. The total exercise time is 32 min (figure 2A).

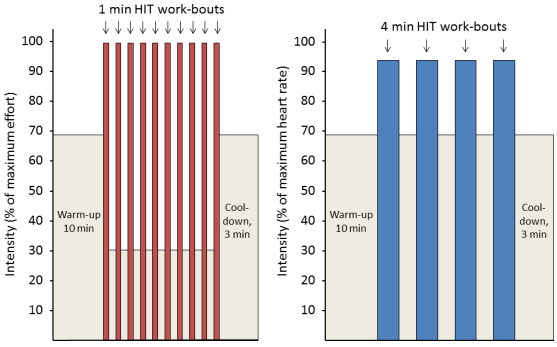

Figure 2 HIT protocol. Panel A: low-volume hit; Panel B: high-volume hit. HIT, high-intensity interval training.

The HV-HIT protocol consists of a 10 min warm-up at 60%–70% of HR_{max}, followed by four 4 min work bouts reaching 90%–95% of HR_{max}, separated by a 3 min active recovery of running/walking at 60%–70% of HR_{max}. The training session is terminated after a 3 min cool-down at 60%–70% of HR_{max}. The total exercise time is 38 min (figure 2B).

The LV-HIT and HV-HIT are not matched for time, mean workload and energy expenditure. HIT will be performed as treadmill walking or running. Heart rate monitors (Polar M400) will be used on all sessions, including on the home-based sessions, and the exercise intensity will be estimated based on their HR_{max} during the VO_2max test at baseline.[42] Where required (due to injury, pain or discomfort), the participants will be able to exercise on other ergometers (bikes or elliptical machines). Participants can also choose to perform some of the sessions as outdoor running/uphill walking. We will adjust the absolute workload of both LV-HIT and HV-HIT throughout the intervention period to account for improvements in fitness.

Outcome measures

Outcomes will be assessed at baseline, at 16 weeks (post intervention) and at 12 months from baseline (follow-up) in Trondheim, Norway. In Melbourne, Australia, measurements will be assessed at baseline and after 16 weeks, and only questionnaires and menstrual frequency will be assessed until 12 months from baseline. Participants will be asked not to exercise for >48 hours prior to the test visits in the laboratory and to abstain from caffeine intake for 24 hours prior to the tests. Measurements will be undertaken during the follicular phase of the menstrual cycle (1–7 days after first bleeding) in women with a regular menstrual cycle.

Menstrual frequency

The primary outcome measure is menstrual frequency. Participants will register the first day of their menstrual cycle and length (number of days) during the 12-month study period. They will send their menstruation dairy to the study personnel after each menstrual cycle, and the study personnel will send out reminders to fill out the diary. In addition, participants will complete a questionnaire about their menstrual cycle at each assessment point (baseline, 16 weeks and 12 months). We will compare the number of menstrual bleedings between the groups, measured as the number of observed menstrual bleedings divided by the number of expected menstrual bleedings (n=13, assuming a cycle interval of 28 days) during a 12-month period. The menstrual frequency is to be measured as a proxy for ovulation. Menstrual frequency assessments will be undertaken non-blinded for the study personnel.

Ovarian morphology

Ovarian morphology will only be assessed at the site in Norway. The ultrasound assessments of the ovaries include

ovarian volume in mL (with and without a dominating follicle), dominating follicle volume in mL, number of follicles in each ovary, diameter and distribution of follicles. We will use a multifrequency transvaginal transducer on all measurements. An experienced gynaecologist (MAHR) will perform all imaging and measurements. Ovarian morphology assessments will be undertaken blinded for group allocation.

Fertility and pregnancies

Background information regarding fertility will be registered by questionnaires. These include questions about number of children, natural or assisted fertilisation, if the participants are actively seeking pregnancy or have tried to become pregnant, for how long they have tried to become pregnant, and if they have experienced miscarriages or had abortions. We will report the number of pregnancies during the intervention period, including during the 12 months of follow-up. Questionnaires will be undertaken blinded, whereas the number of pregnancies will be undertaken non-blinded for the study personnel.

Blood biochemistry and insulin sensitivity

Blood samples will be obtained after an overnight fast (no food or fluid intake except water 12 hours prior to assessments) at each time point. Analysis of fasting venous blood samples will include measurements of 17-OH progesterone (baseline only), prolactin (baseline only), haemoglobin, glycated haemoglobin, blood lipids (total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglycerides), blood glucose, white blood cell count (neutrophils, basophils, eosinophils, lymphocytes and monocytes) and leucocytes. Additional blood (including buffy coat) will be stored for later analyses. These later analyses will likely include, but are not limited to, androstenedione, anti-Müllerian hormone, sex hormone-binding globulin, testosterone, insulin, leptin, adiponectin and micro-RNAs. We will also obtain two Tempus RNA tubes from each participant for later assessments of gene expression.

After fasting blood samples, glycaemic control will be measured using a 2-hour oral glucose tolerance test (OGTT). The participants will consume 75 g of glucose diluted in 250 mL water. Blood will be sampled for insulin and glucose measurements at 0 (prior to the OGGT), 30, 60, 90 and 120 min from an indwelling catheter. Glycaemic control will be calculated as total area under the curve (AUC) and incremental area under the curve (iAUC; using fasting concentrations as baseline values) using the trapezoid method, for glucose and insulin concentrations, and peak concentrations during the OGTT. Insulin sensitivity will be estimated using the HOMA-IR; fasting serum insulin in µU/mL × fasting plasma glucose in mmol/L/22.5.[43]

Biological materials collected at NTNU will be stored in the Regional Research Biobank at St Olav's Hospital (Biobank1, https://biobank1.no/nb/). Biological materials from collected at ACU will be stored locally until data collection is completed in Australia, and later sent to Norway. Sample collection and handling are performed in accordance with hospital/laboratory standard procedures. Blood sampling and OGTT will be undertaken blinded in Norway and unblinded in Australia.

Cardiorespiratory fitness and substrate utilisation

Peak oxygen uptake will be measured on a treadmill, using indirect calorimetry (Oxycon Pro, Jaeger, Germany in Norway/TrueOneRMR, Parvo Medics, USA in Australia). Participants will walk or run until voluntary exhaustion using an individualised protocol. After a 10 min walking warm-up, the test will start by walking at moderate intensity for 3 min. The speed or inclination will then be increased every 1–2 min, by 0.5–1.0 km/h or 1%–2%. The cardiorespiratory fitness test will be considered successful with a plateau in VO_2 with a further increase in workload, and a respiratory exchange ratio ≥1.05. Peak oxygen uptake will be calculated as the highest consecutive 30 s measured, both absolute (mL/min) and relative (mL/min/kg). The maximal heart rate obtained during the exercise test at baseline will serve as the basis for calculating the intensity during the HIT sessions.

After an overnight fast (no food or fluid intake except water 12 hours prior to assessments), participants will complete a submaximal test on a treadmill. The protocol includes a 20 min warm-up, followed by 20 min steady-state expired gas sampling at 60% of peak oxygen uptake. We will ask participants to record their diet the day before the baseline test of oxidative capacity and to repeat this diet before the subsequent measures (at 16 weeks and 12 months). Fat oxidation rates (g/min) will be calculated from 5 min of stable oxygen uptake at the final stage of the test (during the last 10 min), using the following equation: $1.695 \times VO_2 - 1.701 \times VCO_2$,[44] where VO_2 is oxygen uptake and VCO_2 is the volume of expired carbon dioxide. Assessments of these outcomes will be undertaken non-blinded for the study personnel.

Adipose tissue composition, morphology and function

Abdominal and gluteal subcutaneous adipose tissue biopsies (~300–500 mg) will be collected under local anaesthesia (1% xylocaine) using a 14-G sterile needle. For a subsample of participants, ~80 mg of each biopsy will be allocated for immediate analyses of mitochondrial respiration using high-resolution respirometry (Oxygraph-2K, Oroboros, Austria). Approximately 200–300 mg will be snap-frozen in liquid nitrogen and stored at −80°C for later analyses, such as micro RNAs, gene expression and protein profiling. The remaining tissue will be immediately fixed in phosphate-buffered formalin, processed and embedded in paraffin and sectioned at 4 µm for morphology and inflammation analyses. The sections will be incubated with CD45 and CD68 antibodies to detect leucocytes and macrophages. These sections will be captured with an EVOS FL Auto 2 Imaging System (ThermoFisher Scientific, USA) and analysed with the

open-source software ImageJ (Fiji).[45] Assessments of these outcomes will be undertaken blinded.

Physical activity and diet

We will monitor physical activity by questionnaires and 5-day activity monitoring (Sensewear Armband, APC Cardiovascular, UK) and record diet through a 4-day diet recall, at each measurement point. Assessments of physical activity and diet will be undertaken blinded.

Quality of life

Quality of life will be assessed using the Polycystic Ovary Syndrome Questionnaire.[46] Assessment of this outcome will be undertaken blinded.

Anthropometrics and body composition

Anthropometric measurements will be conducted when participants are fasted (no food or fluid intake except water 12 hours prior to assessments). Height will be measured standing, without shoes using a standard stadiometer. Total body mass and body composition will be measured wearing light clothing with no metal items and without shoes or socks using bioelectrical impedance analysis (InBody720 bioimpedance scale, Biospace CO, Korea) in Norway and dual-energy X-ray absorptiometry (DXA; GE Lunar iDXA Pro, encore software version 16, General Electric, Boston, Massachusetts, USA) in Australia. For the DXA scan, participants are required to lay supine on the scanning bed for the duration of the scan, which is approximately 15 min with one or two scans depending on the body shape of the participant. Waist and hip circumference will be measured to the nearest 0.5 cm horizontally at the level of the umbilicus, while standing and at normal expiration, using a metric tape. Assessments of these outcomes will be undertaken non-blinded for the study personnel.

Blood pressure and resting heart rate

Blood pressure and resting heart rate will be measured in the seated position after 15 min rest with an automatic blood pressure device three times on the left arm (diastolic and systolic, in mm Hg). The mean of the three measurements will be used to calculate blood pressure and resting heart rate. Assessments of these outcomes will be undertaken blinded in Norway and unblinded in Australia.

Endothelial function and intima-media thickness

Endothelial function and intima-media thickness (IMT) will be assessed at the Norwegian site only. Following a 20 min supine rest period, the diameter of the brachial artery will be imaged (12 MHz Doppler probe, GE Vingmed Ultrasound AS, Horten, Norway). A cuff will be placed around the forearm (immediately distal to the olecranon) to produce the stimulus of forearm ischaemia. When an optimal image is obtained, the probe will be held stable and the ultrasound parameters will be set to optimise the longitudinal, B-mode image of the lumen–arterial wall interface. The ultrasound will also be used to attain simultaneous continuous Doppler velocity using the lowest possible insonation angle (60°). A recording of resting diameter and velocity will be taken for 1 min, then the forearm cuff will be inflated (>200 mm Hg) for 5 min. Both diameter and velocity recordings will resume 30 s before cuff deflation and continue for 3 min after deflation.[47] This flow-mediated dilation (FMD) test is a measure of endothelial function. We will also measure carotid artery IMT in three angles: transversal, longitudinal and anterolateral using ultrasound (12 MHz Doppler probe, GE Vingmed Ultrasound AS, Horten, Norway). Measurements of the diameter will be imaged 1 cm below the bifurcation on the right side. Images will be obtained where the near wall is clearly visualised, with a double-line pattern and at the minimal diameter during the cardiac cycle as previously described elsewhere.[48] The same person (IAK) will perform all the FMD and IMT measurements. Assessments of these outcomes will be undertaken non-blinded for the study personnel.

Enjoyment

A subgroup of participants (the last 40 to be included in the trial) allocated to one of the training groups will complete a Physical Activity Enjoyment Scale (PACES)[49] as well as The Borg's scale[50] to assess enjoyment and perceived exertion at one supervised weekly training session. Assessments of these outcomes will be undertaken non-blinded for the study personnel.

Sample size and statistical analysis

We computed the sample size for a one-way analysis of variance test with three groups. In women with PCOS, a menstrual frequency of on average 4.5 menstrual bleeding during 1 year is expected.[51] With a statistical power of 80%, a significance level of 0.05 and a SD of two menstrual bleeding during a 12-month period, we calculated that 48 women will be required to detect an increase in menstrual bleeding to 7.5 in the intervention groups.[26] Because of the non-normality of menstrual frequency, it may be necessary to use a non-parametric test. Non-parametric tests require more participants and we, therefore, added 15% to the required sample size. Additionally, to allow for expected dropouts of 10%–15%, we aim to include 64 women in the study.

We will perform all the statistical analyses blinded for group allocation. The primary analysis will be a comparison between groups for the number of menstrual bleeding during 12 months. We will adjust for the self-reported menstrual frequency at baseline. We will include all women who have reported menstrual frequency in the primary analysis, independent of adherence to the interventions, that is, intention-to-treat analysis. The number of pregnancies during the 16 weeks of intervention and during the 12-month follow-up will be compared between groups. Secondary outcome measures will be compared between groups after 16 weeks and after 12 months and adjusted for baseline values. We will do additional 'per protocol' analyses where we include women in the HIT

groups that have completed >75% of the scheduled exercise sessions during the intervention period (for comparisons after 16 weeks) and a mean minimum of one HIT session/week during the 12-month follow-up (for comparisons at the end of follow-up).

Results will be reported as means with 95% CIs and/or SD. We will report the effect of HIT as mean changes from baseline to 16 weeks of intervention and after 12 months of follow-up. We will use mixed models to test differences between groups. P values <0.05 will be considered significant for both primary and secondary outcomes. We will also perform subgroup analyses to investigate differences between BMI categories and compare adherence rates and enjoyment of the two HIT protocols.

Blinding

We are unable to blind group allocation to participants or study personnel due to the nature of the intervention (supervised exercise training). All baseline assessments will be undertaken prior to randomisation and some assessments will be undertaken blinded for group allocation (as outlined for each outcome measure).

Monitoring

The investigators are responsible for the documentation of any adverse event or serious adverse event. Participants will be told to contact the investigator if they have any unusual symptoms. We will record all medical events during the study in the Case Report Form. In addition, we record serious adverse events in a Serious Adverse Events Report Form. All serious adverse events will be reported to the sponsor within 24 hours after the site has gained knowledge of the event.

Patient and public involvement

No patients were involved in the development of the research question or design of the study. All participants will be invited to a 1-hour educational session about healthy diet and physical activity. We will also give general information about PCOS in these meetings and encourage the participants to ask questions and give us feedback about relevant topics or issues related to their disorder and to the trial implementation. Individual test results will be disseminated to each participant after testing. We will also send out a summary of the study results to all participants at the completion of the study.

ETHICS AND DISSEMINATION

The study is approved by the Regional Committee Medical Research Ethics in Mid-Norway and The ACU Human Research Ethics Committee and has its origin in the Declaration of Helsinki and is consistent with ICH/Good Clinical Practice and applicable regulatory requirements. All protocol modification must be approved by the Regional Committee Medical Research Ethics in Mid-Norway and by The ACU Human Research Ethics Committee.

We will publish the results from the study as peer-reviewed articles in international journals. Our results will also be communicated through the Norwegian society of infertility, through social media channels as well as at national and international conferences.

DISCUSSION

Infertility and risk of lifestyle-related diseases (obesity, metabolic, cardiovascular and psychological diseases) are the main health challenges associated with PCOS, both for the individuals affected by the syndrome, the healthcare systems and society.[2 4] To date, no treatment fully reverses or cures the symptoms of PCOS[40] and lifestyle interventions are recommended as first-line therapy according to recent guidelines.[4 52] Nevertheless, there is limited research available to give women with PCOS sufficient recommendations on physical activity and exercise. Some studies have demonstrated positive effects in reproductive outcomes after exercise, with concomitant improvements in insulin sensitivity and/or visceral fat.[20–24] However, more knowledge is needed about the underlying mechanisms for improvement in fertility after exercise training, as discussed in several systematic reviews on lifestyle interventions and fertility.[12 25 53 54] HIT has shown to be time efficient, safe and well tolerated, with positive effects on metabolic and cardiovascular risk factors in several studies.[29 55 56] Larger studies investigating the adherence to HIT are lacking among women in general, and among women with PCOS in particular. This is of great interest, as we need to find exercise and lifestyle programmes that are feasible over time and that can be easily implemented in everyday life. Exercise programmes that are efficient in a research setting are not necessarily effective in a real-life setting. Thus, there is a great need for studies with a longer intervention and follow-up period.

Our trial has some limitations. The use of menstrual frequency as a proxy for ovulation/reproductive function is one of these. Due to practical reasons, we are not able to undertake ovulation monitoring. The Inbody 720 and DXA are both reliable methods to measure fat-free mass (FFM), body fat percentage (BF%) and fat mass (FM) but it has been shown that the InBody 720 overestimates FFM and underestimates BF% and FM compared with DXA .[57] For each individual participant, the same measurement method will be used (InBody 720 in Norway and DXA in Australia) and our purpose is to evaluate changes in body composition (ie, delta changes). Assessing enjoyment of exercise only at the supervised sessions could imply a bias. On the supervised sessions, the participants will receive motivational support from the researchers and potentially also social support from other participants that can affect their ratings of enjoyment. The enjoyment ratings are, however, implemented to compare the perceived enjoyment of the two HIT protocols. The semi-supervised nature of the trial could imply that some participants choose to undertake all sessions supervised, whereas others choose to exercise at home for most

sessions. We chose to implement a semi-supervised exercise programme to account for the individual preferences of the participants and to be able to include women living further away from the study centres. Such protocols can both control the exercise intervention and motivate participants, and at the same time give more flexibility based on participants' preferences.

The IMPROV-IT trial will determine the effect of HIT on reproductive, cardiovascular, metabolic and psychological health in women with PCOS. The trial will contribute evidence of more specific exercise protocols in the treatment and management of PCOS for clinicians and answer some of the current limitations addressed by both the International evidence-based guidelines for the assessment and management of PCOS[4] and several systematic reviews.[2 12 25 58] This trial is highly relevant and important as PCOS is a major burden for the women with PCOS, the health services and society. As for now, we do not have an optimal treatment.

Trial status

We have recruited 64 women with PCOS to the IMPROV-IT study, with the last included participant in March 2019. The last follow-up will be in March 2020.

Author affiliations
[1]Department of Circulation and Medical Imaging, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim, Norway
[2]Department of Obstetrics and Gynaecology, St Olavs Hospital, Trondheim University Hospital, Trondheim, Norway
[3]Exercise and Nutrition Research Programme, Mary Mackillop Institute for Health Research, Australian Catholic University, Melbourne, Victoria, Australia
[4]Research Institute for Sport and Exercise Science (RISES), Liverpool John Moores University, Liverpool, UK
[5]Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim, Norway
[6]Unit of Applied Clinical Research, Department of Public Health and Nursing, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim, Norway

Twitter Trine Moholdt @trinemoholdt

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Contributors IAK: drafted the manuscript. IAK, TM, EV, ØS and HJ: conceived and contributed to the design of the study. IAK, SL, TM and EP coordinated the study at the two sites and supervised the exercise training. IAK, SL, TM, MAHR and EP: performed measurements on test-days. EV: provided medical advice and support during the study. All authors provided feedback and approved the final manuscript.

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ORCID iD
Ida Almenning Kiel http://orcid.org/0000-0001-9926-4994

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Paper II

Paper III

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Circulating and Adipose Tissue miRNAs in Women With Polycystic Ovary Syndrome and Responses to High-Intensity Interval Training

*Sofie Lionett[1,2,3], Ida A. Kiel[1,2], Donny M. Camera[4], Eszter Vanky[2], Evelyn B. Parr[3], Stian Lydersen[5], John A. Hawley[3] and Trine Moholdt[1,2]**

[1] Department of Circulation and Medical Imaging, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim, Norway, [2] Department of Obstetrics and Gynecology, St. Olav's Hospital, Trondheim, Norway, [3] Exercise and Nutrition Research Program, Mary MacKillop Institute for Health Research, Australian Catholic University, Melbourne, VIC, Australia, [4] Department of Health and Medical Sciences, Swinburne University of Technology, Melbourne, VIC, Australia, [5] Regional Centre for Child and Youth Mental Health and Child Welfare, Norwegian University of Science and Technology, Trondheim, Norway

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Antonio Paoli, University of Padua, Italy

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***Correspondence:**
Trine Moholdt
trine.moholdt@ntnu.no

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MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression post-transcriptionally. In women with polycystic ovary syndrome (PCOS), several miRNAs are differentially expressed compared to women without PCOS, suggesting a role for miRNAs in PCOS pathophysiology. Exercise training modulates miRNA abundance and is primary lifestyle intervention for women with PCOS. Accordingly, we measured the expression of eight circulating miRNAs selected *a priori* along with miRNA expression from gluteal and abdominal adipose tissue (AT) in 12 women with PCOS and 12 women matched for age and body mass index without PCOS. We also determined the miRNA expression "signatures" before and after high-intensity interval training (HIT) in 42 women with PCOS randomized to either: (1) low-volume HIT (LV-HIT, 10 × 1 min work bouts at maximal, sustainable intensity, n = 13); (2) high-volume HIT (HV-HIT, 4 × 4 min work bouts reaching 90–95% of maximal heart rate, n = 14); or (3) non-exercise control (Non-Ex, n = 15). Both HIT groups trained three times/week for 16 weeks. miRNAs were extracted from plasma, gluteal and abdominal AT, and quantified via a customized plate array containing eight miRNAs associated with PCOS and/or exercise training responses. Basal expression of circulating miRNA-27b (c-miR-27b), implicated in fatty acid metabolism, adipocyte differentiation and inflammation, was 1.8-fold higher in women with compared to without PCOS (P = 0.006) despite no difference in gluteal or abdominal AT miR-27b expression. Only the HV-HIT protocol increased peak oxygen uptake (VO_2peak L/min; 9%, P = 0.008). There were no changes in body composition. In LV-HIT, but not HV-HIT, the expression of c-miR-27b decreased (0.5-fold, P = 0.007). None of the remaining seven circulating miRNAs changed in LV-HIT, nor was the expression of gluteal or abdominal AT miRNAs altered. Despite increased cardiorespiratory fitness, HV-HIT did not alter the expression of any circulating, gluteal or abdominal AT miRNAs. We conclude that women with PCOS have a higher basal

expression of c-miR-27b compared to women without PCOS and that 16 weeks of LV-HIT reduces the expression of this miRNA in women with PCOS. Intense exercise training had little effect on the abundance of the selected miRNAs within subcutaneous AT depots in women with PCOS.

Keywords: exercise, miRNA-27b, insulin resistance, epigenetic modifications, cardiorespiratory fitness, female

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-aged women, affecting up to 13% of females globally (Bozdag et al., 2016). Beyond causing infertility (Teede et al., 2011), PCOS is associated with whole-body insulin resistance and metabolic disorders such as obesity and type 2 diabetes mellitus (Anderson et al., 2014; Cassar et al., 2016; Orio et al., 2016). Despite the high prevalence and adverse health implications of PCOS, the etiology and optimal treatments for women with PCOS are unclear. Insulin resistance is an underlying feature of PCOS (Teede et al., 2011). Indeed, insulin sensitivity is 27% lower in women with PCOS compared to healthy controls (Cassar et al., 2016), with up to 85% of women with PCOS being insulin resistant (Stepto et al., 2013). Lifestyle interventions, including exercise training, are recommended as first-line therapy for women with PCOS (Teede et al., 2011, 2018). In this regard, a growing body of evidence demonstrate that high-intensity interval training (HIT) confers greater health benefits compared to moderate intensity exercise in clinical cohorts (Weston et al., 2014; Cassidy et al., 2017). Such superior effects of vigorous intensity exercise are also evident among women with PCOS (Patten et al., 2020). Thus, a greater knowledge of the molecular mechanisms underlying the efficacy of exercise interventions such as HIT on metabolic health may help to understand the pathophysiology of PCOS.

MicroRNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional regulators by binding with specific mRNA transcripts and preventing protein and gene translation or by degrading the target mRNA (Chen X. et al., 2012). miRNAs can function within the tissue of origin or adjacent tissues (Chen X. et al., 2012), thus playing crucial regulatory roles in many biological processes. Several studies in women with PCOS have reported altered expression of miRNAs in the circulation (Murri et al., 2013, 2018; Long et al., 2014; Sorensen et al., 2014, 2019; Ding et al., 2015; Sathyapalan et al., 2015; Arancio et al., 2018; Chen et al., 2019), adipose tissue (AT) (Chen et al., 2013, 2019; Sorensen et al., 2014; Chuang et al., 2015), and follicular fluid and granulosa cells (Sorensen et al., 2014; Butler et al., 2019; Chen et al., 2019), suggesting a potential role for miRNAs in the pathophysiology of this condition. Specifically, abnormal expression of miRNAs with putative roles in regulating glucose metabolism in adipocytes in insulin resistant women with PCOS have been observed (Chen et al., 2013; Wu et al., 2014; Chuang et al., 2015). Furthermore, studies have reported significantly abnormal expression of miRNAs in subcutaneous abdominal AT in insulin resistant women, regardless of PCOS status (Wu et al., 2014; Chuang et al., 2015).

Women with PCOS have larger adipocytes and reduced adipocyte insulin sensitivity compared to women matched for body mass index (BMI) without PCOS (Dunaif et al., 1992; Manneras-Holm et al., 2011). As such, miRNAs could play a role in these AT abnormalities in women with PCOS. Abdominal obesity is associated with increased risk of insulin resistance and cardiovascular disease (Karpe and Pinnick, 2015) and women with PCOS have greater abdominal fat (Fauser et al., 2012). AT from different depots express unique molecular, cellular and metabolic properties and respond in distinct ways to exercise and nutritional challenges (Tan et al., 2004; You et al., 2014; Tsiloulis et al., 2017). Therefore, in order to understand these differential expression profiles, interrogation of miRNAs from different AT depots in women with PCOS is necessary. However, to the best of our knowledge, such profiles are currently lacking. Accordingly, the primary aim of the present investigation was to compare the expression of selected miRNAs in the circulation, gluteal and abdominal AT in women with PCOS to an age- and BMI-matched control group of women without PCOS. Secondary aims were to investigate the effects of two chronic protocols of HIT on the "expression signature" of these miRNAs in women with PCOS, and to assess differences in the selected miRNA expressions in gluteal and abdominal AT and whether the two AT depots responded in distinct ways to HIT. Our main hypotheses were that there would be differential expression patterns in circulating and AT miRNA's in women with, compared to those without PCOS, and that the expression signature would be modulated by chronic exercise training in women with PCOS.

MATERIALS AND METHODS

Participants and Study Design

Forty-two women with PCOS and 12 women without PCOS (Non-PCOS) were recruited for this study, which was part of the Improving Reproductive Function in Women with Polycystic Ovary Syndrome with High-Intensity Interval Training (IMPROV-IT) trial (Kiel et al., 2020) (ClinicalTrials.gov Identifier: NCT02419482) and of the Adipose Tissue Function and Response to Exercise Training in Women With and Without Polycystic Ovary Syndrome trial (HIT-FAT; ClinicalTrials.gov Identifier NCT02943291). The IMPROV-IT trial is a two-center randomized controlled trial undertaken at The Norwegian University of Science and Technology (NTNU) in Trondheim, Norway and at The Australian Catholic University (ACU) in Melbourne, Australia. The study protocol for the IMPROV-IT trial has been published previously (Kiel et al., 2020). Non-PCOS participants were recruited only in Norway. We selected 12

women without PCOS (Non-PCOS) as a control group, who were individually matched to 12 women with PCOS. Women with and without PCOS were individually matched by age and BMI with an age difference of 5 years and BMI difference within 2 kg/m^2. PCOS was defined according to the Rotterdam criteria, where a minimum of two of the following three conditions must be present: (1) polycystic ovary morphology (12 or more 2–9 mm follicles or >10 mL in volume in at least one ovary), (2) hyperandrogenism (either clinical signs such as acne or hirsutism, or biomedical) and/or oligo/amenorrhea (Rotterdam, 2004). Hirsutism was defined with a Ferriman Gallwey score ≥8 (Ferriman and Gallwey, 1961). The cut-off values for biochemical hyperandrogenism were defined as a testosterone concentration of >3.0 nmol/L, calculated free testosterone concentration of >32 pmol/L, sex hormone binding globulin (SHBG) concentration of <30 nmol/L, or free androgen index (FAI as 100× testosterone concentration (nmol/L)/SHBG concentration (nmol/L) >5% (Al Kindi et al., 2012). Oligo/amenorrhea was defined as an intermenstrual interval >35 days and ≤9 menstruations in the past year. Amenorrhea was defined as absent menstruations in the past 90 days.

To be eligible for inclusion into the study, women had to be aged between 18 and 45 years. Exclusion criteria were; if women were undertaking regular endurance training ≥2 sessions/week, cardiovascular diseases or other endocrine disorders, pregnancy or breastfeeding within the last 24 weeks, physical ailments or injuries that would hinder exercise performance, or undergoing concurrent treatments with hormonal contraceptives, insulin sensitizers or drugs known to affect gonadotropin or ovulation (with a wash-out period of 3 months prior to inclusion). Women were not excluded based on dietary intake and were encouraged to continue with their habitual diet during the study period. For women without PCOS, inclusion and exclusion criteria were the same as for women with PCOS, but they were normally menstruating women with no evidence of hyperandrogenism or polycystic ovaries.

Ethical Approval

The study was performed according to the Helsinki declaration and approved by The Regional Committee for Medical and Health Research Ethics in Central Norway (REK-midt 2015/468 and 2016/545) and the ACU Human Research Ethics Committee (2017-260H). Participants were informed about the experiments and potential risks verbally and their written consent was obtained prior to study entry.

Pre- and Post-intervention Testing

Figure 1 displays an overview of the experimental protocol. Participants with a regular menstrual cycle were tested during the early follicular phase (day 1–7 of the menstrual cycle) whereas women with oligo/amenorrhea were tested independent of the time of their cycle. Participants performed an incremental test to exhaustion on a treadmill to measure peak oxygen uptake (VO_2peak) and estimate maximal heart rate (HR_{max}) using an individualized protocol. Following a 10 min warm-up and 3 min at moderate-intensity, the treadmill speed or incline was increased every 1–2 min by 0.5-1.0 km/h or 1–2% until volitional fatigue. After an overnight fast (≥12 h) and refraining from exercise for ≥48 h, participants returned to the laboratory and body fat percentage (BF%) was estimated. In Australia, the BF% was estimated using dual-energy X-ray absorptiometry (DXA; GE Lunar iDXA Pro, Encore software version 16, General Electric, Boston, MA, United States). In Norway, the BF% was estimated using bioelectrical impedance analysis (InBody720, Biospace CO, South Korea). Waist and hip circumference were also measured to the nearest 0.5 cm in duplicate. A resting blood sample was collected in a 4 mL EDTA and a 5 mL serum tube from the antecubital vein. The EDTA tube was immediately spun at 2,200 rpm at 20°C for 10 min while the serum tube rested 30 min before it was spun with the same protocol. Plasma was collected and stored at −80°C for subsequent analysis. On the same day, abdominal and gluteal subcutaneous AT biopsies (300–500 mg) were collected using a 14-gauge needle under local anesthesia with 1% xylocaine and washed on gauze with saline. Blood and connective tissue were removed before approximately 100 mg was snap-frozen in liquid nitrogen and stored at −80°C for subsequent miRNA analyses. Non-PCOS women were tested for all the measures described at baseline, whereas all women with PCOS were tested at baseline and after the 16 weeks exercise intervention (**Figure 1**). To avoid any residual effects of the last exercise session on miRNA expression, resting plasma and AT were sampled ≥48 h following the last exercise session.

Training Protocols

Women with PCOS (n = 42) were randomized to 16 weeks of HIT or a non-exercising control group. Participants were stratified for a BMI < or ≥27 kg/m^2 and randomly assigned in a 1:1:1 manner to one of three groups: (1) LV-HIT (n = 13); (2) HV-HIT (n = 14); (3) Non-exercise (Non-Ex, n = 15). The training program has been described previously (Kiel et al., 2020). Briefly, exercise training was performed on a treadmill or as outdoor running/walking three times/week. Women assigned to the LV-HIT group completed 10 × 1 min work bouts at the maximal intensity they could sustain, separated by 1 min of passive recovery or low-intensity walking. Women in the HV-HIT group completed 4 × 4 min work bouts reaching 90–95% HR_{max} during the first two minutes of each bout, separated by 3 min of active recovery at ∼70% of HR_{max}. All training sessions included a 10 min warm-up and a 3-min cool-down period. At least one weekly session was supervised at the laboratory, and the participants wore HR monitors (Polar M400) during all sessions to ensure compliance with both exercise protocols. Women in the Non-Ex group were advised to continue their habitual physical activity and informed about the current recommendations for physical activity in adults.

Circulating miRNA Extraction and Reverse Transcription

After centrifuging thawed plasma samples for 10 min at 4°C in order to remove cellular debris, circulating miRNAs (c-miRs) were extracted using the miRNeasy Serum/Plasma Advanced Kit (217204; Qiagen, Australia), which allows for extraction and purification of small (<200 nt) cell-free RNA. A total of 200 μL

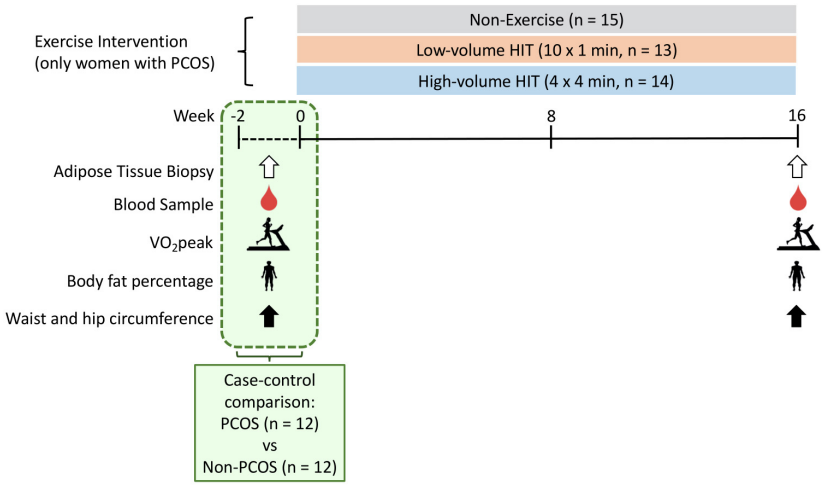

FIGURE 1 | Study protocol. The testing days included: VO_2peak, body fat percentage, waist and hip circumference measurements as well as blood and adipose tissue sampling. Non-PCOS (n = 12) women were only tested at baseline, whereas all women with PCOS (n = 42) were tested at baseline and after the 16 weeks exercise intervention. The 12 PCOS women in the case-control comparison also took part in the exercise intervention. Non-PCOS were age- and BMI-matched to these 12 women with PCOS.

supernatant was then transferred to a new sterile tube. To assist with determining the yield of template recovered, 3.5 μL of a miRNeasy Serum/Plasma Spike-In Control (219610; Qiagen, Australia) was added to all samples prior to extraction of RNA. Input amounts of RNA were standardized using the same initial volume (2 μL) for all samples following the manufacturer's instructions. RNA was reverse transcribed into cDNA by using the miRCURY LNA RT kit (339340; Qiagen, Australia) in a BioRad thermal cycler (BioRad, Australia). The resulting cDNA was stored at −20°C.

Adipose Tissue miRNA Extraction and Reverse Transcription

MicroRNAs were extracted from frozen abdominal and gluteal AT samples using the miRNeasy Mini (217004; Qiagen, Australia) and RNeasy MinElute Cleanup (74204; Qiagen, Melbourne, VIC, Australia) Kits according to manufacturer's instructions. Approximately 100 mg of AT was homogenized in QIAzol Lysis Reagent and phase separated with chloroform. Samples were then loaded into spin columns, washed with ethanol and eluted in 14 μL of RNAse-free water. Extracted miRNAs were quantified on a Qubit 4 Fluorometer using the Qubit microRNA Assay Kit (Q32881; Life Technologies, Australia). Samples were then equilibrated with RNase-free water and reverse transcribed to cDNA using the miRCURY LNA RT Kit (339340; Qiagen, Australia) in a BioRad thermal cycler (BioRad, Australia). The resulting cDNA was stored at −20°C.

Targeted MicroRNA Quantification

Quantification of both circulating and AT miRNAs were performed on a Qiagen customized 96-well miScript miRNA PCR Array (339330; Qiagen, Australia) with miScript SYBR using a BioRad CFX96 (BioRad, Australia) following manufacturer's instructions. The array contained eight miRNAs selected *a priori* based on previous reports of altered c-miRs profiles in women with PCOS and/or in response to exercise training (Murri et al., 2013; Sorensen et al., 2014; Improta et al., 2018; Da Silva et al., 2019). The selected c-miRs were hsa-miR-21-5p, hsa-miR-27b-5p, hsa-miR-93-5p, hsa-miR-146a-5p, hsa-miR-155-5p, hsa-miR-222-3p, hsa-miR-223-3p, and hsa-miR-103a-3p. These miRNAs have been shown to be implicated in hormone secretion and metabolism, inflammation, adipogenesis, and lipid and glucose metabolism (Chen W. et al., 2012; Murri et al., 2013; Sorensen et al., 2014; Chuang et al., 2015). The same eight miRNAs were investigated in the subcutaneous abdominal and gluteal AT (n = 8 for each group) to explore any "cross-talk" between the circulation and a potential target tissue, as accumulating evidence demonstrates that c-miRs can be transferred to and taken up by recipient cells and tissues where they can regulate their specific targets (Fabbri et al., 2012; Russell and Lamon, 2015).

PCR arrays were run using a miScript SYBR Green PCR Kit (339346; Qiagen, Australia). A quantification cycle above 40 in >50% of the samples was considered as absence of expression of the miRNA. Moreover, samples that exhibited low abundance (relative threshold cycle above 40) were excluded from analysis as indicated in **Table 3**. The array also contained an inter-plate calibrator, along with UniSp3 and UniSp6 RNA Spike-in controls, RNU1A1 and SNORD44. Due to low circulating expression of SNORD44, c-miR expression was normalized to the geometric mean of UniSp3, UniSp6, and RNU1A1. In contrast, AT miRNAs were normalized to the geometric mean of SNORD44, UniSp3, UniSp6, and RNU1A1. The $2^{\Delta\Delta}$CT method of relative

quantification was used to calculate the relative abundance of miRNAs in plasma and AT (Livak and Schmittgen, 2001).

Blood Biochemistry and Insulin Sensitivity

Plasma glucose and serum insulin concentrations and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) were determined for the case-control study (PCOS and Non-PCOS). Plasma glucose concentration was measured using a Roche Moduclar P (Roche, Switzerland), while serum insulin concentration was measured in duplicates using an enzyme-linked immunosorbent assay (ELISA; IBL-International, Germany). HOMA-IR was used as a method for quantifying insulin resistance; fasting serum (μU/mL) × fasting plasma glucose (mmol/L) divided by 22.5 (Matthews et al., 1985).

Statistics

Descriptive statistics are presented as means ± SD. Group means for PCOS vs Non-PCOS were compared by Student's two-tailed *t*-test for independent samples. We used linear mixed models with participant as random factor, and with two dummy variables to uniquely identify the two exercise groups (LV-HIT and HV-HIT) and their interactions, with time as fixed factors. We adjusted for the baseline values for the outcome variables as recommended by Twisk et al. (2018). In this model, the coefficients for the interaction terms give the estimated exercise training effects in LV-HIT and HV-HIT compared to the Non-Ex group. Paired *t*-tests were used to test for differences in miRNA expression in AT depots (gluteal versus abdominal) at baseline. To test if miRNA expression in the two AT depots would respond in distinct ways to the two HIT protocols, changes in miRNA expression were calculated by using post-intervention minus pre-intervention values (corresponding to the ΔmiR expression in **Table 6**). We used linear mixed models as detailed but with the two exercise groups' interaction with AT depots as fixed factors. Due to previously observed abnormal expression of miRNAs in subcutaneous AT in insulin resistant women regardless of PCOS status, we also analyzed the gluteal and abdominal subcutaneous AT miRNA expression based on insulin resistance. Insulin resistance was estimated as HOMA-IR, with similar cut-off points as previously reported (Chuang et al., 2015); HOMA-IR value <2.5 was considered normal whereas HOMA-IR ≥ 2.5 indicated insulin resistance. Group means for insulin resistant and non-insulin resistant women were compared by Student's two-tailed *t*-test for independent samples. Normality of residuals was evaluated using visual inspection of Q-Q plots and logarithmic transformation (log10) of the dependent variable was performed when necessary to obtain normality. Due to multiple hypotheses, we considered *P-values* <0.01 as statistically significant. Pearson correlation between miRNAs different between groups (PCOS and Non-PCOS) and baseline VO_2peak, BMI, fat percentage or waist/hip ratio was calculated using GraphPad Prism version 8.1.2 (GraphPad Software, United States). All other analyses were carried out using SPSS version 25.0 (SPSS Inc., United States).

RESULTS

Characteristics: PCOS vs Non-PCOS Women

Characteristics of the participants included in the case-control comparison are presented in **Table 1**. Apart from greater waist/hip ratio in women with PCOS (0.90 ± 0.06 versus 0.83 ± 0.05, $P = 0.005$), there were no differences between groups (**Table 1**). There was no difference in HOMA-IR between the groups, although nine of the women with PCOS versus five women without PCOS had HOMA-IR ≥ 2.5 (**Table 1**).

Physiological Responses to High-Intensity Interval Training

There were no changes in body weight, BMI, body fat percentage, waist, and hip circumference or waist/hip ratio for either LV-HIT or HV-HIT post intervention (**Table 2**). Although not significant, it is worth noting that the mean fat percentage decreased by 1.4% in the Non-Ex group. This is not a systemic decrease in fat percentage in the Non-Ex group but a result of two participants in this group decreasing by 6% in fat percentage. Following 16 weeks of HIT, absolute, but not relative, VO_2peak increased by 9% ($P = 0.008$) in HV-HIT (**Table 2**).

miRNA Expression Profile in PCOS vs Non-PCOS

Basal expression of circulating miRNA-27b (c-miR-27b) was higher in women with PCOS compared to Non-PCOS (1.8-fold, $P = 0.006$; **Figure 2B**), with no significant differences in the expression of c-miR-21, −93, −103a, −146a, −155, −222, and −223 (**Figures 2A–H**). No differences in basal gluteal and

TABLE 1 | Baseline characteristics of PCOS and Non-PCOS women.

	Non-PCOS	PCOS	*P-value*
n	12	12	
Age (years)	30 ± 7	30 ± 7	0.98
Body weight (kg)	83.0 ± 18.5	85.2 ± 19.7	0.78
BMI (kg/m^2)	29.3 ± 5.8	29.8 ± 6.5	0.85
Body fat percentage (%)	36.4 ± 9.2	36.8 ± 9.0	0.91
Waist circumference (cm)	94 ± 14	102 ± 14	0.16
Hip circumference (cm)	113 ± 13	113 ± 11	0.99
Waist/Hip ratio	0.83 ± 0.05	0.90 ± 0.06	**0.005**
VO_2peak (L/min)	2.83 ± 0.38	2.73 ± 0.38	0.54
VO_2peak (mL/min/kg)	34.9 ± 7.0	33.2 ± 6.1	0.53
Glucose (mmol/L)	4.9 ± 0.4	5.0 ± 0.6	0.52
Insulin (μU/mL)	14.2 ± 10.6	15.7 ± 6.9	0.71
HOMA-IR	3.1 ± 2.3	3.6 ± 1.8	0.58

Values are mean ± SD. PCOS, polycystic ovary syndrome; BMI, body mass index; VO_2peak, peak oxygen uptake; HOMA-IR, homeostatic model assessment of insulin resistance. In the Non-PCOS group, not enough blood was collected for analyzing insulin concentration, so n = 11 for insulin concentration and HOMA-IR in Non-PCOS. The meaning of a bold value is that the P-value is statistically significant.

TABLE 2 | Participant characteristics before and after 16 weeks of high-intensity interval training.

	Pre		Post		Difference (time × group)		
	n	Mean ± SD	*n*	Mean ± SD	Estimate	95% CI	*P-value*
Age (years)							
Non-Ex	15	28 ± 5					
LV-HIT	13	31 ± 5					
HV-HIT	14	30 ± 5					
Body weight (kg)							
Non-Ex	15	86.2 ± 20.1	15	84.1 ± 19.3			
LV-HIT	13	84.6 ± 19.1	13	81.8 ± 20.2	−0.75	−5.28 to 3.78	0.74
HV-HIT	14	92.6 ± 22.6	14	91.9 ± 23.4	1.66	−2.78 to 6.11	0.45
BMI (kg/m^2)							
Non-Ex	15	31.4 ± 6.9	15	30.6 ± 6.5			
LV-HIT	13	28.9 ± 6.6	13	28.8 ± 6.7	0.65	−0.08 to 1.38	0.08
HV-HIT	14	33.1 ± 7.4	14	32.8 ± 7.6	0.59	−0.13 to 1.30	0.11
Body fat percentage (%)							
Non-Ex	15	41.0 ± 8.3	15	39.6 ± 8.2			
LV-HIT	13	35.5 ± 10.7	13	35.3 ± 10.4	0.98	−0.75 to 2.72	0.26
HV-HIT	14	41.8 ± 7.9	14	41.2 ± 9.0	0.82	−0.88 to 2.52	0.34
Waist circumference (cm)							
Non-Ex	15	101 ± 16	15	99 ± 16			
LV-HIT	13	98 ± 17	12	95 ± 18	−0.63	−5.83 to 4.56	0.81
HV-HIT	14	110 ± 17	14	105 ± 16	−2.46	−7.44 to 2.53	0.33
Hip circumference (cm)							
Non-Ex	15	115 ± 14	15	114 ± 12			
HV-HIT	13	112 ± 14	12	111 ± 12	0.01	−3.32 to 3.35	0.99
LV-HIT	14	117 ± 14	14	115 ± 15	−1.22	−4.42 to 1.98	0.45
Waist/Hip ratio							
Non-Ex	15	0.88 ± 0.07	15	0.87 ± 0.09			
LV-HIT	13	0.87 ± 0.07	12	0.85 ± 0.09	−0.005	−0.05 to 0.04	0.81
HV-HIT	14	0.94 ± 0.06	14	0.91 ± 0.08	0.01	−0.03 to 0.05	0.65
VO_{2peak} (L/min)							
Non-Ex	15	2.73 ± 0.49	15	2.63 ± 0.45			
LV-HIT	13	2.85 ± 0.29	13	2.85 ± 0.39	0.13	−0.07 to 0.32	0.20
HV-HIT	14	2.81 ± 0.34	13	2.94 ± 0.38	0.27	0.07 to 0.46	**0.008**
VO_{2peak} (mL/kg/min)							
Non-Ex	15	32.2 ± 6.9	15	32.5 ± 7.5			
LV-HIT	13	34.4 ± 8.6	13	35.5 ± 8.0	0.88	−1.45 to 3.20	0.45
HV-HIT	14	31.7 ± 6.5	13	34.6 ± 8.0	1.97	−0.36 to 4.30	0.10

The difference (time × group) is the exercise training effect in LV-HIT and HV-HIT compared to the Non-Ex group. n, number of participants; SD, standard deviations; CI, confidence interval; Non-Ex, non-exercising group; LV-HIT, 10 × 1 min exercise group; HV-HIT, 4 × 4 min exercise group; BMI, body mass index; VO_{2peak}, peak oxygen uptake. The meaning of a bold value is that the P-value is statistically significant.

abdominal AT miRNA expression between women with PCOS and non-PCOS were observed (**Figures 3A–H**, **4A–H**). No correlations were observed between basal c-miR-27b expression and baseline VO_2peak, BMI, fat percentage, or waist/hip ratio (data not shown).

Effect of HIT on miRNA Expression Profile

The expression of the selected miRNAs in the circulation, gluteal and abdominal AT before and after 16 weeks of HIT in women with PCOS are summarized in **Tables 3–5**. In LV-HIT, the expression of c-miR-27b decreased significantly after training (0.5-fold, $P = 0.007$; **Table 3**), while the expression of c-miR-155 showed a tendency to decline ($P = 0.06$, **Table 3**). There were no changes in circulating miR-21, -93, -103a, -146a, -155, -222, and -223 in either of the groups. Chronic exercise training did not alter the expression of any of the selected miRNAs in the two AT depots in women with PCOS (**Tables 4**, **5**).

At baseline, none of the selected miRNAs were differentially expressed in gluteal compared to abdominal AT (data not shown). Furthermore, there were no differences in how the selected miRNAs changed in abdominal versus gluteal AT in response to either of the two HIT interventions (**Table 6**).

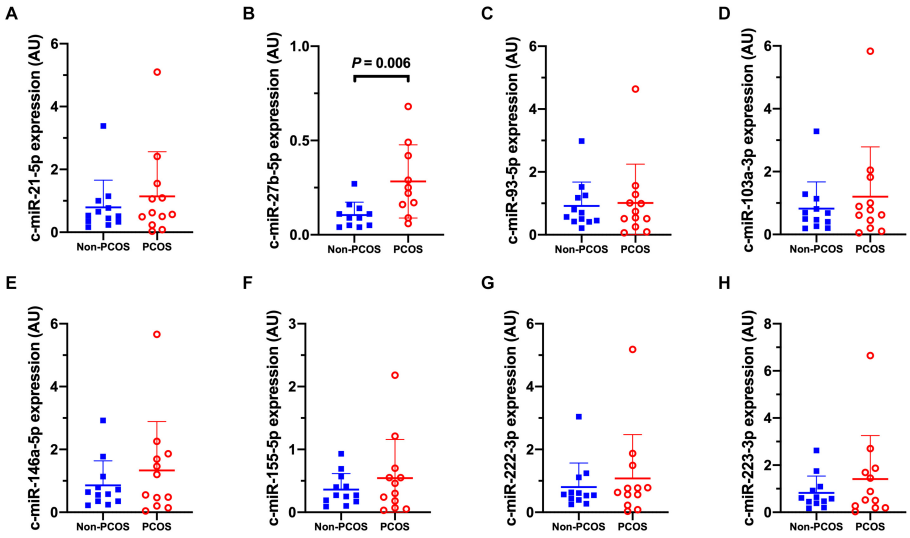

FIGURE 2 | Circulatory microRNA expression patterns. **(A)** c-miR-21-5p, **(B)** c-miR-27b-5p, **(C)** c-miR-93-5p, **(D)** c-miR-103a-3p, **(E)** c-miR-146a-5p, **(F)** c-miR-155-5p, **(G)** c-miR-222-3p, **(H)** c-miR-223-3p abundance in women with (PCOS; open, red circles) and without PCOS (Non-PCOS; blue squares). Values are arbitrary units expressed relative to the geometric mean of UniSp3, UniSp6 and RNU1A1. Individual data with group means and SD are displayed. PCOS, polycystic ovary syndrome; c, circulating; miR, microRNA; AU, arbitrary units.

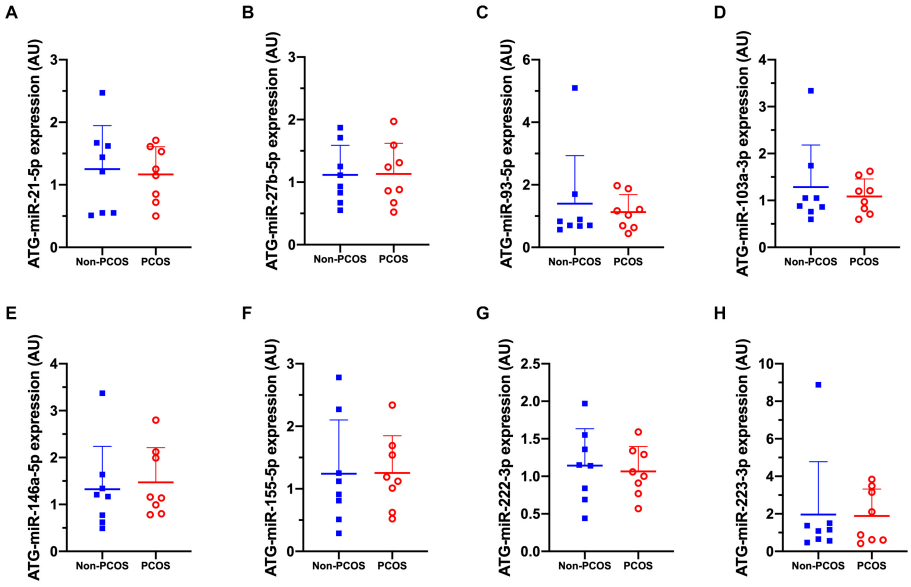

FIGURE 3 | Gluteal adipose tissue (ATG) microRNA expression patterns. **(A)** ATG-miR-21-5p, **(B)** ATG-miR-27b-5p, **(C)** ATG-miR-93-5p, **(D)** ATG-miR-103a-3p, **(E)** ATG-miR-146a-5p, **(F)** ATG-miR-155-5p, **(G)** ATG-miR-222-3p, **(H)** ATG-miR-223-3p abundance in women with (PCOS; open, red circles) and without PCOS (Non-PCOS; blue squares). Values are arbitrary units expressed relative to the geometric mean of SNORD44, UniSp3, UniSp6, and RNU1A1. Individual data with group means and SD are displayed. PCOS, polycystic ovary syndrome; ATG, gluteal adipose tissue; miR, microRNA; AU, arbitrary units.

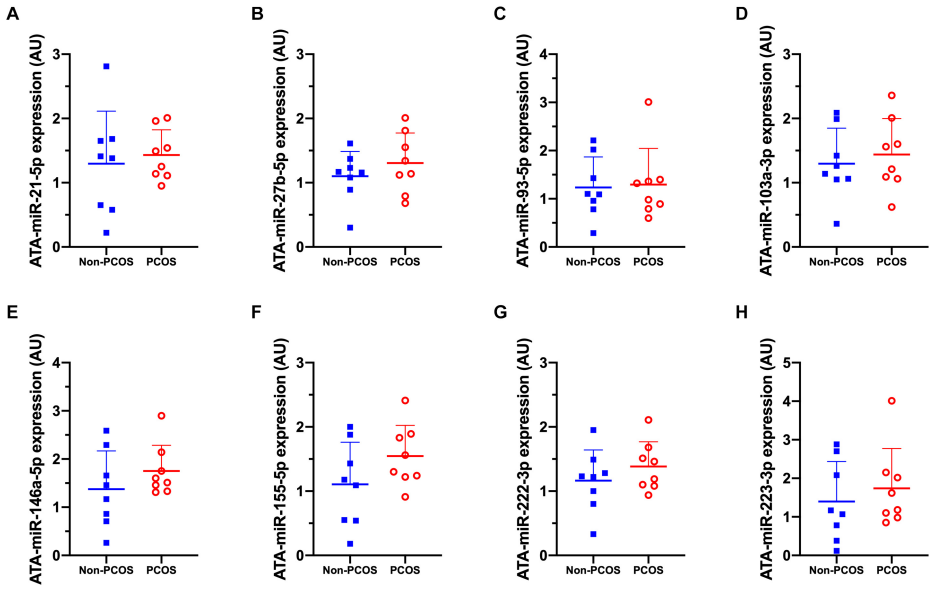

FIGURE 4 | Abdominal adipose tissue (ATA) microRNA expression patterns. **(A)** ATA-miR-21-5p, **(B)** ATA-miR-27b-5p, **(C)** ATA-miR-93-5p, **(D)** ATA-miR-103a-3p, **(E)** ATA-miR-146a-5p, **(F)** ATA-miR-155-5p, **(G)** ATA-miR-222-3p, **(H)** ATA-miR-223-3p abundance in women with (PCOS; open, red circles) and without PCOS (Non-PCOS; blue squares). Values are arbitrary units expressed relative to the geometric mean of SNORD44, UniSp3, UniSp6, and RNU1A1. Individual data with group means and SD are displayed. PCOS, polycystic ovary syndrome; ATA, abdominal adipose tissue; miR, microRNA; AU, arbitrary units.

Gluteal and Abdominal AT miRNA Expression Profile in Insulin Resistant and Non-insulin Resistant Women

There were no differences in basal gluteal and abdominal AT miRNA expression between insulin resistant and non-insulin resistant women regardless of PCOS status (data not shown). miR-103a tended to be lower in insulin resistant women in gluteal subcutaneous AT (P = 0.09) and miR-155 tended to be higher in insulin resistant women in abdominal subcutaneous AT (P = 0.08).

DISCUSSION

We report novel data regarding miRNA expression profiles in women with and without PCOS, along with responses to 16 weeks of HIT undertaken by women with PCOS. We show that basal expression of c-miR-27b was higher in women with PCOS compared to age- and BMI-matched women without PCOS. We also show that 16 weeks of exercise training induced a significant decrease in c-miR-27b in women with PCOS, despite no changes in the expression profile of the other targeted c-miRs. Finally, we show that miRNA expression from the two separate subcutaneous AT sites was unaffected by exercise training. Collectively, our findings provide new data regarding the effects of chronic exercise training and selected miRNAs responses in women with PCOS.

The first important finding from the current investigation was that basal c-miR-27b expression was higher in women with PCOS compared to age- and BMI-matched women without PCOS. miRNA-27b has been implicated in several cellular and metabolic processes including fatty acid metabolism, adipocyte differentiation, substrate metabolism, and inflammation (Fernandez-Valverde et al., 2011; Chen W. et al., 2012; Murri et al., 2013). Murri et al. (2013) reported that women without PCOS with a BMI > 30 kg/m^2 had lower basal whole blood miR-27b expression compared to women with normal weight (BMI < 25 kg/m^2), whereas obesity was associated with higher expression of miR-27b compared to normal weight in women with PCOS. These findings, together with our data, indicate divergent expression of basal c-miR-27b in women with and without PCOS. Previous work showed that overexpression of miR-27b attenuated the abundance of regulators of adipogenesis such as peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer-binding protein α (C/EBPα) (Lin et al., 2009), with PPAR-γ confirmed as a direct target of miR-27b (Karbiener et al., 2009). Similarly, work in diabetic rats and 3T3-L1 adipocytes revealed elevated expression of the closely related miR-27a within the retroperitoneal fat pad and in the presence of high glucose, respectively (Herrera et al., 2010). While these findings indicate a role for miR-27b in the etiology of adipogenesis and obesity, we found no relationship between basal c-miR-27b expression and BMI, fat percentage or waist/hip ratio, possibly indicating the cellular effects of miR-27b are likely

TABLE 3 | Circulatory miRNA expression before and after 16 weeks of high-intensity interval training.

	Pre		Post		Difference (group × time)
	n	Mean ± SD	*n*	Mean ± SD	*P-value*
c-miR-21b-5p					
Non-Ex	14	1.22 ± 0.59	15	2.03 ± 2.83	
LV-HIT	13	1.68 ± 1.84	13	1.61 ± 1.75	0.46
HV-HIT	14	1.61 ± 1.76	14	1.28 ± 0.94	0.30
c-miR-27b-5p					
Non-Ex	14	0.73 ± 0.50	15	1.15 ± 1.25	
LV-HIT	12	0.39 ± 0.79	12	0.19 ± 0.20	**0.007**
HV-HIT	11	0.23 ± 0.23	10	0.20 ± 0.10	0.12
c-miR-93-5p					
Non-Ex	14	1.07 ± 0.58	15	1.46 ± 1.57	
LV-HIT	13	1.61 ± 1.56	13	1.49 ± 1.34	0.83
HV-HIT	14	1.44 ± 1.46	14	1.83 ± 2.44	0.70
c-miR-103a-3p					
Non-Ex	14	1.13 ± 0.63	15	1.73 ± 1.99	
LV-HIT	13	1.75 ± 1.86	13	1.55 ± 1.55	0.59
HV-HIT	14	1.64 ± 1.79	14	1.46 ± 1.33	0.45
c-miR-146a-5p					
Non-Ex	14	1.91 ± 0.93	15	2.90 ± 3.46	
LV-HIT	13	1.61 ± 1.71	13	1.45 ± 1.28	0.19
HV-HIT	14	1.58 ± 1.72	14	1.33 ± 1.05	0.10
c-miR-155-5p					
Non-Ex	14	0.85 ± 0.53	15	1.25 ± 1.41	
LV-HIT	12	0.94 ± 0.92	13	0.69 ± 0.82	0.06
HV-HIT	14	0.70 ± 0.76	13	0.58 ± 0.39	0.37
c-miR-222-3p					
Non-Ex	14	0.95 ± 0.45	15	1.33 ± 1.27	
LV-HIT	13	1.56 ± 1.61	13	1.33 ± 1.30	0.59
HV-HIT	14	1.51 ± 1.59	14	1.39 ± 1.13	0.54
c-miR-223-3p					
Non-Ex	14	2.03 ± 1.17	15	3.16 ± 3.80	
LV-HIT	13	1.64 ± 1.86	13	1.55 ± 1.55	0.17
HV-HIT	14	1.69 ± 2.03	14	1.41 ± 1.03	0.14

The difference (time × group) is the exercise training effect in LV-HIT and HV-HIT compared to the Non-Ex group. n, number of participants; SD, standard deviations; Non-Ex, non-exercising group; LV-HIT, 10 × 1 min exercise group; HV-HIT, 4 × 4 min exercise group; c, circulating; miR, microRNA. The meaning of a bold value is that the P-value is statistically significant.

TABLE 4 | Gluteal adipose tissue miRNA expression before and after 16 weeks of high-intensity interval training.

	Pre		Post		Difference (group × time)
	n	Mean ± SD	*n*	Mean ± SD	*P-value*
ATG-miR-21-5p					
Non-Ex	8	1.07 ± 0.42	8	2.18 ± 3.09	
LV-HIT	8	1.07 ± 0.39	8	1.03 ± 0.54	0.25
HV-HIT	8	1.41 ± 1.25	8	1.49 ± 1.52	0.48
ATG-miR-27b-5p					
Non-Ex	8	1.07 ± 0.41	8	1.43 ± 1.46	
LV-HIT	8	1.12 ± 0.55	8	0.96 ± 0.25	0.53
HV-HIT	8	1.02 ± 0.56	8	1.29 ± 1.28	0.69
ATG-miR-93-5p					
Non-Ex	8	1.17 ± 0.78	8	1.94 ± 2.92	
LV-HIT	8	1.09 ± 0.47	8	0.99 ± 0.58	0.41
HV-HIT	8	0.91 ± 0.54	8	0.89 ± 0.59	0.19
ATG-miR-103a-3p					
Non-Ex	8	1.09 ± 0.52	8	2.12 ± 3.25	
LV-HIT	8	1.07 ± 0.36	8	0.97 ± 0.47	0.18
HV-HIT	8	0.95 ± 0.37	8	1.05 ± 0.69	0.15
ATG-miR-146a-5p					
Non-Ex	8	1.03 ± 0.30	8	2.01 ± 2.91	
LV-HIT	8	1.19 ± 0.79	8	0.94 ± 0.64	0.20
HV-HIT	8	1.72 ± 1.73	8	1.52 ± 1.24	0.89
ATG-miR-155-5p					
Non-Ex	8	1.04 ± 0.33	8	2.79 ± 4.38	
LV-HIT	8	1.10 ± 0.56	8	1.13 ± 0.69	0.19
HV-HIT	8	1.47 ± 1.37	8	1.47 ± 1.22	0.43
ATG-miR-222-3p					
Non-Ex	8	1.06 ± 0.41	8	2.10 ± 3.13	
LV-HIT	8	1.07 ± 0.41	8	0.95 ± 0.35	0.16
HV-HIT	8	1.02 ± 0.55	8	1.06 ± 0.65	0.18
ATG-miR-223-3p					
Non-Ex	8	1.63 ± 2.03	8	1.87 ± 2.60	
LV-HIT	8	1.46 ± 1.40	8	1.21 ± 1.37	0.39
HV-HIT	8	1.31 ± 1.06	8	1.11 ± 0.79	0.48

The difference (time × group) is the exercise training effect in LV-HIT and HV-HIT compared to the Non-Ex group. n, number of participants; SD, standard deviations; Non-Ex, non-exercising group; LV-HIT, 10 × 1 min exercise group; HV-HIT, 4 × 4 min exercise group; ATG, gluteal adipose tissue; miR, microRNA.

to be confined more locally to within particular AT depots rather than whole body in such adipogenic models (Karbiener et al., 2009; Herrera et al., 2010).

Accumulating evidence demonstrates the capacity of vesicle-carrying miRNAs within the circulatory system to be released into the cytosol of recipient cells where they can regulate specific mRNA targets (Mittelbrunn et al., 2011; Fabbri et al., 2012). As such, we investigated miRNA expression within the subcutaneous abdominal and gluteal AT depots of women with PCOS. Somewhat surprisingly, and in contrast to one of our original hypotheses, we found no differences in miRNA expression signatures between women with and without PCOS in either of the subcutaneous adipose sites. Previously, Chuang et al. (2015) reported significantly higher miR-223 expression in subcutaneous abdominal AT in insulin resistant women regardless of PCOS status. However, when data from insulin resistant (HOMA-IR $\geq$ 2.5) and insulin sensitive (HOMA-IR $\leq$ 2.5) women were pooled, there was no difference in miR-223 expression between women with and without PCOS (Chuang et al., 2015). Similarly, the expression patterns of other subcutaneous abdominal AT miRNAs have been reported to be similar between women with and without PCOS, but to be different between insulin resistant (HOMA-IR $\geq$ 2.5) and insulin sensitive (HOMA-IR $<$ 2.5) women (Wu et al., 2014). We found no differences in either gluteal or abdominal AT miRNA expression profiles between insulin resistant and non-insulin resistant women (regardless of PCOS status). Our ability to detect

TABLE 5 | Abdominal adipose tissue miRNA expression before and after 16 weeks of high-intensity interval training.

	Pre		Post		Difference (group × time)
	n	Mean ± SD	*n*	Mean ± SD	*P-value*
ATA-miR-21-5p					
Non-Ex	8	1.18 ± 0.76	8	0.86 ± 0.39	
LV-HIT	8	1.34 ± 0.93	8	1.06 ± 0.56	0.57
HV-HIT	8	1.33 ± 0.62	8	1.73 ± 2.00	0.19
ATA-miR-27b-5p					
Non-Ex	8	1.05 ± 0.41	8	0.90 ± 0.37	
LV-HIT	8	1.23 ± 0.71	8	0.87 ± 0.33	0.93
HV-HIT	8	1.01 ± 0.42	8	1.01 ± 0.66	0.75
ATA-miR-93-5p					
Non-Ex	8	1.03 ± 0.28	8	1.10 ± 0.73	
LV-HIT	8	1.33 ± 1.01	8	1.15 ± 0.60	0.67
HV-HIT	8	1.08 ± 0.28	8	1.60 ± 1.37	0.33
ATA-miR-103a-3p					
Non-Ex	8	1.04 ± 0.31	8	0.96 ± 0.34	
LV-HIT	8	1.19 ± 0.67	8	1.18 ± 0.33	0.26
HV-HIT	8	1.23 ± 0.40	8	1.38 ± 0.66	0.15
ATA-miR-146a-5p					
Non-Ex	8	1.10 ± 0.50	8	0.94 ± 0.39	
LV-HIT	8	1.44 ± 1.10	8	1.38 ± 1.10	0.52
HV-HIT	8	1.47 ± 0.74	8	2.16 ± 2.00	0.07
ATA-miR-155-5p					
Non-Ex	8	1.14 ± 0.57	8	0.80 ± 0.29	
LV-HIT	8	1.38 ± 0.98	8	1.36 ± 1.01	0.22
HV-HIT	8	1.40 ± 0.65	8	1.56 ± 0.87	0.07
ATA-miR-222-3p					
Non-Ex	8	1.03 ± 0.27	8	1.05 ± 0.42	
LV-HIT	8	1.19 ± 0.55	8	1.04 ± 0.31	0.89
HV-HIT	8	1.15 ± 0.48	8	1.27 ± 0.78	0.49
ATA-miR-223-3p					
Non-Ex	8	1.11 ± 0.60	8	1.50 ± 1.22	
LV-HIT	8	1.75 ± 1.82	8	2.54 ± 3.60	0.73
HV-HIT	8	1.83 ± 1.16	8	1.79 ± 1.58	0.91

The difference (time × group) is the exercise training effect in LV-HIT and HV-HIT compared to the Non-Ex group. n, number of participants; SD, standard deviations; Non-Ex, non-exercising group; LV-HIT, 10 × 1 min exercise group; HV-HIT, 4 × 4 min exercise group; ATA, abdominal adipose tissue; miR, microRNA.

TABLE 6 | Adipose tissue depots differences (gluteal versus abdominal) in miRNA expression response to high-intensity interval training.

	Gluteal AT		Abdominal AT		Difference (group × AT depots)
	n	Mean ± SD	*n*	Mean ± SD	*P-value*
ΔmiR-21-5p					
Non-Ex	8	1.11 ± 3.13	8	−0.32 ± 0.52	
LV-HIT	8	−0.04 ± 0.52	8	−0.28 ± 1.01	0.96
HV-HIT	8	0.08 ± 2.19	8	0.40 ± 1.77	0.73
ΔmiR-27b-5p					
Non-Ex	8	0.36 ± 1.72	8	−0.15 ± 0.43	
LV-HIT	8	−0.16 ± 0.57	8	−0.37 ± 0.68	0.80
HV-HIT	8	0.27 ± 1.50	8	0.00 ± 0.79	0.78
ΔmiR-93-5p					
Non-Ex	8	0.77 ± 0.07	8	0.06 ± 0.57	
LV-HIT	8	−0.10 ± 0.87	8	−0.18 ± 1.26	0.98
HV-HIT	8	−0.02 ± 0.88	8	0.52 ± 1.27	0.77
ΔmiR-103a-3p					
Non-Ex	8	1.03 ± 3.35	8	−0.08 ± 0.32	
LV-HIT	8	−0.09 ± 0.61	8	−0.01 ± 0.86	0.87
HV-HIT	8	0.10 ± 0.87	8	0.15 ± 0.56	0.95
ΔmiR-146a-5p					
Non-Ex	8	0.97 ± 2.86	8	−0.17 ± 0.43	
LV-HIT	8	−0.26 ± 0.67	8	−0.07 ± 1.19	0.97
HV-HIT	8	−0.20 ± 2.34	8	0.68 ± 2.09	0.48
ΔmiR-155-5p					
Non-Ex	8	1.75 ± 4.42	8	−0.33 ± 0.49	
LV-HIT	8	0.03 ± 0.78	8	−0.03 ± 1.15	0.63
HV-HIT	8	0.00 ± 2.04	8	0.16 ± 0.91	0.84
ΔmiR-222-3p					
Non-Ex	8	1.04 ± 3.24	8	0.02 ± 0.44	
LV-HIT	8	−0.13 ± 0.49	8	−0.15 ± 0.69	0.71
HV-HIT	8	0.04 ± 1.00	8	0.13 ± 0.94	0.92
ΔmiR-223-3p					
Non-Ex	8	0.24 ± 1.09	8	0.39 ± 1.17	
LV-HIT	8	−0.25 ± 2.02	8	0.79 ± 2.77	0.53
HV-HIT	8	−0.19 ± 1.46	8	−0.04 ± 1.57	0.52

n, number of participants; SD, standard deviations; Non-Ex, non-exercising group; LV-HIT, 10 × 1 min exercise group; HV-HIT, 4 × 4 min exercise group; AT, adipose tissue; miR, microRNA. ΔmiR corresponds to the changes in miRNA expression calculated by using post-intervention minus pre-intervention values.

significant differences between these cohorts may, in part, be due to a lack of statistical power because of low subject numbers in the present study.

Despite women with PCOS having significantly higher basal expression of c-miR-27b, there were no differences in the tissue abundance of this miRNA in either subcutaneous abdominal or gluteal AT. This "disconnect" in miRNA expression between the circulatory system and AT could be due to several reasons. First, the origin of c-miR-27b is unknown. Second, the subcutaneous AT depots may lack the specific membrane transporters to import this miRNA from the circulation (Icli and Feinberg, 2017). Third, temporal differences in miRNA expression are likely to exist between different body compartments (i.e., in the systemic circulation versus various tissues/organs). Future work will be required to underpin the mechanistic basis of miRNA "cross-talk" between the circulation and target tissues, and may provide important information linking PCOS and adipogenesis.

Another finding from the current investigation was the decrease in c-miR-27b expression in women with PCOS after 16 weeks of low-, but not high-volume, HIT. It is difficult to explain such a divergent response, especially as only the high-volume HIT protocol was associated with a significant increase in cardiorespiratory fitness. While little is known about c-miR-27b expression patterns following exercise training, Barber et al. (2019) recently reported that 20 weeks of moderate-intensity endurance training in middle aged men and non-PCOS women was associated with a two-fold increase in c-miR-27b (Barber et al., 2019). The discrepancy between our current work and the

findings by Barber et al. (2019) may be explained by differences in exercise modality. HIT versus moderate-intensity continuous endurance training can differentially influence cardiometabolic risk factors including blood pressure, low- and high-density lipoproteins, body weight and insulin sensitivity in patients with cardiometabolic diseases (Weston et al., 2014). Additionally, diverse sampling timepoint (24 h post last training session in the study by Barber et al. (2019) compared to≥48 h in our study) and study participants (middle aged men and women without PCOS compared to women with PCOS in our study) may, in part, help explain these divergent findings. Parr et al. (2016), Denham et al. (2018), Barber et al. (2019), and Da Silva et al. (2019) have previously reported that chronic exercise training programs can significantly modulate c-miRNA expression in healthy individuals and in those with overweight/obesity. The physiological significance of such responses are unclear and difficult to explain due to a variety of different miRNA quantification techniques reported, differences in sampling time points, a range of exercise protocols, and divergent clinical cohorts (Gomes et al., 2015). Time-course studies are needed to span the acute and chronic sampling points for a more comprehensive coverage of miRNA expression profiles.

We found no changes in miRNA expression in either subcutaneous abdominal or gluteal AT after high- or low-volume HIT protocols in women with PCOS. This observation is in agreement with recent data from Tsiloulis et al. (2017) who reported no effect of 6 weeks of endurance exercise training (four supervised sessions/week; three moderate intensity sessions; and one HIT session) on the expression of 526 miRNAs in abdominal and gluteal AT in overweight males. Collectively, these findings indicate miRNAs are stably expressed in both abdominal and gluteal AT and are largely unaltered in response to exercise training protocols lasting up to several months. Furthermore, at baseline, we found no difference in expression of the eight selected miRNAs in basal gluteal versus abdominal AT. Our findings are largely supported by the results of Tsiloulis et al. (2017), who reported only four out of the 526 identified miRNAs to be differentially expressed between gluteal and abdominal adipocytes in overweight males. In addition, we found no differentially expressed miRNAs between the AT depots following exercise training. Other studies have reported that AT from different depots express unique molecular, cellular, and metabolic properties and respond in distinct ways to exercise and nutritional challenges (Tan et al., 2004; You et al., 2014). However, this does not seem to be the case for selected miRNA expression.

A major strength of the present study is the concurrent investigation of miRNA expression patterns in the circulation and abdominal and gluteal AT. Some miRNAs exert specificity in tissue expression within the body and can either be released or taken up by these tissues from the bloodstream (Russell and Lamon, 2015). Thus, investigation of targeted miRNAs from both the circulation and a "target" tissue allowed us to compare expression patterns between these tissues. We also acknowledge study limitations: we used a targeted approach to selectively investigate a small number of miRNAs and therefore may have missed changes in expression patterns of other miRNAs. Finally, larger subject numbers would have conferred greater statistical power to detect small, but potential differences in miRNA expression.

In conclusion, we report that the basal expression of circulating miR-27b was higher in women with PCOS compared to women without PCOS. We were unable to detect any differences in the eight targeted miRNAs in subcutaneous abdominal and gluteal AT. While 16 weeks of low- but not high-volume HIT altered the expression of c-miR-27b in women with PCOS, the chronic exercise training protocols employed in this investigation did not induce alterations in the expression of the other targeted miRNAs within the circulation or the miRNAs in subcutaneous abdominal and gluteal AT in women with PCOS. Further studies are required to ascertain miR-27b's role and association with obesity, inflammation and adipogenesis in PCOS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Regional Committee for Medical and Health Research Ethics in Central Norway (REK-midt 2015/468 and 2016/545) and the Australian Catholic University Human Research Ethics Committee (2017-260H). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SL and DC drafted the manuscript, performed the analyses, and analyzed the data. SL and SLy performed statistical analyses. SL, IK, DC, EV, JH, and TM were responsible for study conception and design. SL, IK, EP, and TM coordinated the study at the two sites, performed measurements on test-days, and supervised the exercise training. All authors provided feedback and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper IV

Absent exercise-induced improvements in fat oxidation in women with polycystic ovary syndrome after high-intensity interval training

Sofie Lionett[1,2], Ida A. Kiel[1,2], Ragnhild Røsbjørgen[1], Stian Lydersen[3], Steen Larsen[4,5] and Trine Moholdt[1,2*]

[1]Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trondheim, Norway

[2]Department of Obstetrics and Gynecology, St. Olav's Hospital, Trondheim, Norway

[3]Regional Centre for Child and Youth Mental Health and Child Welfare, Department of Mental Health, Norwegian University of Science and Technology, Trondheim, Norway

[4]Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark

[5]Clinical Research Centre, Medical University of Bialystok, Bialystok, Poland

*** Correspondence:**

Dr. Trine Moholdt

Trine.Moholdt@ntnu.no

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Abstract

Background: Polycystic ovary syndrome (PCOS) and metabolic inflexibility are linked to insulin resistance, and women with PCOS appear to be metabolic inflexible in the rested, insulin-stimulated state. Exercise training is a primary lifestyle intervention in PCOS. Exercise training improves whole-body fat oxidation during submaximal exercise in healthy women, yet little is known about the effect on this outcome in women with PCOS.

Methods: We measured whole-body fat oxidation during submaximal exercise before and after 16 weeks of high-intensity interval training (HIT) in women with PCOS randomly allocated to either: low- or high-volume HIT (n=41; low-volume HIT, 10 x 1 min work bouts at maximal, sustainable intensity and high-volume HIT, 4 x 4 min work bouts at 90-95% of maximal heart rate) or non-exercise control (n=23), and in women without PCOS (Non-PCOS) allocated to low- or high volume HIT (n=15). HIT was undertaken three times weekly. In a subset of women with and without PCOS, we measured mitochondrial respiration in abdominal and gluteal subcutaneous adipose tissue using high-resolution respirometry, as well as fat cell sizes in these tissues.

Results: At baseline, women with PCOS had lower whole-body fat oxidation and mitochondrial respiration rates in abdominal adipose tissue compared to Non-PCOS. Peak oxygen uptake (mL/min/kg) increased in women with PCOS (~4%, p = 0.006) and Non-PCOS (~6%, p = 0.003) after 16 weeks of HIT. Whole-body fat oxidation only improved in Non-PCOS after HIT. No changes were observed in mitochondrial respiration and cell size in abdominal and gluteal adipose tissue after HIT in either group of women.

Conclusion: We observed exercise-induced improvements in whole-body fat oxidation during submaximal exercise in Non-PCOS, but not in women with PCOS, after 16 weeks of HIT, suggesting metabolic inflexibility in women with PCOS.

Clinical Trial Registration: www.clinicaltrials.gov, identifier NCT02419482 and NCT02943291.

Abstract word count: 295 words.

1.0 Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-age women, affecting up to 13% of women globally (Bozdag et al., 2016). PCOS is associated with increased risk of infertility, insulin resistance, type 2 diabetes and cardiovascular diseases (Moran et al., 2010, de Groot et al., 2011, Teede et al., 2010). Despite the high prevalence and adverse health complications of PCOS, the underlying mechanisms and optimal treatment are still unclear. Insulin resistance and the compensatory hyperinsulinemia is proposed to play a central role in the pathophysiology, contributing to the metabolic and reproductive features of PCOS (Diamanti-Kandarakis and Papavassiliou, 2006).

Metabolic flexibility is the ability to alter substrate use in response to a physiological stimulus, including the transition from fasting to fed states/insulin stimulation or exercise (Goodpaster and Sparks, 2017). Metabolic inflexibility, characterized by distorted nutrient sensing, blunted substrate switching, and impaired energy homeostasis, is linked to insulin resistance (Goodpaster and Sparks, 2017). Women with PCOS appear to have higher metabolic inflexibility in the rested, insulin-stimulated state compared to unaffected women (Rimmer et al., 2020). Previous studies have suggested that the insulin resistance observed in PCOS may be linked to aberrant adipose tissue morphology and function including enlarged adipocytes and decreased insulin-stimulated rates of glucose utilization in adipocytes (Dunaif et al., 1992, Manneras-Holm et al., 2011).

Lifestyle modification including regular physical activity is regarded as first-line therapy in women with PCOS (Teede et al., 2018). Exercise training induces a multitude of

positive, health-related outcomes in women with PCOS (Kite et al., 2019), and superior effects of vigorous intensity exercise compared to moderate intensity training, have been observed among women with PCOS (Greenwood et al., 2016, Patten et al., 2020).

The aims of this study were first to compare whole-body fat oxidation during submaximal exercise, cell size and mitochondrial respiration of adipose tissue in women with and without PCOS, and second to assess the response to 16 weeks of HIT.

2.0 Materials and Methods

2.1 Study design

The present study is a secondary analysis of a two-center, randomized controlled trial; IMproving Reproductive function in women with Polycystic OVary syndrome with high-intensity Interval Training (IMPROV-IT (Kiel et al., 2020); ClinicalTrials.gov identifier: NCT02419482) conducted at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway and the Australian Catholic University (ACU) in Melbourne, VIC, Australia, and a randomized, uncontrolled trial undertaken at NTNU (The Adipose Tissue Function and Response to Exercise Training in Women With and Without Polycystic Ovary Syndrome trial (HIT-FAT); ClinicalTrials.gov identifier: NCT02943291). The detailed study design for the IMPROV-IT trial has been published elsewhere (Kiel et al., 2020). In brief, after stratification for BMI $<$ or $\geq$ 27 kg/m^2 and study center, women with PCOS were randomized in a 1:1:1 manner to 16 weeks of semi-supervised HIT or a no-exercise control

group: 1) Low-volume HIT (LV-HIT), 2) High-volume HIT (HV-HIT), or 3) Non-exercise (Non-Ex).

In the HIT-FAT trial, women without PCOS were selected as a control group, and individually matched by age (± 5 years) and BMI (± 2 kg/m^2) to women with PCOS in the IMPROV-IT trial. Women without PCOS were randomly allocated (1:1), after stratification for BMI < or ≥ 27 kg/m^2, to 16 weeks of semi-supervised LV-HIT or HV- HIT. For the purpose of the analyses in this report and because no differences were observed between the LV-HIT and HV-HIT groups, we pooled the LV-HIT and HV-HIT groups into one group for women with PCOS (PCOS HIT) and one group for women without PCOS (Non-PCOS HIT).

2.2 Ethical approval

The studies were performed according to the Helsinki declaration and approved by The Regional Committee for Medical and Health Research Ethics in Central Norway (REK-midt 2015/468 and 2016/545), and the ACU Human Research Ethics Committee (2017-260H). Participants were informed about the experiments and potential risks verbally and in writing before their written consent was obtained.

2.3 Participants

Sixty-four previously inactive women with PCOS and 15 previously inactive women without PCOS were included in this study. PCOS was defined according to the Rotterdam criteria (Rotterdam, 2004), with at least two of the following three features present: polycystic ovary

morphology (12 or more 2-9 mm follicles or >10 ml in volume in at least one ovary), hyperandrogenism (either clinical signs such as acne or hirsutism, or biomedical) and/or oligo/amenorrhea. Hirsutism was defined as a modified Ferriman-Gallwey score of ≥8 (Ferriman and Gallwey, 1961). The PCOS diagnosis was ruled out in all the women without PCOS as they were normally menstruating, with no evidence of hyperandrogenism or polycystic ovaries.

To be eligible for inclusion, the women had to be between 18 and 45 years old and were excluded if they were undertaking regular endurance training ≥ 2 sessions/week, had any cardiovascular diseases or endocrine disorders, were pregnant, had been breastfeeding within the last 24 weeks, or if they were using hormonal contraceptives, insulin sensitizers or drugs known to affect gonadotropin or ovulation (with a washout period of 3 months prior to inclusion).

2.4 Interventions

The exercise training was semi-supervised during the 16 weeks intervention period, and participants attended at least one weekly supervised training session, with the opportunity to perform the two remaining weekly sessions either supervised at the study centers or unsupervised (total of three exercise sessions per week).

The exercise training protocols have been described previously (Kiel et al., 2020). Briefly, participants walked or ran on treadmills during the supervised exercise sessions, while they could choose to perform the unsupervised exercise sessions walking or running on treadmills or outdoors. The LV-HIT protocol consisted of 10 × 1 min intervals at the

maximal intensity the participants could sustain, interspersed by 1 min of passive recovery or low-intensity walking. The HV-HIT protocol comprised 4 × 4 min intervals at an intensity corresponding to 90-95% maximal heart rate (HR_{max}) interspersed by 3 min active recovery at ~70% of HR_{max}. All training sessions included 10 min warm-up and 3 min cool-down. Participants wore HR monitors (Polar M400) during all exercise sessions, and registered their exercise sessions via an online exercise-training diary (Polar Flow) which the researchers had access to. Thereby, the researchers could supervise adherence to the protocols.

Women with PCOS assigned to the Non-Ex group were advised to continue their habitual physical activity and informed about the current recommendations of at least 150 min weekly of moderate intensity physical activity. All participants were instructed to maintain their habitual diet throughout the intervention period, and dietary intake was controlled using a 4-day diet recall at baseline and in the last week of the intervention. Physical activity level was monitored using activity monitors (Sensewear Armband, APC Cardiovascular, UK) for five days at baseline and during the last week of the intervention period.

2.5 Outcomes

Figure 1 displays an overview of the study protocol. Outcomes were assessed at baseline and after 16 weeks of intervention. All assessments were performed in the early follicular phase (day 1-7 after first bleeding) of the participants' menstrual cycle in women with a regular menstrual cycle while women with oligo/amenorrhea were tested independent of their cycle day.

Participants visited the laboratory on three separate occasions at baseline and after 16 weeks. On the first visit, participants performed an incremental test to exhaustion on a treadmill. We used an individualized protocol to measure peak oxygen uptake (VO_2peak) and maximal heart rate (HR_{max}); after 10 min warm-up and 3 min at moderate intensity, the treadmill speed or inclination was increased every 1-2 min by 0.5-1.0 km/h or 1-2% until volitional exhaustion. The recorded HR_{max} was used to calculate the intensity for the HIT sessions (Berglund et al., 2019).

On the second visit, participants returned to the laboratory after an overnight fast (≥12 hours) and refraining from exercise >48 hours prior. In Norway, body composition was estimated using bioelectrical impedance analysis (InBody 720, Biospace CO, Korea) while Dual-energy X-ray absorptiometry (DXA; GE Lunar iDXA Pro, Encore software version 16, General Electric, Boston, MA, USA) was used in Australia. Body composition was estimated using the same method in each individual at each timepoint. Waist and hip circumference were measured in duplicate to the nearest 0.5 cm. A resting blood sample was collected in a 5 mL serum tube and rested for 30 min in room temperature before it was spun at 2,200 rpm at 20°C for 10 min. Serum was collected and stored at -80 °C for further analysis. On the same day, abdominal and gluteal subcutaneous adipose tissue biopsies were obtaining using a 14-gauge needle under local anesthesia (1 % Xylocaine) excising ~300-500 mg of tissue from each depot. The adipose tissue was washed on gauze with saline, and capillaries and connective tissues were removed. Approximately 80 mg was allocated for immediate analysis of mitochondrial respiration using high-resolution respirometry (Oxygraph-2K, Oroboros, Innsbruck, Austria). Some of the remaining tissue (~200-300 mg) was snap-frozen

in liquid nitrogen and stored at -80 °C for later analysis, whereas some was immediately fixed in phosphate-buffered formalin for subsequent fat cell size analysis. We were not always able to obtain adipose tissue biopsies from both depots and/or unable to excise enough adipose tissue for all analyses, and this is why the number of samples is lower in mitochondrial respiration and morphology measurements.

On the third visit and following an overnight fast, participants performed a submaximal test on a treadmill. The protocol included 20 min warm-up without gas sampling, followed by 20 minutes of steady-state workload at 60% of VO_2peak with sampling of the expired gas. Participants recorded their dietary intake the day prior to baseline testing and repeated this diet before the subsequent measures at 16 weeks. Fat oxidation rates (g/min) was calculated from 5 min of steady oxygen uptake during the last 10 min of the test; 1.695 x VO_2 – 1.701 x VCO_2, where VO_2 is oxygen uptake and VCO_2 is expired carbon dioxide (Jeukendrup and Wallis, 2005).

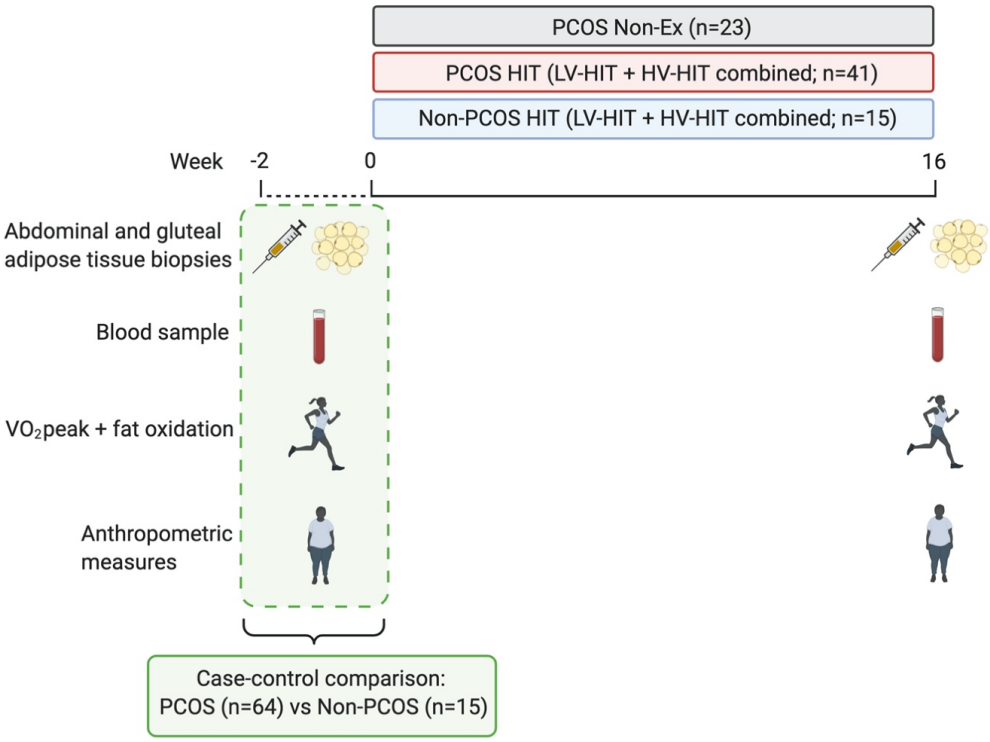

Figure 1: Study protocol. The testing days included: abdominal and gluteal subcutaneous adipose tissue biopsies, fasting blood sampling, VO_2peak, submaximal exercise test to measure fat oxidation rates, body composition measures, and waist and hip circumference. The adipose tissue was used to measure mitochondrial respiration and cell size. The testing days were repeated after a 16 weeks intervention period of either performing high-intensity interval training or no exercise. PCOS, Polycystic ovary syndrome; Non-Ex, no exercise; HIT, high-intensity interval training; LV-HIT, low-volume high-intensity interval training; HV-HIT, high-volume high-intensity interval training; VO_2peak, peak oxygen uptake. Created with BioRender.com.

2.6 Blood analyses

Plasma glucose concentrations were determined using a Roche Moduclar P (Roche, Switzerland) and serum insulin concentrations were measured in duplicate using an enzyme-linked immunosorbent assay (ELISA; IBL-International, Germany). We estimated insulin resistance using the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR); fasting serum insulin (μIU/mL) x fasting plasma glucose (mmol/L) divided by 22.5 (Matthews et al., 1985). HOMA-IR < 2.5 was considered normal while HOMA-IR ≥ 2.5 indicated insulin resistance. These cut-off points for insulin resistance have been used previously in the PCOS literature (Chuang et al., 2015). HbA1c was analyzed on Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 version 5.24 in Norway and using Cobas b 101 system (Roche Diagnostics) in Australia. We measured testosterone and sex-hormone-binding globulin (SHBG) concentrations in women with PCOS. Total testosterone concentrations were analyzed with Agilent 1290 with 6410 Triple Quad LC/MS-MS detector (Agilent, Santa Clara, United States) SHBG concentrations were analyzed with Advia Centaur XPT (Siemens, Erlangen, Germany).

2.7 Mitochondrial respiration

Mitochondrial respiration in abdominal and gluteal subcutaneous adipose tissue was measured in a subset of participants. We measured mitochondrial respiration in the abdominal adipose tissue from 10 Non-PCOS women and 16 women with PCOS, and in gluteal adipose tissue from 14 Non-PCOS women and 18 women with PCOS.

Mitochondrial respiration measurements were carried out in duplicate in ~40 mg abdominal and gluteal subcutaneous adipose tissue using high-resolution respirometry (Oxygraph-2K respirometer; Oroboros, Innsbruck, Austria). Each of the two chambers of this instrument contained 2 mL Buffer Z (1 mM EGTA; 5mM $MgCl_26H_2O$; 105 mM K-Mes; 10 mM KH_2PO_4; 5 mg/mL BSA; pH 7.1 at 37°C). All measurements were performed at 37°C and oxygen concentrations > 100 nmol/mL.

We used a protocol modified from Kraunsoe et al. (Kraunsoe et al., 2010). Amplex Ultra Red (10 µM), Peroxidase from horseradish (HRP; 1 U/mL) and Superoxide dismutase (SOD; 5 U/mL) were titrated into the chambers and stabilized before ~40 mg adipose tissue was added. Digitonin (2 µM) was added to the chambers to permeabilize the cells and baseline respiration was measured before substrates and inhibitors were added. Malate (2 mM) and Octanoyl carnitine (1.5 mM) were added together for measurement of a stable respiration with electron input through complex I and from ß-oxidation. Thereafter, Adenosine diphosphate (ADP; 5 mM) was added to stimulate phosphorylation and obtain maximum electron flow through electron transporting flavoprotein. Pyruvate (5 mM) and Glutamate (10 mM) were then added to measure state 3 respiration specific to complex I, followed by addition of Succinate (10 mM) for measurements of the maximal coupled state 3 respiration. Oligomycin (2.5 mM) was added as an Adenosine 5'-triphosphate (ATP)-synthase inhibitor, and thereafter Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was titrated (starting with 2 µM and followed by steps of 1 µM) to obtain maximal uncoupled respiration. Chambers were then opened slightly for 2-3 min to increase the oxygen concentration in the chamber and avoid oxygen concentrations < 100 nmol/mL for the rest

of the protocol. Rotenone (0.5 µM) was added to inhibit the flow of electrons through complex I, followed by Malonic acid (MnA; 5 mM) to inhibit complex II, and later Antimycin A (2.5 µM) to inhibit complex III. Finally, Ascorbate (2 mM) and Tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.5 mM) were added together to donate electrons directly to cytochrome c oxidase (COX), and shortly after Sodium azide (≥100 mM) was added as a COX inhibitor. Hydrogen peroxide (H_2O_2; 0.1 µM) was added before the following steps: adipose tissue, digitonin, ADP, Oligomycin, Rotenone and Ascorbate to measure H_2O_2 flux. All substrate and inhibitor concentrations are final concentrations.

2.8 Adipose tissue cell size

Adipose tissue was fixated in phosphate-buffered formalin immediately after sampling, before it was embedded in paraffin and cut into 4 µm sections. These sections were mounted on glass slides and dried at 37^0C overnight in an incubator. The sections were stained with CD68 (Mouse monoclonal anti-CD68 (Dako, M0814)) in a Dako Autostainer (EnVision™ + Systems HRP (DAB) Mouse (Dako, K4007)). Digital images were captured with an EVOS FL Auto 2 Imaging System (ThermoFisher Scientific, USA) at x10 objective. Images from the EVOS were analyzed with Fiji (Schindelin et al., 2012). The investigators were blinded for group (PCOS/Non-PCOS, HIT/Non-Ex) and time-point (baseline/16 weeks). The area of ≥200 adipocytes was measured per sample. Fat cell sizes in abdominal adipose tissue was measured from 7 Non-PCOS women and 22 women with PCOS, and in gluteal adipose tissue from 13 Non-PCOS women and 29 women with PCOS.

2.9 Statistical analysis

We calculated the sample size in the IMPROV-IT trial based on the primary outcome; menstrual frequency (Kiel et al., 2020). A one-way analysis of variance test with three groups with a 5% level of significance, a standard deviation of 2 menstrual cycles/year and statistical power of 0.80 gave a target study population of 48 women to detect an increase of three menstrual cycles during a 12-month period. We added 15% to the sample size owing to the non-normality of menstrual frequency, and another 15% to allow for expected dropout, and aimed to include 64 women with PCOS. We did not perform an *a priori* sample size calculation for the outcomes reported in the present paper, but the number of participants in the groups included in our study corresponds to previous studies with similar outcomes (Larsen et al., 2015, Dohlmann et al., 2018, Despres et al., 1984, Talanian et al., 2007, Perry et al., 2008). The number of Non-PCOS women from whom we measured abdominal fat cell size (n=7) was lower compared with previous studies (Despres et al., 1984, Manneras-Holm et al., 2011).

To determine between-group differences in the LV-HIT and HV-HIT groups (for PCOS and Non-PCOS women, respectively) in all reported outcomes, we used linear mixed models with participants as random factor and the effect of time and group allocation as fixed effects with these levels: PCOS baseline, Non-PCOS baseline, PCOS Non-Ex post intervention, PCOS LV-HIT post intervention, PCOS HV-HIT post intervention, Non-PCOS LV-HIT post intervention and Non-PCOS HV-HIT post intervention. Since there were no between-group differences between the two HIT groups, we pooled these groups to increase

statistical power, leaving us with 3 groups post intervention; 1) a PCOS Non-Ex group (n=23), 2) a PCOS HIT group (n=41) and 3) a Non-PCOS HIT group (n=15).

We used linear mixed models with participants as random factor and the effect of time and group allocation as fixed effects with these levels: PCOS baseline, Non-PCOS Baseline, PCOS Non-Ex post intervention, PCOS HIT post intervention and Non-PCOS HIT post intervention. We adjusted for baseline values as recommended by (Twisk et al., 2018). Descriptive statistics at baseline are reported as mean ± SD, and comparisons within and between groups are reported as estimated means with 95% confidence intervals. Normality of residuals was evaluated using Q-Q plots. For two of the dependent variables, the residuals were not normally distributed (insulin concentration and HOMA-IR) and logarithmically transformed to obtain normality. As the results were substantially the same after log-transformation, we report the results for the non-transformed variables to improve interpretation. Group means for weekly exercise training sessions for PCOS HIT and Non-PCOS HIT were compared using Student's t-test for independent samples, and presented as mean ± SD.

We considered two-sided *P*-values <.05 as statistically significant. All analyses were carried out using SPSS version 25.0 (SPSS Inc., United States).

3.0 Results

The distribution of the four PCOS phenotypes (Azziz et al., 2009) were as follows for the Non-Ex and PCOS HIT groups, respectively: 39% and 24% with phenotype A (oligo/amenorrhea + hyperandrogenism + polycystic ovaries), 0% and 15% with phenotype

B (oligo/amenorrhea + hyperandrogenism), 39% and 25% with phenotype C (hyperandrogenism + polycystic ovaries), and 22% and 37% with phenotype D (oligo/amenorrhea + polycystic ovaries).

3.1 Comparisons between women with and without PCOS at baseline

Table 1 shows baseline characteristics of the Non-PCOS women and women with PCOS. Women with PCOS had greater waist/hip ratio and lower fat oxidation rates compared with Non-PCOS women (**Figures 2A & B**). When we compared fat oxidation rates between the 15 women with PCOS who were individually matched with the 15 Non-PCOS women, there were no difference in fat oxidation rates; 7.81 ± 2.14 versus 8.52 ± 1.26 mg/FFM/min (p = .28) for women with PCOS and Non-PCOS women, respectively. There was no statistically significant difference in HOMA-IR between the groups, although 43 women with PCOS (68%) versus five Non-PCOS women (42%) had HOMA-IR ≥ 2.5. Abdominal adipose tissue oxygen flux was higher in Non-PCOS women compared with women with PCOS (**Figure 2C**). No differences were observed in gluteal adipose tissue oxygen flux (**Figure 2D**). Nor were there any differences in metabolic outcomes, or abdominal and gluteal adipose tissue cell size between these groups (**Figures 2E & F)**.

Table 1. Baseline characteristics of PCOS and Non-PCOS women.

	PCOS		Non-PCOS		*P-values*
	n	Mean ± SD	*n*	Mean ± SD	
Age (years)	64	30 ± 5	15	31 ± 6	.50
Body weight (kg)	64	85.1 ± 19.6	15	81.2 ± 17.1	.48
BMI (kg/m^2)	64	30.5 ± 6.5	15	28.4 ± 5.6	.24
FFM (kg)	64	50.7 ± 6.8	15	51.0 ± 5.6	.89
Body fat percentage (%)	64	39.2 ± 8.9	15	34.6 ± 9.7	.08
Waist circumference (cm)	63	101 ± 17	14	92 ± 13	.22
Hip circumference (cm)	63	113 ± 14	14	112 ± 12	.81
Waist/Hip Ratio	63	0.90 ± 0.09	14	0.82 ± 0.05	**.007**
HbA1c (mmol/mol)	64	32.4 ± 3.2	13	31.3 ± 4.0	.30
VO_2peak (mL/min/kg)	64	33.1 ± 7.2	15	36.0 ± 6.8	.17
VO_2peak (L/min)	64	2.7 ± 0.4	15	2.9 ± 0.3	.21
Glucose (mmol/L)	63	5.0 ± 0.5	13	4.8 ± 0.4	.40
Insulin (pmol/L)	64	117 ± 89	12	84 ± 61	.28
HOMA-IR	63	4.5 ± 3.8	12	3.0 ± 2.3	.24
Total testosterone (nmol/L)	64	1.5 ± 0.6		-	
SHBG (nmol/L)	64	43 ± 23		-	

Values are mean ± SD. PCOS, Polycystic ovary syndrome; BMI, body mass index; FFM, Fat free mass; VO_2peak, peak oxygen uptake; HOMA-IR, Homeostatic model assessment of insulin resistance; SHBG, Sex Hormone Binding Globulin. Testosterone and SHBG concentrations were only measured in women with PCOS. Statistically significant P-values are in bold.

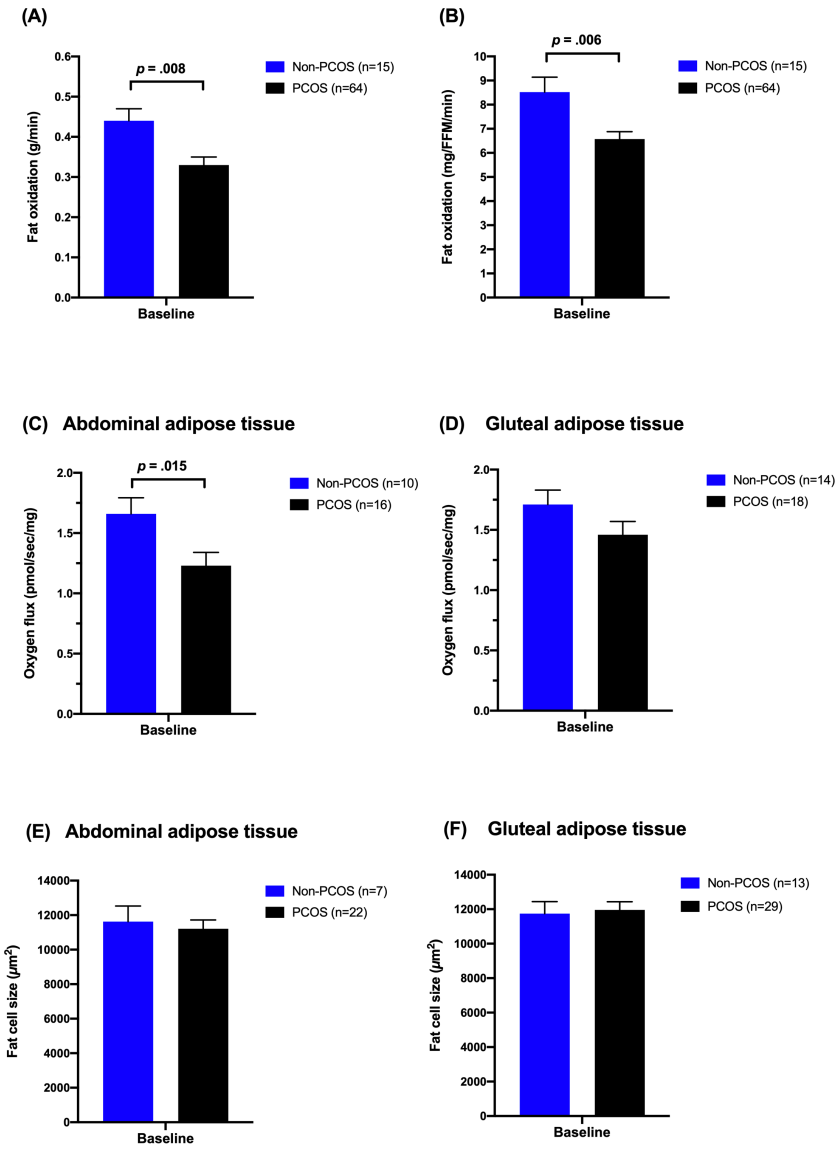

Figure 2: Comparisons between women with PCOS and Non-PCOS women at baseline. Whole-body fat oxidation during submaximal exercise (**A** and **B)**, oxygen flux with complex I + II linked substrates in subcutaneous abdominal (**C**) and gluteal adipose tissue (**D**), and abdominal (**E**) and gluteal (**F**) adipose tissue cell size in Non-PCOS (blue bars) and women with PCOS (black bars). The bars and error bars represent estimated means and SE based on linear mixed models.

3.2 Physiological, metabolic and adipose tissue responses to high-intensity interval training

On average, women in the PCOS HIT group exercised 2.2 ± 0.5 weekly sessions while women in the Non-PCOS HIT group exercised 2.6 ± 0.4 weekly sessions (p = .025), which corresponds to ~6 exercise sessions less in total over the 16 weeks. Seven women with PCOS and one woman in the Non-PCOS group did not register with Polar Flow, therefore we were unable to include their exercise training data.

Table 2 shows the changes in anthropometric measures, metabolic variables, cardiorespiratory fitness and adipose tissue fat cell size from baseline to after 16 weeks of HIT. The PCOS Non-Ex group reduced body weight and body mass index (BMI) more than PCOS HIT (p = .021 and p = .031, respectively), despite no differences in physical activity level or self-reported daily energy intake (data not shown). Non-PCOS women reduced their body weight and BMI after 16 weeks of HIT, with no between-group differences compared with the PCOS HIT group. Waist circumference and waist/hip ratio decreased in the PCOS HIT group (p = .011 and p = .007, respectively), with no between group-differences.

Cardiorespiratory fitness increased by ~6% in the Non-PCOS HIT group (p = .003) and ~4% in the PCOS HIT group (p = .006) after 16 weeks of HIT. The PCOS HIT group improved their absolute VO_2peak (L/min), but not relative (mL/min/kg), significantly more than the PCOS Non-Ex group (p = .002). Fat oxidation rates increased in the Non-PCOS HIT group, with no change in the PCOS HIT or PCOS Non-Ex group (**Figures 3A & B**). There were no between-group differences in changes in oxidation rates after 16 weeks, but a

tendency of higher increase in fat oxidation rates (mg/kg/min) were observed in the Non-PCOS HIT group compared with the PCOS HIT group (p = .075).

We observed no between-group differences in metabolic outcomes, abdominal and gluteal adipose tissue oxygen flux (**Figure 3C &D)**, or cell size (**Figure 3E & F**) after 16 weeks of HIT.

Table 2. Effects of 16 weeks of non-exercise or high-intensity interval training. Estimated mean difference from baseline values (95% confidence interval) based on linear mixed models.

	Non-PCOS HIT		**PCOS Non-Ex**		**PCOS HIT**		**Between PCOS group difference** *(group x time interaction)*		**Between HIT group difference** *(group x time interaction)*	
	Estimate (CI)	*P*	Estimate (CI)	*P*	Estimate (CI)	*P*	Estimate (CI)	*P*	Estimate (CI)	*P*
Body weight (kg)	-1.52 (-2.88 to -0.17)	**.028**	-2.19 (-3.35 to -1.03)	**<.001**	-0.44 (-1.36 to 0.49)	.35	1.75 (0.27 to 3.24)	**.021**	-1.09 (-2.73 to 0.55)	.19
BMI (kg/m^2)	-0.54 (-1.00 to 0.06)	**.029**	-0.79 (-1.20 to -0.37)	**<.001**	-0.20 (-0.53 to 0.13)	.13	0.58 (0.06 to 1.11)	**.031**	-0.34 (-0.92 to 0.25)	.25
FFM (kg)	0.32 (-0.36 to 1.00)	.35	-0.28 (-0.86 to 0.30)	.33	-0.09 (-0.55 to 0.37)	.70	0.19 (-0.55 to 0.93)	.60	0.41 (-0.41 to 1.23)	.32
Body fat percentage (%)	-0.46 (-1.60 to 0.69)	.43	-1.03 (-2.01 to -0.05)	**.04**	-0.29 (-1.07 to 0.49)	0.47	0.74 (-0.51 to 1.99)	.24	-0.19 (-1.58 to 1.20)	.79
Waist circumference (cm)	-0.62 (-4.11 to 2.88)	.73	-0.89 (-3.72 to 1.95)	.53	-2.96 (-5.19 to -0.72)	**.011**	-2.07 (-5.65 to 1.52)	.25	2.35 (-1.81 to 6.51)	.26
Hip circumference (cm)	-0.46 (-3.05 to 2.14)	.73	-0.49 (-2.59 to 1.61)	.64	-0.64 (-2.30 to 1.02)	0.44	-0.15 (-2.82 to 2.51)	.91	0.15 (-2.93 to 3.24)	.92
Waist/Hip Ratio	-0.00 (-0.03 to 0.03)	.92	-0.01 (-0.04 to 0.01)	.20	-0.03 (-0.04 to -0.01)	**.007**	-0.01 (-0.04 to 0.02)	.47	0.02 (-0.01 to 0.06)	.15

HbA1c (mmol/mol)	0.67 (-0.80 to 2.15)	.37	0.43 (-0.69 to 1.55)	.45	1.75 (0.81 to 2.68)	**<.001**	1.32 (-0.10 to 2.73)	.07	-1.12 (-2.88 to 0.63)	.21
VO_2peak (mL/min/kg)	2.20 (0.76 to 3.65)	**.003**	0.35 (-0.85 to 1.55)	.56	1.41 (0.43 to 2.39)	**.006**	1.06 (-0.48 to 2.59)	.17	0.81 (-0.94 to 2.56)	.36
VO_2peak (L/min)	0.13 (0.02 to 0.23)	**.016**	-0.06 (-0.15 to 0.02)	.15	0.11 (0.04 to 0.18)	**.002**	0.17 (0.06 to 0.28)	**.002**	0.01 (-0.10 to 0.14)	.82
Glucose (mmol/L)	0.10 (-0.13 to 0.32)	.38	-0.09 (-.26 to 0.08)	.30	-0.11 (-0.25 to 0.03)	.11	-0.03 (-0.24 to 0.19)	.81	0.20 (-0.06 to 0.47)	.13
Insulin (pmol/L)	-11.5 (-41.3 to 18.3)	.44	-25.1 (-47.2 to -3.0)	**.026**	-13.6 (-31.6 to 4.4)	.14	11.5 (-16.5 to 39.4)	.42	4.1 (-30.8 to 39.1)	.81
HOMA-IR	-0.35 (-1.72 to 1.01)	.61	-1.10 (-2.11 to -0.10)	**.032**	-0.66 (-1.48 to 0.16)	.11	0.44 (-0.83 to 1.71)	.49	0.39 (-1.21 to 1.99)	.63

PCOS, Polycystic ovary syndrome; CI, Confidence interval; BMI, body mass index; FFM, Fat free mass; VO_2peak, peak oxygen uptake; HOMA-IR, Homeostatic model assessment of insulin resistance. Statistically significant P-values are in bold.

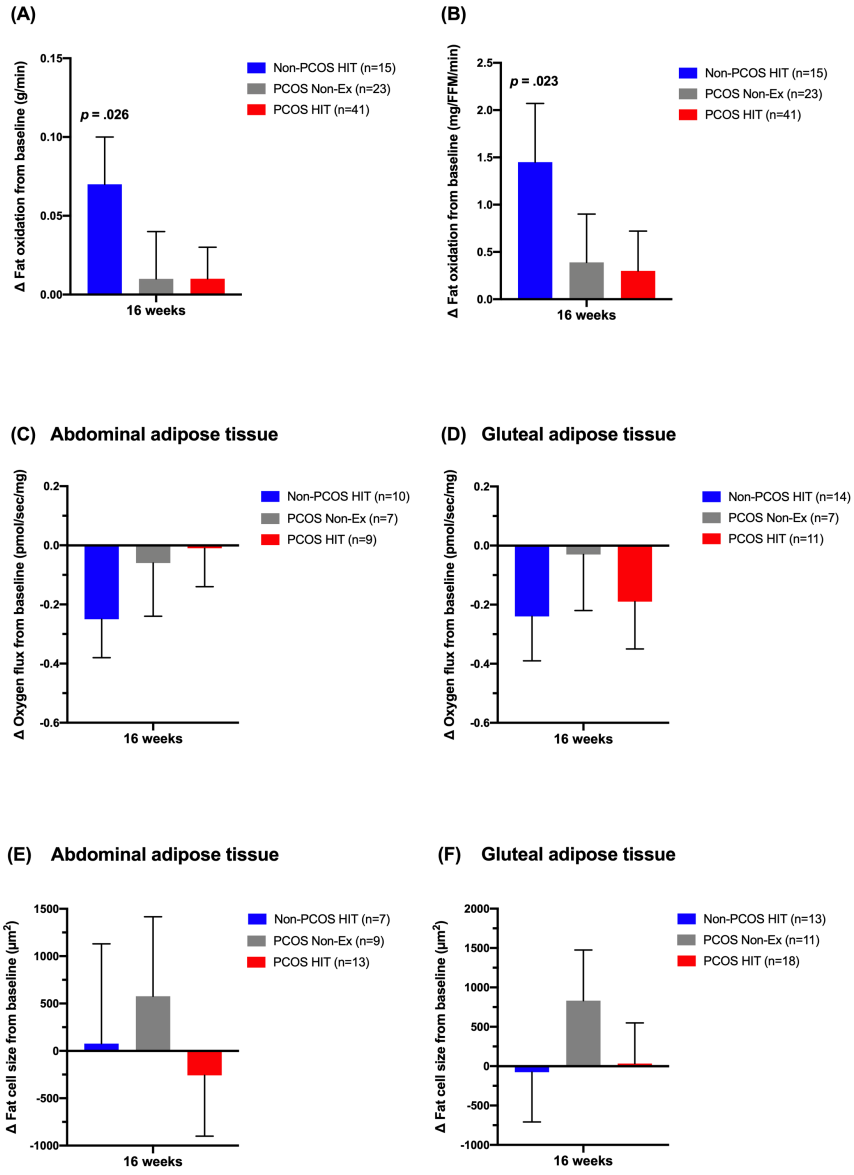

Figure 3: Effects of 16 weeks of non-exercise or high-intensity interval training. Whole-body fat oxidation during submaximal exercise (**A** and **B**), oxygen flux with complex I + II linked substrates in subcutaneous

abdominal (**C**) and gluteal adipose tissue (**D**), and abdominal (**E**) and gluteal (**F**) adipose tissue cell size in Non-PCOS HIT (blue bars), PCOS Non-Ex (grey bars), and PCOS HIT (red bars) after the 16 weeks intervention. The bars and error bars represent estimated means and SE based on linear mixed models. *P* values are for within-group comparisons after 16 weeks of high-intensity interval training.

4.0 Discussion

Our findings provide new data on whole-body fat oxidation during submaximal exercise, and gluteal and abdominal adipose tissue mitochondrial respiration and cell size in women with PCOS and Non-PCOS women, along with the effects of 16 weeks of HIT on these outcomes. We report that 16 weeks of HIT improved fat oxidation rates during submaximal exercise in Non-PCOS women, but not in women with PCOS, despite similar improvements in VO_2peak. Furthermore, women with PCOS had lower mitochondrial respiration with substrates for complex I + II in subcutaneous abdominal adipose tissue compared to Non-PCOS women. Mitochondrial respiration in both adipose tissue sites were unaffected by 16 weeks of HIT in women with and without PCOS. Finally, subcutaneous gluteal or abdominal adipose tissue cell size did not differ between women with PCOS and Non-PCOS women and 16 weeks of HIT did not alter adipocyte sizes in any group of women.

We report novel findings on absent exercise-induced improvements in fat oxidation during submaximal exercise in women with PCOS, despite improved VO_2peak, suggesting metabolic inflexibility in women with PCOS. Conversely, fat oxidation rates improved in Non-PCOS women after 16 weeks of HIT. A recent systematic review reported metabolic inflexibility in the rested, insulin-stimulated state in women with PCOS compared with healthy women (Rimmer et al., 2020). Metabolic inflexibility has been linked to insulin

resistance and type 2 diabetes (Goodpaster and Sparks, 2017), and Broskey and colleagues found that women with PCOS and obesity (28.8 ± 4.7 years) were as metabolically inflexible as middle-aged women with type 2 diabetes and obesity (58.2 ± 9.9 years) (Broskey et al., 2018). Metabolic inflexibility has also been reported in lean women with PCOS (Hansen et al., 2019) and adolescent girls with PCOS (Kim et al., 2018), suggesting that metabolic inflexibility is associated with PCOS independent of BMI and age.

Similar to our findings in Non-PCOS women, previous studies reported improvements in whole-body fat oxidation during submaximal exercise at 60% of VO_2peak in healthy recreationally active women after seven sessions of HIT (10 x 4 min bouts at 90% of VO_2peak separated by 2 min of rest) (Talanian et al., 2007), and in untrained recreationally active men and women after six weeks of HIT (using the same HIT protocol as Talanian et al. (2007) but for three days/week for 6 weeks) (Perry et al., 2008). In our study, the Non-PCOS women exercised significantly more on a weekly basis compared to the women with PCOS (2.6 ± 0.4 versus 2.2 ± 0.5 weekly HIT sessions, corresponding to ~6 HIT sessions less in total over the 16 weeks), which may explain the difference observed in training-induced improvements in fat oxidation rates. However, we report similar improvements in VO_2peak in women with PCOS and Non-PCOS women after 16 weeks of HIT, which suggests that the 6 fewer HIT sessions performed by women with PCOS cannot fully explain the difference in training-induced improvements in fat oxidation rates. Absent training-induced improvements in women with PCOS have been reported previously; Hansen and colleagues observed improved insulin sensitivity measured by the gold-standard hyperinsulinaemic-euglycemic clamp in healthy women without PCOS, but not in women

with PCOS after 14 weeks of exercise training (three weekly exercise sessions; two aerobic HIT sessions and one strength training session) (Hansen et al., 2020). Similarly, Hansen and colleagues reported improved incremental area under the oral glucose tolerance test curve for plasma glucose and insulin after exercise training in healthy women, but not in women with PCOS. Their data suggested that the lack of improvements in insulin action after exercise training were due to impaired ability to upregulate glucose uptake in skeletal muscle.

Skeletal muscle and adipose tissue are crucial tissues in energy metabolism and play a major role in metabolic flexibility in humans. However, little research has been undertaken on metabolic flexibility of white adipose tissue. Metabolic flexibility is driven by cellular processes that may be linked to the mitochondria (Goodpaster and Sparks, 2017). We are the first to explore subcutaneous abdominal and gluteal adipose tissue mitochondrial respiration in women with and without PCOS and the responses to HIT. In our study, we observed higher mitochondrial respiration through complex I + II in subcutaneous abdominal adipose tissue from Non-PCOS women compared to women with PCOS at baseline, no such differences were seen in gluteal adipose tissue. Low-grade inflammation may be one mechanism causing the lower mitochondrial respiration in women with PCOS compared to Non-PCOS women at baseline. There is a proposed link between inflammation and mitochondrial dysfunction in adipocytes, in which the proinflammatory response of macrophages may promote mitochondrial dysfunction in adipocytes (Woo et al., 2019). No changes were observed in mitochondrial respiration in either of the adipose tissue depots in either group of women after 16 weeks of HIT, which suggests that mitochondrial respiration through complex I + II in adipose tissue cannot explain the improved fat oxidation rates and metabolic flexibility

observed in Non-PCOS women. Our findings are supported by data from Larsen and colleagues, who showed increased VO_2peak but no change in mitochondrial respiration in subcutaneous abdominal adipose tissue in overweight but otherwise healthy men and women after 6 weeks of LV-HIT (three days/week with 5 x 60 s maximal effort work-bouts) (Larsen et al., 2015). However, there are some indications in the literature for exercise-induced changes in adipose tissue mitochondrial respiration. Dohlmann and colleagues (2018) found decreased mitochondrial respiration in subcutaneous abdominal adipose tissue in healthy men and women after 6 weeks of LV-HIT (18 HIT sessions with 7 x 1 min exercise bouts at ~100% of VO_2peak) (Dohlmann et al., 2018), while Mendham and colleagues (2020) reported increased mitochondrial respiration in subcutaneous abdominal adipose tissue in obese, black South-African women after 12 weeks of combined aerobic and resistance exercise training (four days/week of 40-60 min with aerobic exercise at 75-80% HR_{peak} and resistance training that included upper- and lower-body exercises at 60-70% HR_{peak}) (Mendham et al., 2020). We are not sure of the reasons for these divergent findings, but they may be explained by different exercise modalities and intensities.

We did not observe changes in mitochondrial respiration in adipose tissue after 16 weeks of HIT. We speculate that changes may have occurred in skeletal muscle mitochondrial function as Larsen and colleagues have previously reported increased mitochondrial respiration in skeletal muscle, but not in abdominal adipose tissue, after 6 weeks of HIT in men and women who were overweight but otherwise healthy (Larsen et al., 2015). Possible gains, or lack of gains, in skeletal muscle mitochondrial function could explain the improved whole-body fat oxidation during submaximal exercise in Non-PCOS

women, and lack of improvement in women with PCOS after 16 weeks of HIT. However, we did not obtain any muscle biopsies in our study.

We observed no differences in subcutaneous abdominal or gluteal fat cell size between Non-PCOS women and women with PCOS, nor do we report changes in cell size after 16 weeks of HIT in either group of women. A case-control study on women with PCOS and age- and BMI-matched women without PCOS reported enlarged abdominal adipocyte volumes in women with PCOS, and an association between hypertrophic adipocytes and insulin resistance (Manneras-Holm et al., 2011). The divergent findings in our study and the study by Manneras-Holm may be explained by higher power in their study (27 women in both groups).

Similar to our findings, previous studies have reported no changes in subcutaneous abdominal adipose tissue volume and size after 12 weeks of exercise training in men with overweight or obesity (three days/week; aerobic exercise twice/week cycling for 30 min at 70% maximal watt and resistance training once weekly) (Stinkens et al., 2018), or after 16 weeks of exercise in women with PCOS (three days/week for 30 min at an intensity of faster than normal walking pace) (Stener-Victorin et al., 2012). A longer and more intense training program may be required to change adipose tissue structure. Després and colleagues observed a sex difference in the sensitivity to exercise training, with less responsiveness in female adipose tissue. Twenty weeks of endurance training for 40 min at 80% HR_{max} 4-5 times weekly did not influence fat percentage or adipose cell weight in women, whereas males showed reductions in both fat percentage and adipose cell weight (Despres et al., 1984).

Our data indicate that women with PCOS allocated the non-exercising control group most likely introduced some lifestyle changes during the study period as we observed reductions in body mass, BMI and body fat percentage and improvements in fasting insulin concentration and HOMA-IR in this group. Although we detected no changes in self-reported daily energy intake or physical activity level, these were only recorded for a brief period of time (4-5 days at each timepoint). At study inclusion, women with PCOS assigned to the control group were informed about the current recommendations of at least 150 min weekly of moderate intensity physical activity, which could have affected their physical activity behavior. Furthermore, individuals who volunteer for exercise studies usually hope to be allocated to the exercise intervention, and women allocated to the control group have most likely been motivated to introduce lifestyle changes.

Subgroup analyses from a systematic review and meta-analysis on the effectiveness of exercise compared to control in women with PCOS showed greater improvements in cardiometabolic outcomes in supervised versus unsupervised exercise interventions (Kite et al., 2019), and a supervised rather than semi-supervised exercise protocol in our study could possibly have resulted in greater gains for women with (and without) PCOS. Furthermore, including a more varied exercise protocol with a combination of LV-HIT and HV-HIT sessions could have induced larger improvements, as shown previously (Almenning et al., 2015).

Major strengths of our study are the pair-wise age- and BMI-matching of women with PCOS and Non-PCOS women, the rigorous inclusion and exclusion criteria, and that we explored two adipose tissue depots in women with and without PCOS. We also acknowledge

study limitations. We used different methodologies to estimate body composition at the two study centers, limiting the baseline comparison for these outcomes between women with and without PCOS. The Inbody 720 scale that we used for participants in Norway underestimates body fat and overestimates FFM compared with DXA (McLester et al., 2018). Due to limited adipose tissue biopsy volumes, we were unable to compare mitochondrial respiration and fat cell size between the women with PCOS and Non-PCOS women who were individually age- and BMI-matched. Furthermore, due to limited adipose tissue volumes and statistical power, we were unable to run meaningful statistical analyses for LV-HIT and HV-HIT groups, which made us unable to detect differences in the effects of LV-HIT and HV-HIT on mitochondrial respiration and fat cell size. Instead, data for LV-HIT and HV-HIT were pooled to increase the statistical power and to investigate the effect of HIT.

In conclusion, we observed exercise-induced improvements in whole-body fat oxidation during submaximal exercise in Non-PCOS women but not in women with PCOS after 16 weeks of HIT. These findings suggest metabolic inflexibility in women with PCOS. Mitochondrial respiration was lower in subcutaneous abdominal, but not gluteal, adipose tissue in women with PCOS compared to Non-PCOS women, and 16 weeks of HIT did not alter adipose tissue mitochondrial respiration in either group of women. Further studies are required to ascertain the underlying mechanisms behind the absent exercise-induced improvements in fat oxidation and metabolic inflexibility observed in women with PCOS.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

SLi drafted the manuscript. SLi, RR and SLa analyzed the data. SLi and SLy performed statistical analyses. SLi, IK and TM were responsible for study conception and design, coordinated the studies at the two sites, performed measurements on testing days, and supervised the exercise training. All authors provided feedback and approved the final manuscript.

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